The longitudinal transcriptional response to neoadjuvant chemotherapy with and without bevacizumab in breast cancer

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Abstract

Purpose: Chemotherapy induced alterations to gene expression are due to transcriptional reprogramming of tumor cells or subclonal adaptations to treatment. The effect on whole-transcriptome mRNA expression was investigated in a randomized phase II clinical trial to assess the effect of neoadjuvant chemotherapy with the addition of bevacizumab.

Experimental Design: Tumor biopsies and whole-transcriptome mRNA profiles were obtained at three fixed time points with 66 patients in each arm. Altogether, 358 specimens from 132 patients were available, representing the transcriptional state before treatment start, at 12 weeks and after treatment (25 weeks). Pathological complete response (pCR) in breast and axillary nodes was the primary endpoint.

Results: Pathological complete response (pCR) was observed in 15 patients (23%) receiving bevacizumab and chemotherapy and 8 patients (12%) receiving only chemotherapy. In the estrogen receptor positive patients, 11 out of 54 (20%) treated with bevacizumab and chemotherapy achieved pCR, while only 3 out of 57 (5%) treated with chemotherapy reached pCR. In patients with estrogen receptor positive tumors treated with combination therapy, an elevated immune activity was associated with good response. Proliferation was reduced after treatment in both treatment arms and most pronounced in the combination therapy arm, where the reduction in proliferation accelerated during treatment. Transcriptional alterations during therapy were subtype specific, and the effect of adding bevacizumab was most evident for Luminal B tumors.

Conclusion: Clinical response and gene expression response differed between patients receiving combination therapy and chemotherapy alone. The results may guide identification of patients likely to benefit from anti-angiogenic therapy.

Translational Relevance

Neoadjuvant chemotherapy in combination with bevacizumab in breast cancer increases the number of patients achieving pathological complete response. Some studies have demonstrated benefit for hormone receptor positive tumors, other in triple negative tumors. In this trial, tumor sampling before, after 12 weeks and at the completion of the treatment enabled a detailed longitudinal characterization of the gene expression in the individual tumors. The high expression of immune related genes in tumors responding to antiangiogenic therapy, clearly indicate that host immune factors is of importance for treatment response. The results indicate that the proliferative estrogen receptor positive tumors (luminal B like tumors) are most influenced by bevacizumab in combination with chemotherapy. The results emphasize the need for molecular analyses in clinical trials. In this study, the gene expression characteristics may be important for prediction of treatment effect and should be further evaluated for possible clinical use.

Introduction

Vascular Endothelial Growth Factor (VEGF) stimulates angiogenesis by influencing vessel formation through regulation of proliferation, migration and survival of endothelial cells (1-3). Blocking VEGF by bevacizumab, a monoclonal antibody, has been proposed to cause inhibition of neovascularization, regression of existing immature micro-vessels, and normalization of abnormal vasculature (4,5); this has consequences for the flux of oxygen, nutrients, metabolites and therapeutic agents, ultimately preventing tumor growth and resulting in tumor shrinkage. However, addition of bevacizumab to standard chemotherapy in unselected breast cancer patients has resulted only in a modest increase in response rate and progression free survival (6-11). A more in-depth exploration of the effects of angiogenesis blocking agents is important for future efforts to improve patient survival. The effect on the molecular level of bevacizumab administered in combination with chemotherapy has been less studied, and there is a lack of biomarkers for prediction of tumor responsiveness to bevacizumab (12). Breast cancer is a heterogeneous disease with at least five subtypes according to the PAM50 classification, each with distinct biology and clinical outcome (13-15). The frequency and prognostic impact of pathological complete response are known to vary between PAM50 subtypes (16-19). Subtype-specific responses to bevacizumab have not yet been systematically studied. Stratification of patients by molecular subtype may be important to identify patient subpopulations that will benefit from such treatment.

In this study, the effect of bevacizumab in HER2-negative breast carcinomas treated with a neoadjuvant chemotherapy regimen is investigated by comparing gene expression profiles of responding and non-responding tumors. Tumors were also compared before,

during and after treatment in a subtype- and treatment-specific manner, to reveal molecular changes influenced by the therapy.

Patients and methods

Study design

Patients were recruited at two sites in Norway (Oslo University Hospital, Oslo and St. Olav's Hospital, Trondheim), between November 2008 and July 2012. Written informed consents were obtained from all patients prior to inclusion. The study was approved by the institutional protocol review board, the regional ethics committee, the Norwegian Medicines Agency and carried out in accordance with the Declaration of Helsinki, International Conference on Harmony/Good Clinical practice. The study is registered in the http://www.ClinicalTrials.gov/ database with the identifier NCT00773695.

Patients with HER2-negative mammary carcinomas with size ≥ 2.5 cm previously untreated for breast cancer were eligible. Other key inclusion criteria were WHO performance status ≤ 2, adequate hematological and biochemical parameters, and no sign of metastatic disease. Additional prerequisites were normal organ function in general and normal left ventricular ejection fraction. Concomitant medications with anticoagulants, other than low dose acetylsalicylic acid (160 mg or lower) were not allowed. A block randomization procedure was used, and the randomization was performed by the centralized research support facility at Oslo University Hospital. The randomization list was not known to the personnel responsible for providing information or treatment to the patients. The patients were stratified based on their tumor size ($2.5 \leq T \leq 5$ cm, $T > 5$ cm) and hormone receptor status (positive for estrogen (ER), progesterone, or both), and randomized 1:1 to receive bevacizumab and chemotherapy (combination-therapy arm) or chemotherapy alone (chemotherapy arm).

Of the 150 patients enrolled, 138 were assigned to treatment with chemotherapy, and 12 (independently randomized; not reported herein) received endocrine therapy as determined by the responsible oncologist. Of the 138 patients treated with chemotherapy, 66 in each group were included in the primary efficacy analysis (**Figure 1**).

Treatment

The chemotherapy regimen consisted of 4 cycles of FEC100 (5-fluorouracil 600 mg/m2, epirubicin 100 mg/m2 and cyclophosphamide 600 mg/m2) every 3 weeks, followed by docetaxel 100 mg/m2 every 3 weeks or 12 weekly infusions of paclitaxel 80 mg/m2. Bevacizumab was administered intravenously at a dose of 15 mg/kg every third week or 10 mg/kg every other week in patients receiving docetaxel or paclitaxel, respectively.

