

An endothelial gene signature score predicts poor outcome in patients with endocrine treated, low genomic grade breast tumors

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Running head: An endothelial gene signature in breast cancer patients

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Translational relevance

The ability to evaluate the effect of a clinical trial drug is central to its success. Whilst anti-angiogenic drugs have demonstrated modest increases in the progression-free survival of breast cancer patients, they do not prolong life expectancy and importantly, lack a formal predictive biomarker. The ability of vascular gene transcripts to provide treatment predictive information in a breast cancer setting remains unexplored. Here, we utilize a set of genes representative of a normal vascular endothelium to identify a subgroup of endocrine-treated breast cancer patients with better and worse long term distant metastasis-free survival. Moreover, we also note significant increases in signature genes following treatment of metastatic tumors with the anti-angiogenic inhibitor sunitinib, highlighting that evaluation of transcriptional changes in microvascular genes alongside assessment of MVD and angiogenic factors in clinical trials of antiangiogenic compounds may be warranted.

Abstract

Purpose The ability of vascular genes to provide treatment predictive information in breast cancer patients remains unclear. As such, we assessed the expression of genes representative of normal endothelial microvasculature (MV) in relation to treatment-specific patient subgroups.

Experimental Design We used expression data from 993 breast tumors to assess 57 MV genes (summarized to yield an MV score) as well as the Genomic Grade Index (GGI) and PAM50 signatures. MV score was compared to CD31 staining by correlation and gene ontology (GO) analysis, along with clinicopathological characteristics and PAM50 subtypes. Uni- and multivariate analysis was performed in all and treatment specific subgroups as well as a clinical trial cohort of 14 metastatic breast cancer patients, 7 of whom received anti-angiogenic therapy.

Results MV score did not correlate with microvessel density (correlation = 0.096), but displayed enrichment for angiogenic GO terms, and was lower in Luminal B tumors. In endocrine-treated patients, a high MV score was associated with decreased risk of metastasis (HR 0.58; 95%CI, 0.38-0.89), even after adjusting for histological grade, but not GGI or PAM50. Subgroup analysis showed the prognostic strength of the MV score resided in low genomic grade tumors and MV score was significantly increased in metastatic breast tumors after treatment with sunitinib + docetaxel ($P = 0.031$).

Conclusion MV score identifies two groups of better and worse survival in low-risk endocrine-treated breast cancer patients. We also show normalization of tumour vasculature on a transcriptional level in response to an angiogenic inhibitor in human breast cancer samples.

Introduction

Breast cancer remains the most common malignancy and a leading cause of cancer-related death in women (1). Decreasing mortality rates in recent decades have come at a cost of both more extensively applied toxic adjuvant therapies, stressing the importance of finding reliable prognostic and treatment predictive markers. The success of administering trastuzumab to patients with overexpression of the HER2 protein serves as an excellent example (2,3), but equally useful predictive markers for treatment-response are largely lacking for other targeted therapies - including antiangiogenic therapies like bevacizumab and sunitinib.

The last 15 years have seen the application of microarray technology to tumor samples with the aim of finding better prognostic and treatment predictive strategies for breast cancer patients. This research has resulted in a plethora of genomic classifiers ranging from binary good/poor prognosis signatures (4–6) to multi-level classifiers capable of diving breast cancer into prognostically-relevant molecular subgroups (7). Further studies have tested the predictive capacity of gene signatures for both chemotherapy and tamoxifen (8–10) with favorable findings, although given the central role of proliferation-related genes in many classifiers (11,12) their value over traditional immunohistochemical markers such as Ki67 remains unclear.

Recent clinical trials into antiangiogenic therapies (13–15) have served to once again highlight the belief that breast cancer progression can be impeded through targeting of tumour angiogenesis. Despite this continued interest in these therapies, and questions marks over their ability to prolong overall patient survival, the clinical relevance of angiogenesis-related transcription as a treatment predictive factor in breast cancer has remained largely unexplored. Here, we aim to examine a previously published set of 57 gene transcripts (16) (representative of a normal endothelium)

firstly through comparison to traditional microvessel density (MVD) by correlation and gene ontology (GO) analysis and secondly through assessment of the signature in 6 different breast cancer cohorts, with particular focus on its performance in subgroups of endocrine and chemotherapy treated patients.

Materials and methods

Patients and datasets

Internal datasets: We have previously described both the Uppsala (N=253) and Stockholm (N=159) datasets (17–19) with an extensive overview for both cohorts found here (12). Both microarray studies were approved by the ethics committees at Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden, respectively and are publically available at NCBI GEO under accession numbers GSE4922 and GSE1456, respectively. External datasets: Data from the Netherlands Cancer Institute (NKI, N= 295) (20), Erasmus Medical Center (Rotterdam N= 286) (21), and the John Radcliffe and Guys hospitals (Oxford and London, United Kingdom, N= 99 and N=87, respectively) (22) were used, the Oxford and Guys data for further analysis of the findings in relation to endocrine therapy. The NKI dataset is publically available as part of the *breastCancerNKI* R package and the Rotterdam and Oxford/Guys datasets are available under accession numbers GSE2034 and GSE5432. Clinical information for 14 patients treated with sunitinib plus docetaxel or docetaxel alone has been previously described (23), ClinicalTrials.gov identifier NCT00393939, and expression data is publically available at NCBI GEO under accession number GSE54323. For comparison of MV score to lymphovascular invasion (LVI) we used previously published data (24), retrievable under the accession number GSE54323.

RNA extraction and array hybridization

Uppsala and Stockholm cohorts: RNA was extracted from homogenized tumor material with RNeasy spin column kits (Qiagen) and quality was assessed with an Agilent 2100 Bioanalyzer. Two to 5 µg of RNA was used to produce biotinylated cRNA. Hybridization to HGU133 A and B microarrays (Affymetrix, Santa Clara, CA,

USA) and scanning was performed according to Affymetrix protocols – a comprehensive account can be found in Pawitan *et. al* (19).