Tumor evaluation, sampling and assessment of response

Hematological parameters were evaluated before each chemotherapy administration, whereas biochemical parameters were evaluated every third week. The local pathologist, blinded to the treatment assignment, performed histopathological examination of the breast using study-specific guidelines.

Samples were sequentially collected before treatment (core needle biopsies; termed '*week 0* samples'; n = 132), during treatment (core needle biopsies 12 weeks into treatment and minimum three weeks after the last FEC dose; termed '*week 12* samples'; n = 115) and after treatment (at surgery, minimum three weeks after the last taxane dose; termed '*week 25* samples'; n = 112). Altogether 358 specimens were available for further analyses. Matched tumor samples from all three time points were available from 96 patients.

Pathological complete response (pCR), herein defined as complete eradication of all invasive cancer cells in both breast and axillary lymph nodes, was the primary end point.

Gene expression profiling

Gene expression profiling was performed using 40ng total RNA and one color Sureprint G3 Human GE 8x60k Microarrays (Agilent Technologies) following the manufacturer's protocol (details in Data Supplement). Gene expression profiles were successfully obtained for 358 samples; 131 *week 0* samples, 115 *week 12* samples and 112 *week 25* samples. Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4439.

The PAM50 subtyping algorithm developed by Parker *et al.* 2009 (14) was used to assign a subtype label to each sample (details in data supplement). A proliferation score was derived for each sample by computing mean expression values of 11 proliferation related PAM50 genes: *CCNB1, UBE2C, BIRC5, KNTC2, CDC20, PTTG1, RRM2, MKI67, TYMS, CEP55,* and *CDCA1*[14]. *TP53* mutation status was determined by sequencing the entire coding region (exons 2–11), including splice junctions (details in data supplement).

Statistics

All statistical analyses were performed in R version 3.0.3 (20). Associations between variables were assessed with Fisher's exact tests, t-tests and Kruskal–Wallis tests as appropriate. Significance Analysis of Microarrays (SAM) was used to find differentially expressed genes (DEGs) between two groups using the R package *samr* (21,22) and reporting genes called significant at False Discovery Rate (FDR) 5%. Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used for enrichment analysis on the DEGs. A measure of KEGG pathways activity was obtained using the R package *qusage* (23).

Results

Patient and tumor characteristics

The study overview is presented in **Figure 1**. Demographic characteristics were balanced between the treatment arms (**Table 1**). No significant skewness in distribution of important clinical and molecular parameters such as tumor size, grade, lymph node status, hormone receptor status, *TP53* mutation status or PAM50 subtypes (Fisher's exact test and χ^2-test) was observed; neither were any genes differentially expressed in the pre-treatment biopsies between the treatment arms (two-class unpaired SAM).

Febrile neutropenia, proteinuria and hypertension were the most frequent adverse events observed in both treatment arms (**Table 2**). In the combination-therapy arm, significantly higher frequencies of bleeding disorders and hypertension were observed (Fisher's Exact test; $P < 0.001$). Serious adverse events, mainly febrile neutropenia and infection were also significantly more frequent in this arm (Fisher's Exact test; $P < 0.001$ and $P < 0.05$, respectively). One death occurred after 12 weeks in the combination-therapy arm, but despite autopsy, a specific cause could not be established.

Complete responders in the combination-therapy arm have elevated expression of genes involved in immune related processes

A total of 23 of the 132 patients (17%) achieved pCR. The overall pCR rates were significantly higher in ER-negative *vs.* ER-positive tumors, in *TP53*-mutated *vs. TP53*-wildtype tumors, and in Basal-like subtype *vs.* other PAM50 subtypes (Fisher's Exact test and χ^2-test as appropriate; $P < 0.01$). The frequency of pCR was 23% (n = 15) in the combination-therapy arm compared to 12% (n = 8) in the chemotherapy arm (Fisher's Exact test, $P = 0.17$). For ER-positive tumors, bevacizumab-treated patients had a pCR rate of 20% (n = 11), whereas only 5% (n = 3) achieved pCR in the chemotherapy alone

group (Fisher's Exact test, P = 0.02). Patients with ER-negative tumors did not seem to benefit from addition of bevacizumab to chemotherapy in this patient cohort (**Figure 2**).

Comparing tumor gene expression profiles before treatment in the combination-therapy arm in patients that achieved pCR (n = 15) to those that did not (n = 51) identified 720 differentially expressed genes (DEGs; two-class unpaired SAM; **Table S1**). Functional annotation analyses of these genes using DAVID (24) showed that the gene list included known cell surface antigens (eg. *CD3D, CD8B, CD38*), chemokines (eg. *CCL5, CXCL9, CXCL10*), interleukins and interleukin receptors (eg. *IL12B, IL18, IL21*), toll-like receptors (eg. *TLR1, TLR2, TLR6*), and tumor necrosis factors (eg. *TNF, TNFRSF13B, TNFRSF21*). The gene list was significantly enriched for genes involved in immune response related processes and included Gene Ontology (GO) terms such as leucocyte cell-cell adhesion, regulation of lymphocytes, T-cell activation and proliferation and positive regulation of lymphocytic proliferation (**Table S2**).

Similar analyses in the chemotherapy only arm identified 1243 DEGs between the complete responders (n = 8) and the non-complete responders (n = 57) (two-class unpaired SAM; **Table S3**). The gene-list was modestly enriched for genes involved in cell-cycle related processes and included GO terms such as chromatin, nucleus and chromosomal part, cell division and nuclear chromosome segregation (DAVID; **Table S4**). A total of 173 DEGs (24% of DEGs in the combination-therapy arm) between the complete responders and non-complete responders overlapped among the two treatment arms, and were enriched for genes involved in DNA damage and repair, and cell-cycle processes.