Immunohistochemistry and microvessel density

Uppsala cohort: For immunohistochemical analysis, formalin-fixed paraffin-embedded sections (4 µm) were deparaffinized in xylene and rehydrated in graded concentrations of ethanol to TBS. Antigen retrieval by microwave treatment was preformed for 20 minutes in Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.17). CD31 antibody (clone JC70A, DAKO, Glostrup, Denmark) was diluted 1:50, and staining was carried out in a Tech Mate™ Autostainer (DAKO). Slides were counterstained with haematoxylin and dehydrated. Microvessel density was determined using previously described methods (25). Briefly, tumor sections were examined at low power to determine the areas containing the greatest numbers of microvessels. Individual microvessels were then counted in these areas at x400 magnification (three fields per tumor section). Mean counts per high field were then calculated and the resulting value was normalized to yield a Microvessel density (MVD) score of between 0 and 100 (for ease of comparison to Microvascular gene expression score) using the rescale command of the “scales” package in R (26).

Microvasculature signature, PAM50 subtypes and genomic grade

Human homologs for 57 out of 58 the previously published mouse microvasculature transcripts (16) were extracted from the HomoloGene database at the National Center for Biotechnology Information (NCBI, Supplemental Table S1). Datasets were RMA normalized and median centered before the expression levels of signature genes were

added per tumor, and the resulting sum was scaled to yield a Microvasculature signature score (hereafter called MV score) of between 0 and 100 within each dataset using the *rescale* function of the R *genefu* package. The exception to this was the NKI dataset, here, we used the original normalized and median centered data from the *breastCancerNKI* R package for scaling as described above. Of note, mean centering has been demonstrated as sufficient to remove much of the dataset- bias associated with gene expression data from different cohorts, allowing for meaningful prognostic comparisons to be made (27). Fifty-seven of the genes (corresponding to 115 probes) were present on the HGU133 A and B platforms, and could be used for MV score determination in the Uppsala and Stockholm data. In the external data sets, identical methodology was used to determine MV scores and for the NKI, Rotterdam, Oxford, Guys and metastatic tumor data, 49, 46, 57, 57 and 57 signature genes were present on the respective platforms corresponding to 56, 79, 115, 115 and 115 probes, respectively. In the case of multiple probes mapping to the same gene an average expression of probes was taken. When data was pooled for combined analysis, the signature score was calculated and scaled in individual datasets before pooling.

PAM50: Molecular subtyping according to the PAM50 signature was performed as outlined in the original publication (7), using the code provided by Parker *et al.* on the UNC Microarray Database website as a data supplement to the original paper. Of note, we have previously published our code and the PAM50 subtypes for the Uppsala and Stockholm cohorts (12).

Genomic grade index (GGI): GGI was calculated as described in the original publication (6) and we have previously published our code for this signature along with the GGI calls for the Uppsala and Stockholm datasets (12).

Comparison of MV score to MVD and significance analysis for microarrays and Gene Ontology analysis

To assess similarity between the MV score and MVD scores we performed a Pearson correlation comparing a) both scores directly and b) each of the MV score genes to MVD score in 182 patients of the Uppsala cohort. P-values were adjusted for multiple testing using the FDR method as part of the *p.adjust* command of the R *stats* package. To test for enrichment of biological themes, the significance analysis of microarrays (SAM (28); quantitative response) was used to rank genes for association with the MV score (gene expression) and MVD scores (vessel count), respectively. Overrepresentation of gene ontology categories (GO) reflecting biological processes was determined with conditional hypergeometric tests using the R *GOfstats* package. Since many of the MV score genes are annotated as vascular or angiogenesis-related, and by necessity will correlate to the signature, the 57 signature genes were removed from the data prior to GO testing. Estimation of false discovery rate control was performed according to Storey (29) using the R package *qvalue* 2.0.0. All occurring categories in respective gene lists were tested.

Statistical analysis and software

To test for differences in mean expression of the MV score in relation to clinico-pathological parameters Student's test- for comparing means between two groups or ANOVA- for comparisons between more than two groups with post-hoc Tukey were

used as indicated in table legends. Survival analysis was performed using Kaplan–Meier and multivariable proportional hazards (Cox) where only variables with demonstrated prognostic significance in univariable testing were included in the final multivariable model. To ensure consistent survival endpoints across all datasets, distant metastasis-free survival (DMFS) was chosen. Hazard ratio is reported per 25 increments of MV score and MVD score, to simplify interpretation. Receiver operating characteristics were used to determine best MV score cutoff (with DMFS at 5 years as endpoint) in testing datasets before being applied to validation datasets and correlograms were constructed using the Spearman rank correlation metric. C-index calculation was performed in R using the “concordance” output from a Cox regression analysis for the GGI and PAM50 signatures alone or in combination with the MV score. All analyses were performed using the R Statistical language (26), with the *GOSTATS*, *ROCR*, *survival* and *samr* packages.

Results

The MV score is not correlated to traditional microvessel density, but does reflect angiogenic/endothelial gene ontology processes

In order to understand if any similarities exist between genes associated with microvasculature gene expression and traditional microvessel density (MVD) score we stained and scored the tumors of 182 patients from our previously published Uppsala cohort (12,17–19) using the endothelial/MVD marker CD31. Next, we derived a normal microvasculature signature score (MV scores) using gene expression data from the same tumors by adding the mRNA expression levels for 57 out of 58 previously identified (16) endothelial-specific gene transcripts (Supplemental Table

S1). Both the MV and MVD scores were then normalized and scaled (for ease of comparison) to yield values between 0 and 100.

A simple correlation analysis did not reveal any clear overall similarity between the MV and MVD scores (Pearson correlation = 0.096, $P = 0.20$, data not shown) and in individual signature gene analysis, only a weak correlation was found between five MV score genes and the MVD score (Supplemental Table S1). Next, we compiled two lists of the top 200 genes most associated with the MV score (having first removed the 57 signature genes) and MVD scores and assessed whether any biological processes were over-represented in these lists through gene ontology (GO) analysis. The top ten GO terms for both lists are displayed in Table 1, where an enrichment of terms related to angiogenesis and cardiovascular/ blood vessel/ endothelial development is notable amongst MV score correlated genes (Table 1, upper list). Conversely, no angiogenic or vascular development terms are found within MVD (CD31) correlated genes, which rather display an enrichment of terms associated with immune response (Table 1, lower list). These results show firstly that the MV score strongly reflects angiogenic/endothelial processes on a transcriptional level and secondly, based on the CD31 GO terms, that we may be unaware of the extent to which the immune response is involved in blood vessel formation and maintenance.