The gene expression of the DEGs was further correlated with the continuous response ratio (ratio of tumor size at start of treatment to size at surgery). In the ER positive

subset of the combination-therapy arm, 345 out of 720 DEGs remained after correction for multiple testing (Spearman's correlation test, FDR <0.05); the gene-list was highly enriched for genes involved in immune processes. In the ER positive subset of the chemotherapy arm, only one out of 1243 DEGs remained significant after multiple testing correction (FDR < 0.05).

Tumors treated with bevacizumab exhibit accelerated reduction in proliferation score

We next evaluated whether gene expression profiles were differently affected by the two treatment regimes. The *week 12* samples in the combination-therapy arm showed significant lower expression of 42 genes compared to the chemotherapy arm (two-class unpaired SAM; **Table S5**). The gene list was highly enriched for genes involved in cell-cycle related and cellular components maintaining pathways (DAVID; **Table S6**). Thus, at *week 12*, cell-cycle related processes were significantly more suppressed in the combination-therapy arm. An overall decrease in PAM50 proliferation score (14) was observed in both treatment arms over time (Kruskal-Wallis test, $p < 0.001$; **Figure S1**). Notably at *week 12*, the proliferation score was significantly lower in the combination-therapy arm compared to the chemotherapy arm (Kruskal-Wallis test, $p = 0.006$; **Figure 3A**). Stratification by ER-status showed a similar, but more pronounced down-regulation of cell-cycle genes at *week 12* in the ER-positive subset of the combination-therapy arm, with correspondingly larger difference in proliferation score between treatment arms (Kruskal-Wallis test, $P = 0.001$). In the ER negative subset, no significant differences in gene expression profiles were detected between the treatment arms at any time point.

Following treatment, higher proportion of samples were categorized as Luminal-A and Normal-like, indicating a shift towards PAM50 expression profiles associated with better prognosis in response to treatment (**Figure 3B and 3C**).

Changes in gene expression in response to treatment is subtype-specific

Net changes in gene expression and pathway activities in response to anthracycline +/- bevacizumab (from *week 0* to *week 12*) and taxane +/- bevacizumab (from *week 12* to *week 25*) were assessed for matched tumor pairs from individual patients. Analyses were performed separately for the PAM50 subtypes to uncover subtype-specific changes in response to treatment. HER2-enriched and Normal-like subgroups were excluded due to limited numbers.

Changes in response to anthracycline +/- antiangiogenic therapy (Week 0 – Week 12);

In the combination-therapy arm, 2524, 9053, and 4248 DEGs were identified between *week 0* and *week 12* samples in the Basal-like, Luminal-B and Luminal-A subtypes, respectively (**Figure 4; Table S7, S8 and S9**). In the chemotherapy arm, 4210, 2346 and 1082 genes showed differential expression in Basal-like, Luminal-B and Luminal-A subgroups respectively, with an overlap of 57%, 22% and 20% with the corresponding gene lists in the combination-therapy arm (**Figure 4A and Table S10; Figure 4B and Table S11; Figure 4C and Table S12**).

Pathway activity analyses performed using 186 pathways in the KEGG database revealed that corresponding to the highest numbers of genes affected in Luminal-B tumors, several pathways showed significantly lower activity in the combination arm compared to the chemotherapy arm. Pathways involved in DNA replication and repair,

cell cycle, RNA degradation, purine and pyrimidine metabolism were among the top hits. (**Figure 4D; Table S13**). For Luminal-A and Basal-like subgroups, fewer pathways showed significantly differential activity between the two treatment arms.

Changes in response to taxane +/- antiangiogenic therapy; (Week 12 – Week 25);

Changes in gene expression were less substantial from *week 12* to *week 25* and involved fewer DEGs compared to changes from *week 0* to *week 12*. In the combination arm, one, seven and 2241 DEGs were identified in the Basal-like, Luminal-B and Luminal-A subtypes, respectively; the corresponding numbers in the chemotherapy arm were none, 439 and 2545 (**Figure 4A-4C**).

Interestingly, for the Luminal-A subgroup in the chemotherapy arm, the number of DEGs after taxane treatment (between *week 12* and *week 25* tumors*)* greatly exceeded the corresponding number after anthracycline treatment (between *week 0* and *week 12* tumors) (n = 2545 *vs.* n = 1082; **Figure 4C**). A subsequent pathway activity analysis showed significant differences in pathway activity between the two treatment regimens for around 100 pathways, including retinol metabolism, PPAR signaling, ribosome and spliceosome synthesis, cell-cycle and DNA repair pathways (**Figure S2; Table S14**).

Corresponding to few/none DEGs in Basal-like and Luminal-B subgroup after taxane treatment, no significant differences in pathway activities were detected between *week 12* and *week 25* samples.

Discussion

In this study, transcriptional response to treatment with standard chemotherapy, with and without bevacizumab was studied in serial biopsies from 132 patients. More patients achieved pCR in the combination-therapy arm compared to the chemotherapy alone arm, in line with reports from previous studies (6,7,10,11). A significant benefit of adding bevacizumab was observed in the ER-positive subgroup consistent with the larger NSAPB-B40 trial (11), in contradiction to the GeparQuinto trial, where the ER-negative tumors were found to benefit from bevacizumab (10), and the CALGB40603 study, including patients with a low ER expression, or triple negative tumors (6). Different chemotherapy and bevacizumab regimens, pCR definitions and threshold to define ER-positive/negative tumors make cross-study comparisons difficult. However, our results indicate possibilities for use of gene expression profiling for selection of patients who might benefit from bevacizumab.

Interestingly, immune response related pathways were significantly up-regulated in the complete responders in the combination-therapy arm. This is the first unsupervised study to demonstrate a link between activated immune response pathways and pCR under treatment with bevacizumab. Previously, supervised studies have shown association between immune gene modules and tumor infiltrating lymphocytes with standard neoadjuvant treatment response (25-30). Mechanisms such as promotion of immunogenic tumor cell death (31) by stimulating tumor antigen release, stimulation of dendritic cell maturation, proliferation of tumor-specific $CD8^+$ cells, and sensitizing tumor cells to $CD8^+$ T cell-mediated apoptosis (32-34) are suggested. The prominent association of activated immune response pathways and response in the combination-therapy arm indicates that the immunogenic cell death promoted by immune

modulatory effects of chemotherapy may have been enhanced by addition of bevacizumab, ultimately resulting in a higher incidence of pCR in tumors receiving combination-therapy. Verification of this in independent cohorts is necessary to understand if this can be clinically useful for selection of patients likely to benefit from such therapy. The association with activated immune response pathways is particularly interesting, in relation to the growing interest for testing immunological check point inhibiton in breast cancer (35). If the responder population identified in our study has common features with responding patients treated with CTLA-4 or PD-1 check point inhibitors, this should be investigated further.