A low MV score is associated with a luminal B tumor subtype

With the aim of determining the prognostic and treatment predictive capacity of the MV score we extended our analysis to three additional gene expression breast cancer datasets (12,19–21). Again, MV scores were normalized and scaled within each

dataset and the resulting score was assessed in relation to the clinico-pathological parameters shown in Table 2.

We noted a trend towards lower MV scores in grade 3 tumors across all datasets (vs. grade 1 tumors, Table 2) and similarly, a statistically significant low signature score in the estrogen receptor positive Luminal B subtype (vs. Luminal A, Table 2, $p= <0.001$, 0.008, 0.016 and <0.001 for the Uppsala, Stockholm, NKI and Rotterdam datasets, respectively). A trend towards a higher MV score was found in tumors of a Normal-like subtype, which reached statistical significance in 2/4 datasets (vs. Luminal A subtype, Table 2, $p= 0.003$ and 0.047 for the Stockholm and Rotterdam datasets, respectively). Of interest, as information on lymphovascular invasion (LVI) was not available in these cohorts, we analyzed an additional publically available dataset (24) ($n=128$) and could not demonstrate a difference in MV score in the absence or presence of LVI (MV score mean \pm sem = 45.71 ± 2.23 and 49.54 ± 2.15 for tumours with and without LVI, respectively. $p= 0.22$, Welch t-test, data not shown).

A low MV score predicts poor outcome in endocrine treated patients

The prognostic capacity of many first generation gene expression signatures tends to be limited to ER positive breast tumors (30). As such, we assessed the MV score in univariable analysis across all, ER positive, and ER negative patients in each dataset. No consistent relationship to distant metastasis-free survival (DMFS) was found in any of these groupings, however in all patients two out of four datasets demonstrated a lower Hazard Ratio (HR) with increasing MV score (Supplemental Table S2, see “All patients” Uppsala and Stockholm cohorts, HR 0.69; 95% CI, 0.49 to 0.96 and 0.46; 95% CI, 0.24 to 0.87, respectively).

To determine if the type of adjuvant therapy received influenced these results we sub-divided each cohort into patients who did not receive systemic treatment, who received endocrine treatment, or those who received chemotherapy. The MV score did not provide consistent statistically significant information regarding DMFS in untreated, untreated ER-positive, or chemotherapy treated patients (Figure 1, see “Untreated”, “Untreated (ER positive)” and “Chemotherapy”, All). For patients receiving endocrine therapy, a trend towards decreased risk of distant metastasis was observed in two out of three datasets (Figure 1, see “Endocrine treatment”, Uppsala and Stockholm, HR 0.69; 95% CI, 0.42 to 1.12 and 0.45; 95% CI, 0.20 to 1.03, respectively), however this trend did not reach overall statistical significance (Figure 1, see “Endocrine treatment”, All, HR 0.82; 95% CI, 0.57 to 1.18). As the HR for the NKI dataset in endocrine treated patients (Figure 1, HR 1.56; 95% CI, 0.36 to 6.6) was in the opposite direction to that of the Uppsala and Stockholm datasets, and as 50% of the endocrine patients in the NKI datasets also received chemotherapy, we further examined the MV score in an independent dataset of 186 patients collected at the John Radcliffe and Guy’s Hospitals (Oxford/Guys dataset (22)). All patients had ER positive tumors and received adjuvant tamoxifen monotherapy. Here, a higher MV score was associated with a reduced risk of DMFS in endocrine treated patients (Figure 1, see “Additional datasets”, Both, HR 0.58; 95% CI, 0.38 to 0.89), consistent with the trend found in Uppsala and Stockholm endocrine treated patients. This statistical significance remained in multivariable analysis combining the Uppsala, Stockholm, Oxford and Guys data, when considering standard prognostic markers (Table 3, n = 380, see “Histologic Grade”, MV score HR 0.70; 95% CI, 0.50 to 0.97). Taken together these data suggest an endocrine therapy predictive capacity for the MV score.

A low MV score predicts poor outcome in patients with endocrine treated, low genomic grade breast tumors

Whilst numerous gene expression signatures exist for endocrine treated patients (5,9,31), their prognostic capacity relies on proliferation related genes (12,32). To address a potential relationship to proliferation for the MV score, we classified the Uppsala, Stockholm and Oxford/Guys datasets according to the genomic grade index (GGI), a strongly proliferation-related gene signature (12,22). When histopathological grade was replaced by genomic grade in multivariable analysis, the prognostic ability of the MV score was lost (Table 3, see “Genomic Grade”, MV score HR 0.89; 95% CI, 0.65 to 1.21). Similarly, neither MV score nor the PAM50 subtypes were prognostic in a multivariable analysis containing both variables (Supplemental Table S3, MV score HR 0.84; 95% CI, 0.58 to 1.21). To further examine the reason for this loss of prognostic power we firstly identified a cutoff (of 39) for the MV score in our training datasets (Uppsala and Stockholm cohorts) that would perform best for prediction of DMFS in endocrine treated patients (Supplemental Figure S1A iv). Secondly, we used this cutoff to produce Kaplan-Meier curves for both our training and validation datasets (Oxford and Guys cohorts), split according to genomic grade (GG-1 or GG-3). This analysis showed that the strength of the MV score resides in the lowly proliferative genomic grade 1 group of tumors (Figure 2A, GG-1, training group, MV score high/low n= 66/48; Figure 2B, GG-1 validation group, MV score high/low n= 84/26; and Figure 2C, GG-1 both groups, MV score high/low n= 150/74; $P = 0.31, 0.012$ and 0.007 , respectively. Compare to Figure 2D, E and F, GG-3, training, validation and both groups together, respectively.). In this subgroup, as in the full set of endocrine treated tumors, the MV score had independent prognostic

capacity over standard prognosticators (Supplemental Table S4, HR 0.47; 95% CI, 0.26 to 0.84). It is pertinent to highlight here that although the separation of GG-1 curves does not reach formal statistical significance in the training dataset (Figure 2A), there is a crossing of curves after approximately four years, potentially rendering the logrank test underpowered. Moreover this cutoff displays strong prognostic capacity in other treatment subgroups of the training dataset (Supplemental Figure S1B i-iii). Next, we hypothesized that a combination of both signatures (MV and GGI) would provide more prognostic information than either one alone. In order to test this, we calculated the concordance index (c-index) for the GGI, MV and PAM50 signatures alone and in combination in all patients of our original four datasets. In line with our hypothesis, we found that in general the addition of the MV score to the PAM50 and GGI gene signatures may provide more prognostic information all datasets than either signature alone (Supplemental Figure S2, compare green vs. blue bars - PAM50 vs. PAM50 + MV score and red vs. yellow bars - GGI vs. GGI + MV score, in all datasets).

To further explore the relevancy of our signature in a clinical setting we calculated the change in MV score in a previously published cohort of 14 metastatic breast tumors (7 from the control arm and 7 from the treated arm) before and after treatment (14 days) with the angiogenic inhibitor sunitinib (23). These samples were taken as part of a sub-study from a recent phase III clinical trial comparing the efficacy of sunitinib plus docetaxel vs. docetaxel alone in an advanced breast cancer setting (33). A heatmap displaying the intra-patient change in the expression of the MV score genes before and after treatment in both clinical trial arms is shown in Figure 3A, where in general, a greater change in signature genes was found in the combination arm (Figure 3A, red bar). Concomitantly, a significant increase in MV

score was noted after treatment in the combination arm (Figure 3B, right panel, $P = 0.031$ vs. baseline), however, low patient numbers prevent further analysis regarding survival. For the sake of completeness, we also show a table of the change in MV score (14 days – baseline) and RECIST response for all patients, no clear trend is observable (Supplemental Table S5). These results are in line with the concept of vascular normalization following treatment with an angiogenic inhibitor (34,35), but notably, are the first demonstration of this principle on a transcriptional level in human breast tumour samples.

Discussion

In this study, we found that the abundance of normal microvascular transcripts was reproducibly related to both the luminal B breast cancer subtype and the clinical endpoint distant metastasis-free survival. In 993 primary breast carcinomas, a simple summary signature was expressed at lower levels in luminal B tumors and in endocrine treated patients, high expression of the MV score displayed a trend towards a more favorable outcome in 2 out of 3 datasets and a similar finding was noted in a set of 186 patients subjected to tamoxifen monotherapy. Multivariate and subgroup analysis revealed that this association was only present in a subgroup of tumors characterized by low genomic grade. Additionally, a significant increase in signature score was found in 7 metastatic breast tumors after 14 days treatment with sunitinib + docetaxel, an increase that was not present in tumors treated with docetaxel alone.

Whilst others have also produced microvascular gene expression signatures in a breast cancer setting, these signatures have generally been designed to capture the transcriptional differences between microdissected normal vs. tumor microvasculature. In short, these signatures likely represent a tumor endothelium that is increasingly thought of as angiogenically active and chronically inflamed (36). This

is in contrast to our signature score that is derived from a physiologically normal microvasculature and as such is highly expressed in low-risk tumors. These differences are further emphasized when comparing the overlap of our module genes with other published angiogenesis signatures: number of genes from our module present in Bhati *et al.* signature, 1 out of 48; in Masiero *et al.* signature 13/43; Pepin *et al.* 0/494; and Mannelqvist *et al.* 0/18 (data not shown) (37–40). Of note, two recently published endothelial metagenes do display a greater degree of overlap with our signature, those being “signature 4” and “signature 5” from Winslow *et al.* (41) (overlap = 4/9 and 2/3 respectively). Interestingly these metagenes were derived through correlation analysis to a core set of genes enriched in tumor stromal compartments. Related to this, we also characterised the MV score in the context of other published signatures and gene expression modules (42) and found that that the MV score (Supplemental Figure S3 A-D, MV_SIG, red arrows) is strongly inversely correlated to the AURKA proliferation-related gene module when considering all patients (Supplemental Figure S3 A-D, AURKA, blue arrows). Of note, whilst we also see moderate correlations to the GGI, PLAU and Stomal modules in all patients, these become weakly correlated or not statistically significant in the endocrine treated, low genomic grade subgroup (data not shown). A second link between our signature and proliferation was in evidence when we examined the MV score within the PAM50 molecular subgroups. Here, the MV score was consistently lower in the luminal B tumors of all four tested datasets relative to luminal A tumors. It has previously been demonstrated that the one of the main factors distinguishing these two tumors groups is level of proliferation with higher levels found in luminal B tumors (43). However, given that our signature retains prognostic significance in the lowly proliferative GG1 tumor subgroup (Supplemental Table S4), it is reasonable to

state that the prognostic capacity of signature extends beyond that of a simple proliferative marker.

Given the vast morphological differences between the endothelium lining the cardiovascular/ lymphatic systems and tumor endothelium, disparities in the quantity and type of genes expressed are to be expected. The tumor endothelium is characterized by atypical cell morphology, bloodflow that can range from chaotic to non-existent (44), and intracellular gaps that leak fluids and blood into the surrounding tissue (45). Taken together, these hallmarks of endothelial dysfunction not only influence gene expression patterns but also make pathological assessments of microvascular density challenging. Indeed, spotty CD31 staining has been highlighted in the tumor endothelium *in vivo*, owing to a lack of expression in some cells and an absence of cells entirely in some areas of the vessel wall (46). This is likely one of the reasons as to why we found no overt similarity when comparing the MV score to microscopic assessment of MVD with CD31 staining. Staining issues notwithstanding, the value of MVD assessment as a prognostic marker has, on the whole, been called into question. In a systematic review of microvascular density and outcome, Uzzan and colleagues reported risk ratios in the range of 1.5-2, concluding that MVD has significant but weak prognostic capacity in breast cancer, and that standardization of MVD assessment is needed (47). Similar weak/negative findings were recently reported by Cheng *et al.* in a recent renal cell carcinoma meta-analysis of MVD (48).

This study had some limitations, the foremost of those being that this is a retrospective study performed in multiple patient cohorts (rather than a single, large, homogeneously-treated cohort) and that the patient numbers in the metastatic cohort are low ($N= 14$), as expected from a feasibility study. Furthermore not all analyses

were pre-specified, our initial aim was to characterize the MV score in terms of its relationship to traditional MVD and to standard breast cancer clinico-pathological parameters in the context of treatment subgroups and DMFS. The subgroup analysis splitting patients into GG1 and GG3 as well as the examination of change in signature score before and after treatment in the metastatic cohort were exploratory in nature.