Overall pCR rates were higher in tumors with unfavorable prognostic features such as ER-negativity, presence of TP53 mutations and Basal-like gene expression profile. Since ER-negative tumors tend to more often be TP53-mutated and Basal-like, it is challenging to evaluate the individual predictive power of these features. Differential response to preoperative chemotherapy in subtypes of breast cancer has been repeatedly reported (10,18,36,37). High efficacy of cytotoxic agents resulting in death and elimination of highly proliferating tumor cell population is likely the reason behind the major shift in transcriptional programming observed in response to treatment; it potentially explains the overall shift in molecular profiles towards Normal-like/Luminal-A subtypes with low proliferating phenotypes as well as higher pCR rates in tumors with proliferating phenotype (14,15).

Following treatment, the proliferation scores were reduced, likely corresponding to elimination of the rapidly proliferating tumor cells. Importantly, the magnitude of reduction in proliferation scores in the combination-therapy arm was significantly higher compared to those in the chemotherapy arm. This accelerated reduction of

proliferation scores also indicates that bevacizumab enhances the effects of chemotherapy. It is, however, difficult to infer whether this enhancement is a result of improved drug delivery due to changes in vascular permeability induced by bevacizumab.

Substantial changes in gene expression were observed in Luminal-A tumors both from *week 0* to *12* and from *week 12* to *25* (**Figure 4C**). In the chemotherapy alone arm, the Luminal-A tumors showed suppressed ribosome and spliceosome synthesis pathways in response to anthracycline treatment, but significantly higher activity in response to taxane treatment (**Figure S2**). Up-regulation of ribosome and spliceosome synthesis pathways may be related to treatment resistance in Luminal-A tumors as efficacy of chemotherapeutic agents has been suggested to involve inhibition of ribosome biogenesis (38).

Differential gene expression and pathway activity between the two treatment arms were most evident in Luminal-B tumors, exhibiting significantly lower activity of pathways involved in DNA repair and cell-cycle **(Figure 4D)**. Elimination of almost all samples of Luminal-B subtype in the combination-therapy arm after treatment with anthracycline (**Figure 3B**) may be related to this radical change in molecular activity. In the Basal-like subtype, very few additional genes were affected by combination-therapy, concordant with the lack of additional clinical response seen in this patient group.

The clinical toxicity increased with bevacizumab treatment, as more patients experienced febrile neutropenia in addition to hypertension and episodes of bleeding. Increases in febrile neutropenia and hypertension have also been seen in similar studies (10,11). The clinical practice guidelines at the time of the study were not recommending

the use of G-CSF for all patients, only those having experienced febrile neutropenia, and this may have increased the frequency of this adverse event.

In this study, immune response was found to be a strong predictor of response in patients treated with bevacizumab in addition to the standard chemotherapy. The effect of addition of bevacizumab at gene expression level was most evident in the ER-positive Luminal-B tumors. In conclusion, the study provides valuable insights into changes in tumor behavior after neoadjuvant chemotherapy with and without bevacizumab, which may aid in identifying patients more likely to benefit from addition of bevacizumab to the standard chemotherapy.

References

1. Li JL, Sainson RC, Oon CE, Turley H, Leek R, Sheldon H*, et al.* DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy in vivo. Cancer research **2011**;71(18):6073-83 doi 10.1158/0008-5472.CAN-11-1704.
2. Rini BI, Small EJ. Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma. J Clin Oncol **2005**;23(5):1028-43 doi 10.1200/JCO.2005.01.186.
3. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocrine reviews **2004**;25(4):581-611 doi 10.1210/er.2003-0027.
4. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nature reviews **2008**;8(8):579-91 doi 10.1038/nrc2403.
5. Goel S, Wong AH, Jain RK. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. Cold Spring Harbor perspectives in medicine **2012**;2(3):a006486 doi 10.1101/cshperspect.a006486.
6. Sikov WM, Berry DA, Perou CM, Singh B, Cirrincione CT, Tolaney SM*, et al.* Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage II to III triple-negative breast cancer: CALGB 40603 (Alliance). J Clin Oncol **2015**;33(1):13-21 doi 10.1200/JCO.2014.57.0572.
7. Earl HM, Hiller L, Dunn JA, Blenkinsop C, Grybowicz L, Vallier AL*, et al.* Efficacy of neoadjuvant bevacizumab added to docetaxel followed by fluorouracil, epirubicin, and cyclophosphamide, for women with HER2-negative early breast cancer (ARTemis): an open-label, randomised, phase 3 trial. The lancet oncology **2015**;16(6):656-66 doi 10.1016/S1470-2045(15)70137-3.
8. Kumler I, Christiansen OG, Nielsen DL. A systematic review of bevacizumab efficacy in breast cancer. Cancer treatment reviews **2014**;40(8):960-73 doi 10.1016/j.ctrv.2014.05.006.
9. Stevenson CE, Nagahashi M, Ramachandran S, Yamada A, Bear HD, Takabe K. Bevacizumab and breast cancer: what does the future hold? Future oncology **2012**;8(4):403-14 doi 10.2217/fon.12.22.
10. von Minckwitz G, Eidtmann H, Rezai M, Fasching PA, Tesch H, Eggemann H*, et al.* Neoadjuvant chemotherapy and bevacizumab for HER2-negative breast cancer. The New England journal of medicine **2012**;366(4):299-309 doi 10.1056/NEJMoa1111065.
11. Bear HD, Tang G, Rastogi P, Geyer CE, Jr., Robidoux A, Atkins JN*, et al.* Bevacizumab added to neoadjuvant chemotherapy for breast cancer. The New England journal of medicine **2012**;366(4):310-20 doi 10.1056/NEJMoa1111097.
12. Lambrechts D, Lenz HJ, de Haas S, Carmeliet P, Scherer SJ. Markers of response for the antiangiogenic agent bevacizumab. J Clin Oncol **2013**;31(9):1219-30 doi 10.1200/JCO.2012.46.2762.
13. Network CGA. Comprehensive molecular portraits of human breast tumours. Nature **2012**;490(7418):61-70 doi 10.1038/nature11412.
14. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T*, et al.* Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol **2009**;27(8):1160-7 doi 10.1200/jco.2008.18.1370.
15. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA*, et al.* Molecular portraits of human breast tumours. Nature **2000**;406(6797):747-52 doi 10.1038/35021093.
16. Bonnefoi H, Litiere S, Piccart M, MacGrogan G, Fumoleau P, Brain E*, et al.* Pathological complete response after neoadjuvant chemotherapy is an independent predictive factor irrespective of simplified breast cancer intrinsic subtypes: a landmark and two-step approach analyses from the EORTC 10994/BIG 1-00 phase III trial. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO **2014**;25(6):1128-36 doi 10.1093/annonc/mdu118.