In summary, we report a microvascular gene signature score representative of a normal endothelium that reproducibly describes differential expression between Luminal A and B molecular subtypes, and identifies a subgroup of endocrine treated patients with worse outcome. Moreover, we show the first evidence of normalization of tumour vasculature on a transcriptional level in response to an angiogenic inhibitor in human metastatic breast cancer samples. In light of these findings, evaluation of transcriptional changes in microvascular genes alongside assessment of MVD and angiogenic factors in clinical trials of antiangiogenic compounds appears warranted.

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Table 1. Gene Ontology terms associated with MV score and CD31 correlated genes

Top 10 gene ontology categories overrepresented in 200 MV score-correlated genes.

Term	Gene Ontology ID	P value	Q-value	Odds Ratio	Expected Count	Count	Category size
Cardiovascular system development	GO:0072358	6.1E-15	3.7E-12	6.7	7.2	34	739
Blood vessel development	GO:0001568	3.0E-13	9.2E-11	7.5	4.6	26	471
Multicellular organismal process	GO:0032501	1.5E-09	3.0E-07	3.4	47.2	78	4836
Endothelium development	GO:0003158	1.7E-08	2.5E-06	16.8	0.7	9	68
Ameboidal cell migration	GO:0001667	2.1E-08	2.5E-06	7.9	2.1	14	216
Epithelium migration	GO:0090132	2.5E-07	2.5E-05	8.7	1.5	11	152
Lymph vessel development	GO:0001945	6.2E-07	5.4E-05	40.9	0.2	5	18
Angiogenesis	GO:0001525	1.2E-06	9.1E-05	7.4	1.7	11	196
Cell adhesion	GO:0007155	1.6E-06	1.1E-04	3.5	8.3	24	849
Positive regulation of locomotion	GO:0040017	1.9E-06	1.1E-04	5.8	2.6	13	267

Top 10 gene ontology categories overrepresented in 200 CD31 correlated genes.

Term	Gene Ontology ID	P value	Q-value	Odds Ratio	Expected Count	Count	Category Size
Response to type I interferon	GO:0034340	5.8E-19	2.7E-16	33.9	0.8	17	74
Type I interferon signaling pathway	GO:0060337	5.8E-19	2.7E-16	33.9	0.8	17	74
Interferon-gamma-mediated signaling pathway	GO:0060333	1.0E-13	3.3E-11	26.3	0.7	13	67
Defense response to virus	GO:0051607	9.5E-13	2.3E-10	12.7	1.7	17	168
Antigen processing and presentation of endogenous antigen	GO:0019883	3.0E-12	5.8E-10	180.9	0.1	7	11
Response to other organism	GO:0051707	6.5E-12	1.0E-09	6.2	5.7	27	556
Response to biotic stimulus	GO:0009607	1.4E-11	1.9E-09	6.0	5.9	27	575
Response to interferon-gamma	GO:0034341	1.7E-11	2.0E-09	16.6	1.0	13	98
Positive regulation of immune response	GO:0050778	2.6E-11	2.7E-09	6.8	4.3	23	418
Defense response	GO:0006952	3.2E-11	3.0E-09	5.1	8.7	32	943

Table 2. Mean MV score in relation to clinico-pathological parameters in four independent datasets

Variable	Uppsala (N=253)			Stockholm (N=159)			NKI (N=295)			Rotterdam (N=286)		
	Mean MV score	±SD	p value	Mean MV score	±SD	p value	Mean MV score	±SD	p value	Mean MV score	±SD	p value
ER												
Positive	48.1	16.8		35.8	15.9		58.6	13.4		51.8	17.6	
Negative	43.3	18.0	0.128	33.6	13.7	0.482	62.5	14.5	0.040	55.6	15.1	0.094
PR							-	-		-	-	
Positive	47.9	17.2		35.1	15.1		-	-		-	-	
Negative	43.5	14.5	0.238	36.1	16.7	0.722	-	-	-	-	-	-
Elston-Ellis grade												
I*	53.2	15.7	-	37.0	12.7	-	60.6	14.2	-	58.4	14.6	-
II	47.8	17.6	0.071	39.9	17.8	0.682	60.2	12.3	0.983	58.7	16.6	0.999
III	39.7	13.8	<0.001	30.9	12.8	0.179	58.2	14.6	0.471	51.6	16.3	0.527
Nodal status												
Negative	49.8	16.9		35.7	15.7		59.3	14.3		-	-	
Positive	43.5	16.2	0.005	35.5	15.8	0.952	59.8	13.2	0.752	-	-	-
Tumor size												
≤20 mm	51.7	17.0		37.0	15.0		59.7	14.1		-	-	
>20 mm	43.1	15.8	<0.001	33.6	15.9	0.175	59.4	13.3	0.830	-	-	-
Age												
≤50	41.3	13.5		32.8	14.6		59.6	13.5		51.9	18.6	
>50	49.2	17.4	0.002	36.7	15.8	0.139	58.8	15.6	0.758	53.5	15.6	0.439
Chemotherapy												
Yes	42.2	12.4		31.7	16.5		60.2	12.5		-	-	
No	48.3	17.0	0.087	36.3	15.2	0.146	59.1	14.4	0.495	-	-	-
Endocrine therapy												
Yes	46.0	17.4		36.2	16.7		59.9	13.5		-	-	
No	48.6	16.3	0.252	33.5	11.9	0.331	59.5	13.8	0.839	-	-	-
PAM50												
Luminal A*	52.5	14.5	-	36.9	12.0	-	60.3	11.2	-	55.7	16.1	-
Luminal B	39.7	14.2	<0.001	26.8	11.2	0.008	53.5	13.9	0.016	45.1	16.6	<0.001
HER2-enriched	42.3	13.1	0.004	32.0	9.5	0.662	62.2	12.1	0.918	52.3	12.9	0.808
Basal-like	42.0	18.7	0.007	35.4	20.4	0.992	62.2	15.8	0.987	52.6	17.2	0.776
Normal-like	57.9	17.5	0.345	49.6	15.1	0.003	61.4	15.9	0.957	65.9	16.7	0.047

SD= Standard deviation; p value calculated using Student's t-test unless otherwise stated

*Reference group, p value based on ANOVA with post-hoc Tukey analysis

Table 3. Multivariable analysis of prognostic markers in the pooled endocrine-treated patients of the Uppsala (N= 80), Stockholm (N= 114), Oxford (N= 99) and Guys (N= 87) cohorts, N= 380 in total

	<u>Histologic Grade (N=324*)</u>			<u>Genomic Grade (N =366*)</u>		
	HR	95% CI	P value	HR	95% CI	P value
Age [§]	1.67	0.76 – 3.70	0.200	1.43	0.69 – 2.99	0.340
Size [‡]	2.30	1.39 – 3.81	0.001	2.09	1.33 – 3.30	0.002
Nodal Status [†]	1.30	0.81 – 2.07	0.270	1.44	0.94 – 2.22	0.090
Histologic grade [¶]	G1	ref	-	ref	-	-
	G2	4.11	1.63 – 10.34	0.003	-	-
	G3	3.38	1.26 – 9.08	0.016	-	-
Genomic Grade [#]	-	-		1.62	1.29 – 2.04	<0.001
MV score (continuous)	0.70	0.50 – 0.97	0.030	0.89	0.65 – 1.21	0.450

* Reduced number of patients, missing cases shown below

Patient numbers in each group (total N= 380):

§Age ≤ 50 years (N= 51) vs. Age > 50 years (N= 329)

‡Size ≤ 20 mm (N = 177) vs. Size > 20 mm (N= 201), missing (N= 2)

†Nodal status, negative (N= 177) vs. positive (N= 191), missing (N= 12)

¶Histological grade, G1 (N = 71) vs. G2 (N = 179) vs G3 (N = 86), missing (N= 44)

#Genomic grade, GG1 (N = 224) vs. GG3 (N = 156)

Distant metastasis-free survival, hazard ratio per 25 increments in MV score

HR= hazard ratio; CI= confidence interval; ref= reference category

In bold: significant P value of < 0.05

Figure legends

Figure 1. Distant metastasis-free survival, MV score and systemic breast cancer treatment. Hazard ratios (HR; Cox proportional hazards regression) are given per 25 increments in MV score expression, for (N) patients in the respective stratum.

Figure 2. Kaplan-Meier survival curves for distant metastasis-free survival in endocrine-treated patients (N = 380). Patients from the Uppsala and Stockholm data sets (A and D); the Guy's and Oxford data sets (B and E); all data sets (C and F). Patients stratified by genomic grade (low genomic grade, GG1; A-C and high genomic grade, GG3; D-F). *P*-values given for a log-rank test of microvascular signature score \geq 39 (MV score high, blue curves) vs. < 39 (MV score low, yellow curves).

Figure 3. Changes in MV signature score gene expression after 14 days treatment with antiangiogenic therapy. (N = 14) The change in MV signature score gene expression before and after 14 days treatment with sunitinib plus docetaxel or docetaxel alone was determined in the metastatic breast tumors of 14 patients. (A) Heatmap showing the intra-patient changes in MV signature score gene expression. Columns represent the difference in signature gene expression for individual patients before and after treatment and rows represent signature genes. Red and green bars highlight different treatment arms; sunitinib plus docetaxel or docetaxel alone, respectively. (B) Boxplots showing MV score at baseline (before treatment) and after 14 days treatment, split by clinical trial treatment arm. Left panel: docetaxel alone arm (n=7), *P* = 0.706 vs. baseline. Right panel: sunitinib plus docetaxel arm, (n=7), *P* = 0.031 vs. baseline. Markers represent individual patients matched across Baseline and

Day 14 boxplots within each treatment arm (e.g. the circle in the Baseline boxplot of the DOC arm is the same patient as the circle in the Day 14 boxplot of the DOC arm).