17. Kim MM, Allen P, Gonzalez-Angulo AM, Woodward WA, Meric-Bernstam F, Buzdar AU*, et al.* Pathologic complete response to neoadjuvant chemotherapy with trastuzumab predicts for improved survival in women with HER2-overexpressing breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO **2013**;24(8):1999-2004 doi 10.1093/annonc/mdt131.
18. von Minckwitz G, Untch M, Blohmer JU, Costa SD, Eidtmann H, Fasching PA*, et al.* Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Oncol **2012**;30(15):1796-804 doi 10.1200/JCO.2011.38.8595.
19. Esserman LJ, Berry DA, Demichele A, Carey L, Davis SE, Buxton M*, et al.* Pathologic Complete Response Predicts Recurrence-Free Survival More Effectively by Cancer Subset: Results From the I-SPY 1 TRIAL--CALGB 150007/150012, ACRIN 6657. J Clin Oncol **2012**;30(26):3242-9 doi 10.1200/JCO.2011.39.2779.
20. Team RC. 2014 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/.>.
21. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proceedings of the National Academy of Sciences of the United States of America **2001**;98(9):5116-21 doi 10.1073/pnas.091062498.
22. AR T, G C, B N, J L. SAM: Significance Analysis of Microarrays. R package version 2.0. **2011**.
23. Yaari G, Bolen CR, Thakar J, Kleinstein SH. Quantitative set analysis for gene expression: a method to quantify gene set differential expression including gene-gene correlations. Nucleic acids research **2013**;41(18):e170 doi 10.1093/nar/gkt660.
24. Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC*, et al.* DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome biology **2003**;4(5):P3.
25. Seo AN, Lee HJ, Kim EJ, Kim HJ, Jang MH, Lee HE*, et al.* Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer. British journal of cancer **2013**;109(10):2705-13 doi 10.1038/bjc.2013.634.
26. Ono M, Tsuda H, Shimizu C, Yamamoto S, Shibata T, Yamamoto H*, et al.* Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer. Breast cancer research and treatment **2012**;132(3):793-805 doi 10.1007/s10549-011-1554-7.
27. West NR, Milne K, Truong PT, Macpherson N, Nelson BH, Watson PH. Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. Breast Cancer Res **2011**;13(6):R126 doi 10.1186/bcr3072.
28. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M*, et al.* Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol **2010**;28(1):105-13 doi 10.1200/JCO.2009.23.7370.
29. Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F*, et al.* Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. J Clin Oncol **2012**;30(16):1996-2004 doi 10.1200/JCO.2011.39.5624.
30. Stoll G, Enot D, Mlecnik B, Galon J, Zitvogel L, Kroemer G. Immune-related gene signatures predict the outcome of neoadjuvant chemotherapy. Oncoimmunology **2014**;3(1):e27884 doi 10.4161/onci.27884.
31. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nature reviews Immunology **2009**;9(5):353-63 doi 10.1038/nri2545.
32. Weir GM, Liwski RS, Mansour M. Immune modulation by chemotherapy or immunotherapy to enhance cancer vaccines. Cancers **2011**;3(3):3114-42 doi 10.3390/cancers3033114.
33. Chen G, Emens LA. Chemoimmunotherapy: reengineering tumor immunity. Cancer immunology, immunotherapy : CII **2013**;62(2):203-16 doi 10.1007/s00262-012-1388-0.
34. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined

treatments against cancer. Cell death and differentiation **2014**;21(1):15-25 doi 10.1038/cdd.2013.67.
35. Bedognetti D, Maccalli C, Bader SB, Marincola FM, Seliger B. Checkpoint Inhibitors and Their Application in Breast Cancer. Breast Care (Basel) **2016**;11(2):108-15 doi 10.1159/000445335.
36. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K*, et al.* Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res **2005**;11(16):5678-85 doi 10.1158/1078-0432.CCR-04-2421.
37. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F*, et al.* The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res **2007**;13(8):2329-34 doi 10.1158/1078-0432.CCR-06-1109.
38. Burger K, Muhl B, Harasim T, Rohrmoser M, Malamoussi A, Orban M*, et al.* Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. The Journal of biological chemistry **2010**;285(16):12416-25 doi 10.1074/jbc.M109.074211.