FIGURE 1

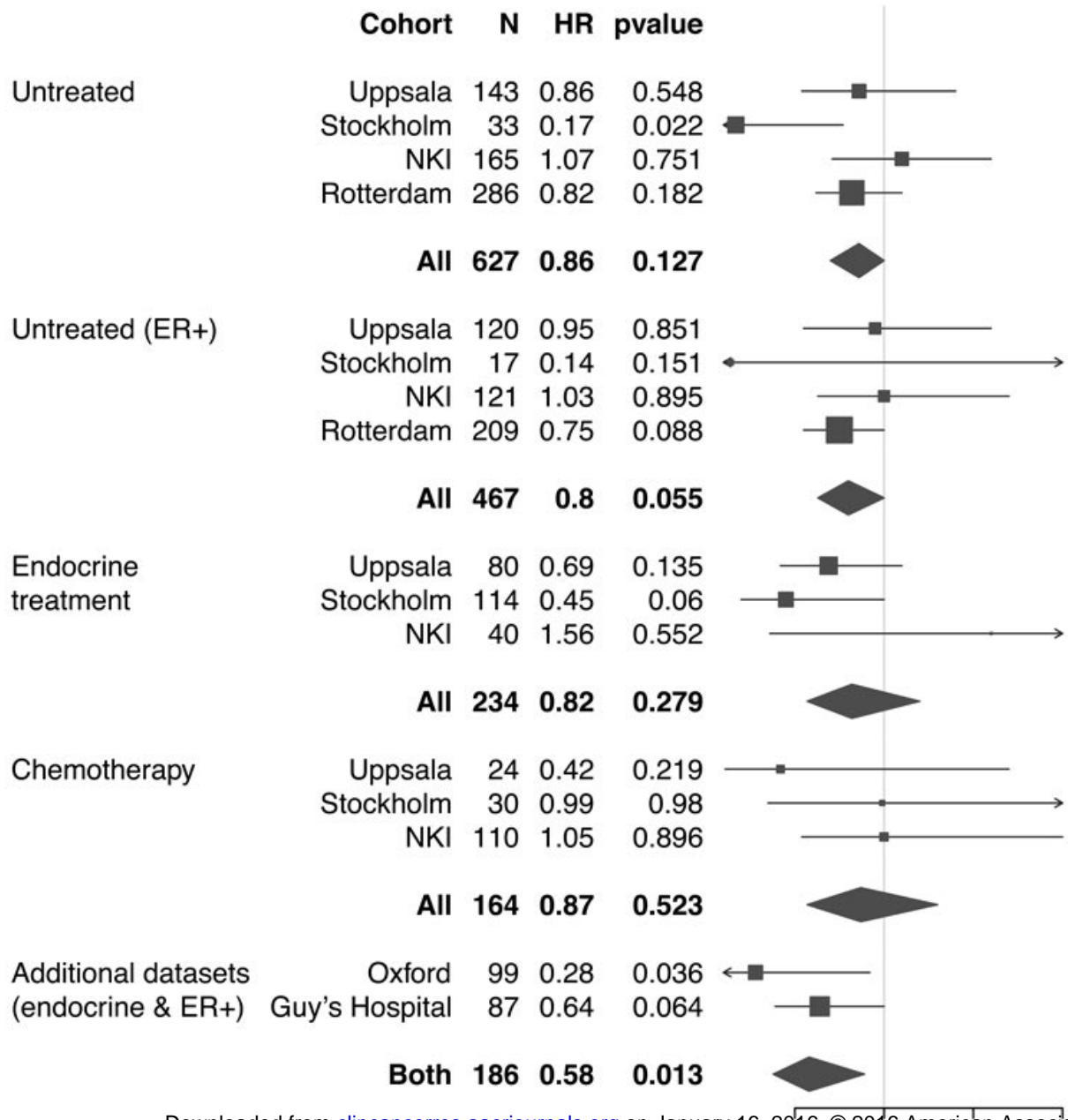


FIGURE 2

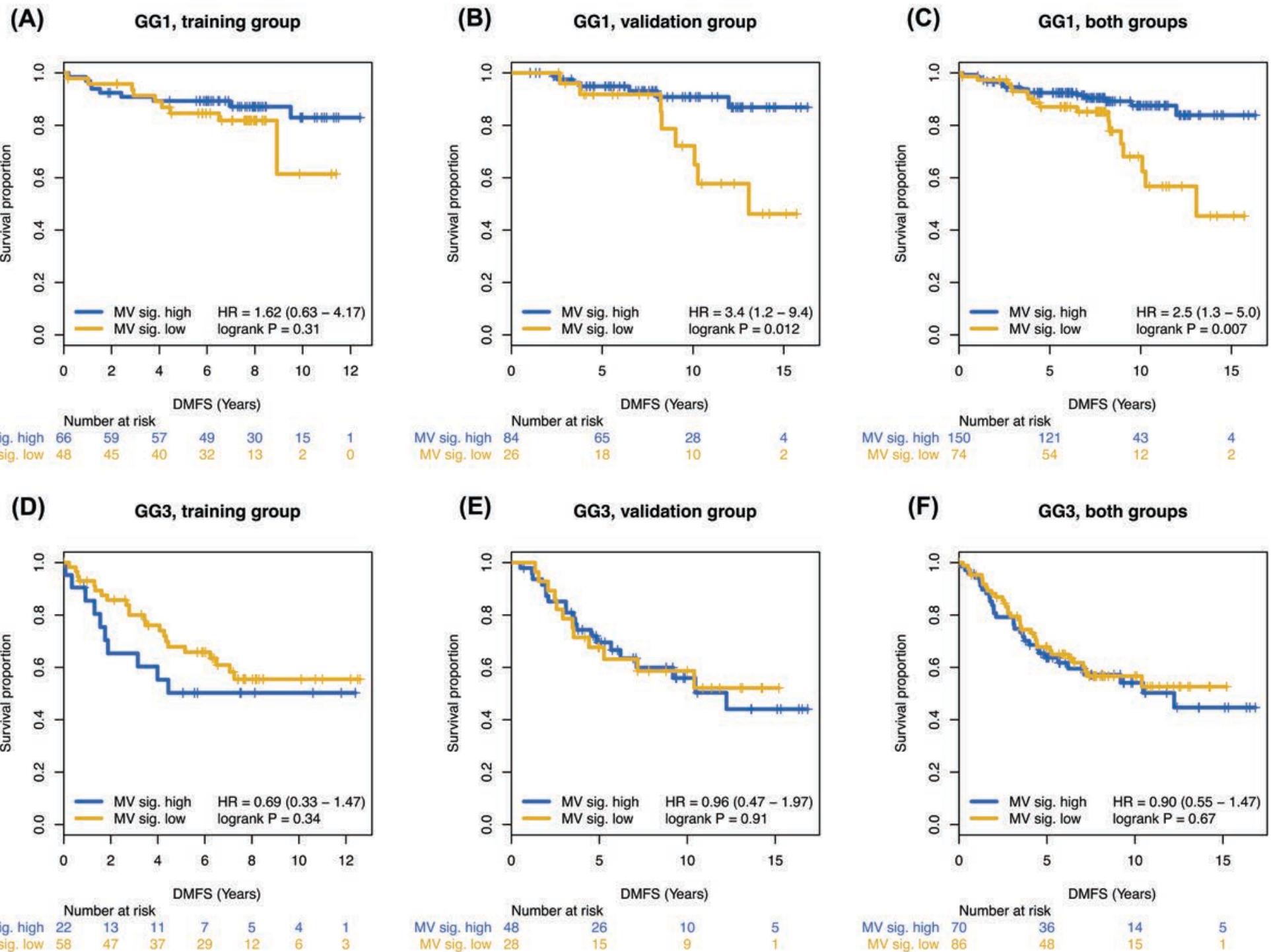
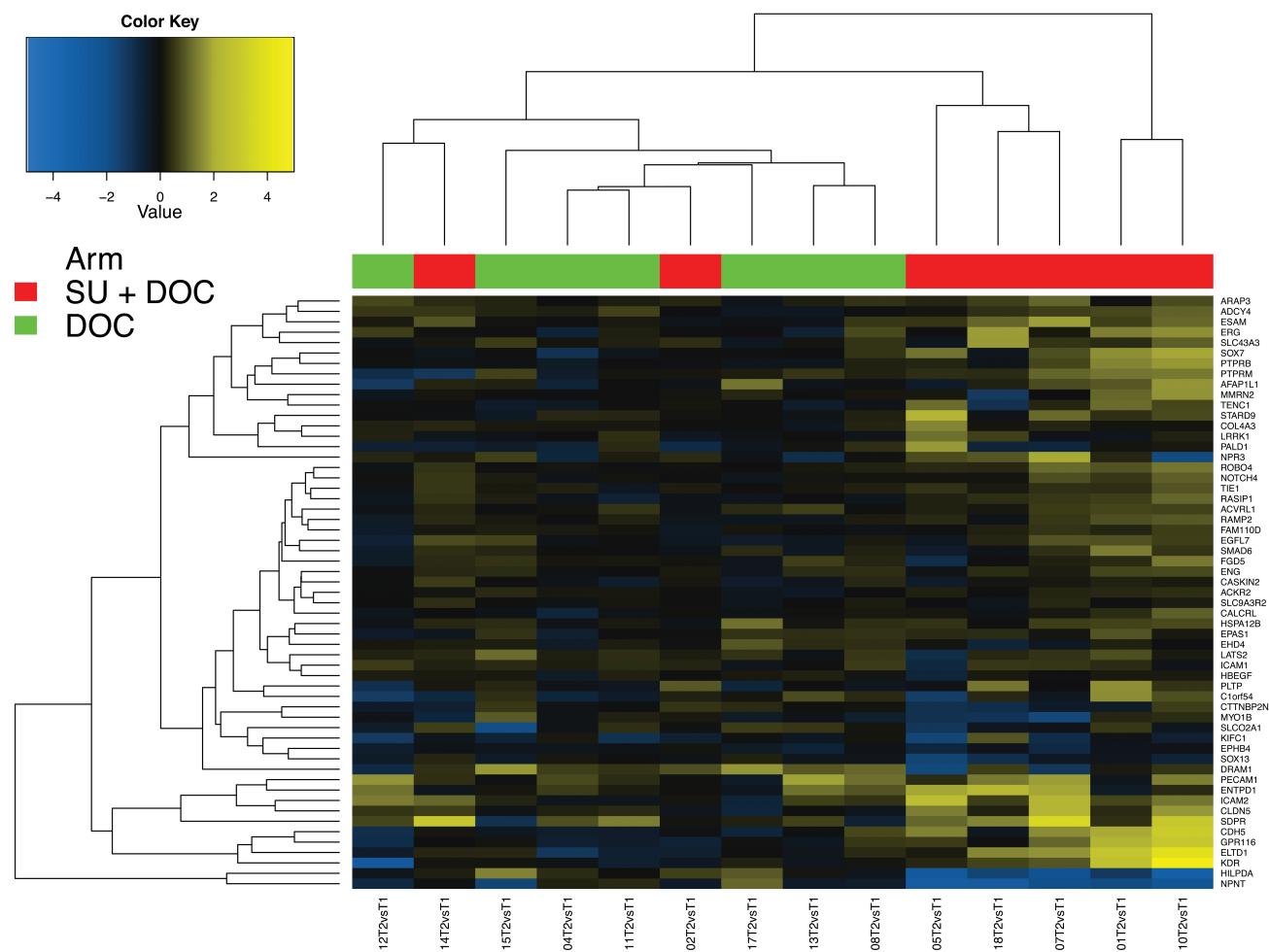


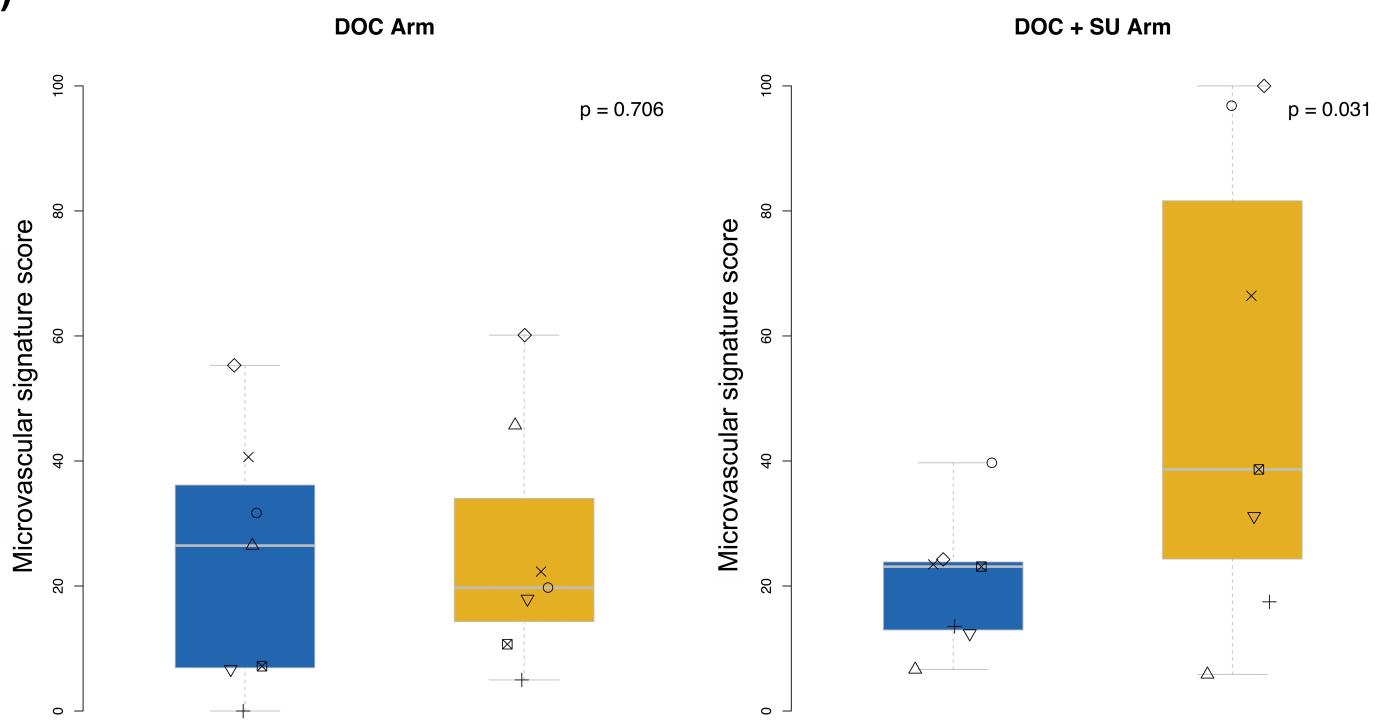
FIGURE 3

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 Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

(A)



(B)



Clinical Cancer Research

An endothelial gene signature score predicts poor outcome in patients with endocrine treated, low genomic grade breast tumors

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