Table 1: Clinicopathological and molecular characteristics of patients

	Chemotherapy arm n (%)	Combination therapy arm n (%)	P-value*
Clinical tumor stage			
T2	21 (32)	19 (29)	
T3	39 (59)	42 (64)	0,86
T4	6 (9)	5 (8)	
Nodal status			
cN0	29 (44)	29 (44)	
cN1-3	10 (15)	11 (17)	0,96
pN1	27 (41)	26 (39)	
Histopathology			
Invasive ductal carcinoma	54 (82)	52 (79)	
Invasive lobular carcinoma	10 (15)	13 (20)	0,68
Other	2 (3)	1 (2)	
Pathological tumor grade			
1	3 (5)	8 (12)	
2	42 (64)	43 (65)	0,28
3	15 (23)	12 (18)	
NA	6 (9)	3 (5)	
Estrogen receptor status			
ER negative	9 (14)	12 (18)	0,66
ER positive	57 (86)	54 (82)	
TP53 mutation status			
TP3 wildtype	44 (67)	41 (62)	
TP53 mutated	18 (27)	21 (32)	0,69
NA	4 (6)	4 (6)	
PAM50 Subtypes			
Luminal-A	28 (42)	27 (41)	
Luminal-B	22 (33)	18 (27)	
HER2-enriched	3 (5)	6 (9)	0,71
Basal-like	10 (15)	11 (17)	
Normal-like	2 (3)	4 (6)	
NA	1 (2)		

*Pearson's Chi-squared test, Fisher's exact test for 2X2 table

Table 2: Adverse events

	All adverse events			Serious adverse events (SAE)		
	Chemotherapy arm	**Combination therapy arm**	P-value**	**Chemotherapy arm**	**Combination therapy arm**	P-value**
Febrile neutropenia	25	53	< 0.001	25	53	< 0.001
Proteinuria	37	43	NS***	0	0	NS
Hypertension	14	37	< 0.001	0	0	NS
Bleeding/Haemorrhage	13	48	< 0.001	1	2	NS
Infection	6	16	0,033	6	15	0,055
Neutropenia	3	5	NS	3	5	NS
Fever	2	3	NS	1	3	NS
Stomatitis	1	2	NS	1	2	NS
Arrhythmia supraventricular	1	2	NS	1	2	NS
Hypersensitivity reaction	2	0	NS	2	0	NS
Syncope	2	0	NS	2	0	NS
Death	0	1	NS	0	1	NS
Other*	21	18	NS	10	12	NS

***Other single SAE: In chemotherapy arm:** Asthenia, Chest pain, Colitis, Constipation, Dehydration, Injection site reaction, Myalgia, Nausea, Periodontitis, Superficial thrombophlebitis. **In combination therapy arm:** Decreased appetite, Dizziness, Extravasation, Hypoesthesia, Left ventricular dysfunction, Nervous system disorder, Non-cardiac chest pain, Esophageal candidiasis, Pancreatitis, Polyneuropathy, Pulmonary embolism, Tubulointerstitial nephritis.

**** Fisher's exact test.**

***** NS (Not significant; p > 0.05)**

Figure legends

Figure 1. The study design: Patients included in the study were randomized to receive chemotherapy with or without bevacizumab.

Figure 2. Pathological complete response (pCR) rates. pCR rates are higher in patient group treated with chemotherapy and bevacizumab (Chemo + bev) compared to the patient group treated with chemotherapy (Chemo). pCR rates are significantly higher in ER negative (ER neg) vs. ER positive (ER pos), inTP53 mutated (TP53 mut) vs. TP53 wildtype (TP53 wt) and Basal-like subtype vs. other subtypes. P- values were obtained from Fisher's exact test (2x2 table) and $\chi 2$ test.

Figure 3. Changes in proliferation score and molecular subtypes in response to treatment. **A.** Proliferation score reduces significantly after treatment in both treatment arms but is more suppressed in the combination therapy arm after 12 weeks of treatment ($p = 0.006$, Kruskal-Wallis test). Molecular subtypes of disease before and after treatment with **B.** Combination therapy and **C.** Chemotherapy.

Figure 4. Changes in gene expression profiles during treatment. Venn diagrams showing number of genes with **s**ignificant alteration in expression between two treatment arms from *week 0* to *week 12* in **A.** Basal-like, **B.** Luminal B and **C**. Luminal A tumors; **D.** Differential pathway activity in the combination therapy arm compared to the chemotherapy arm from *week 0* to *week 12* in Luminal B tumors. The purple and the grey dots represent mean difference in pathway activity in the combination therapy arm and the chemotherapy arm respectively between *week 0* and *week 12.* The bars represent 95% confidence interval for each pathway and color-coded according to their False discovery rate (FDR)-corrrected *p*-values representing the significance of change from *week 0* to *week 12.*

Figure 1

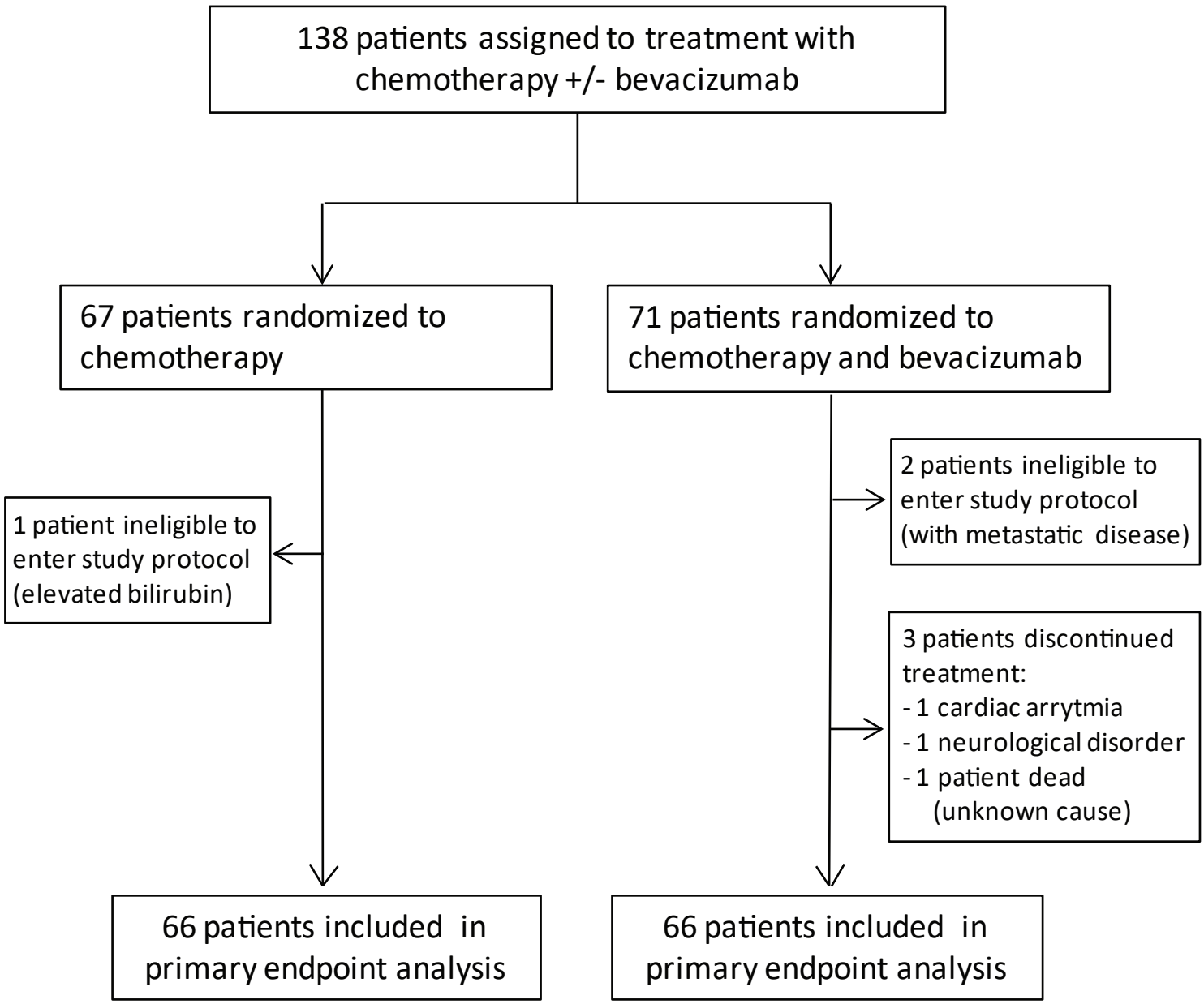

138 patients assigned to treatment with chemotherapy +/- bevacizumab
67 patients randomized to chemotherapy
71 patients randomized to chemotherapy and bevacizumab
1 patient ineligible to enter study protocol (elevated bilirubin)
2 patients ineligible to enter study protocol (with metastatic disease)
3 patients discontinued treatment:
- 1 cardiac arrytmia
- 1 neurological disorder
- 1 patient dead (unknown cause)
66 patients included in primary endpoint analysis
66 patients included in primary endpoint analysis

Figure 2

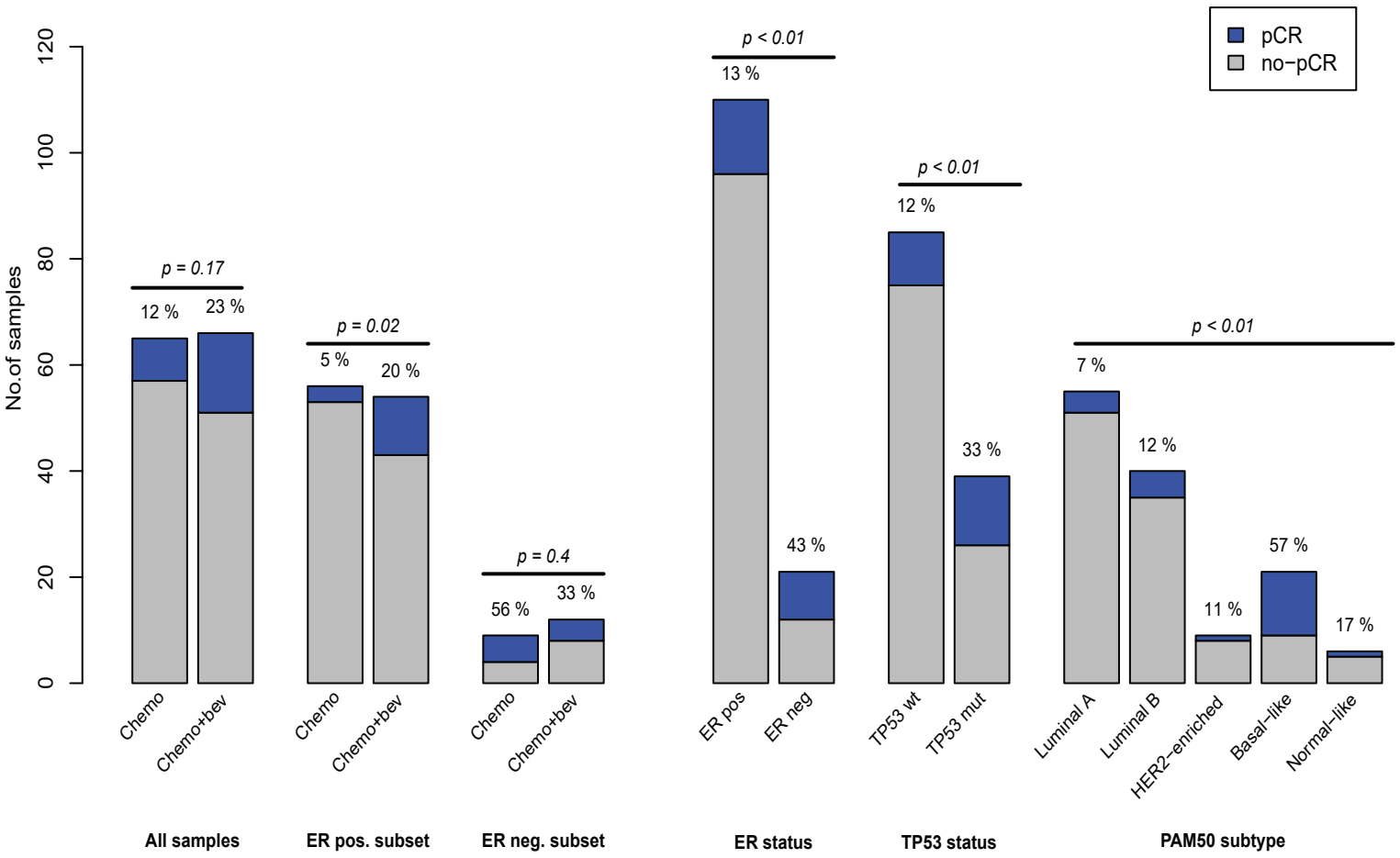
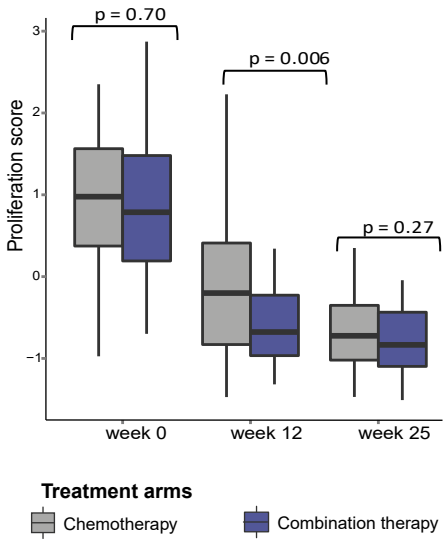
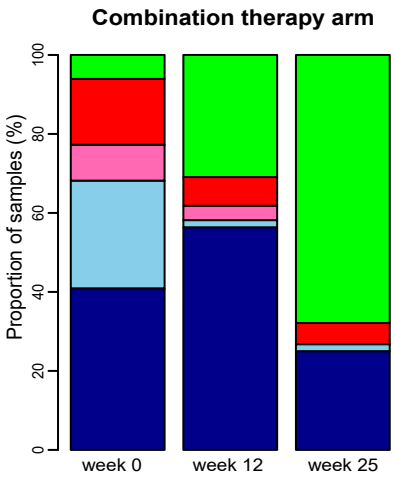

pCR
no−pCR
No.of samples
0
20
40
60
80
100
120
p = 0.17
12 %
23 %
p = 0.02
5 %
20 %
p = 0.4
56 %
33 %
p < 0.01
13 %
43 %
p < 0.01
12 %
33 %
p < 0.01
7 %
12 %
11 %
57 %
17 %
Chemo
Chemo+bev
Chemo
Chemo+bev
Chemo
Chemo+bev
ER pos
ER neg
TP53 wt
TP53 mut
Luminal A
Luminal B
HER2−enriched
Basal−like
Normal−like
All samples
ER pos. subset
ER neg. subset
ER status
TP53 status
PAM50 subtype

Figure 3

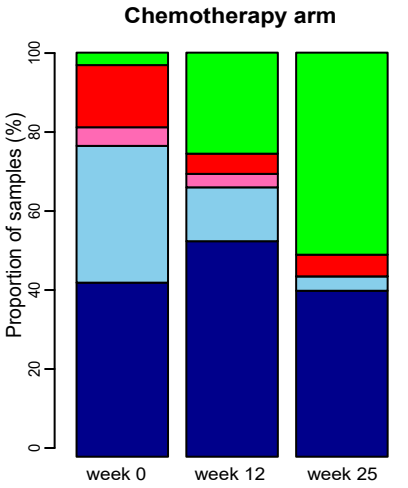
A.


p = 0.70
p = 0.006
p = 0.27
Proliferation score
3
2
1
0
−1
week 0
week 12
week 25
Treatment arms
Chemotherapy
Combination therapy

B.


Combination therapy arm
Proportion of samples (%)
0
20
40
60
80
100
week 0
week 12
week 25

C.


Chemotherapy arm
Proportion of samples (%)
0
20
40
60
80
100
week 0
week 12
week 25

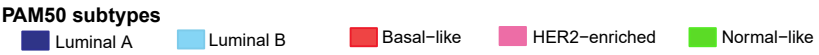
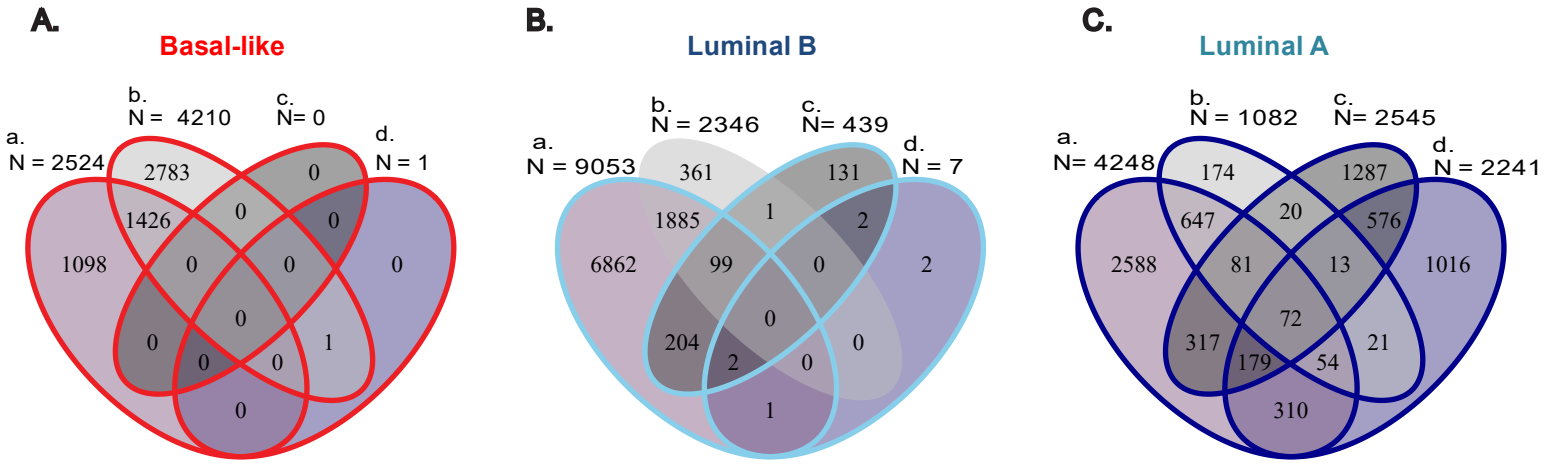

PAM50 subtypes
Luminal A
Luminal B
Basal−like
HER2−enriched
Normal−like

Figure 4



N = No.of differentially expressed genes between
a: week0 - week12 samples in Combination-therapy arm
b: week0 - week12 samples in Chemotherapy arm
c: week12 - week25 samples in Combination-therapy arm
d: week12 - week25 samples in Chemotherapy arm