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ORIGINAL ARTICLE

Identifying predictors of treatment response and molecular changes induced by neoadjuvant chemotherapy and endocrine therapy in hormone receptor-positive/HER2-negative breast cancer: the NEOENDO translational study☆

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Background: Predictors of response to neoadjuvant chemotherapy (NACT) and endocrine therapy (NET) in hormone receptor-positive (HoR+)/human epidermal growth factor receptor 2 (HER2)-negative breast cancer (BC) are required. Also, pathological and molecular changes induced by both strategies and their impact on patients' outcomes have not been reported so far.

Patients and methods: In a cohort of 186 patients with early-stage HoR+/HER2-negative BC treated with NACT or NET, we assessed the association of baseline main clinicopathological features and PAM50 gene expression (GE), intrinsic subtypes (IS) and risk-of-relapse (ROR-P) score with pathological outcomes according to treatment strategy. Molecular NACT/NET-induced changes were described and compared, along with their associations with event-free survival (EFS). Comparison of the two cohorts after propensity score matching (PSM) was used as sensitivity analysis. Molecular changes were confirmed in cell lines.

Results: NACT was associated with higher rates of residual cancer burden (RCB)-0/I than NET in the overall population (38.2% versus 13.5%, $P < 0.001$) and after PSM ($P = 0.036$). PAM50 non-luminal IS were the only independent and positive predictor of RCB-0/I ($P = 0.024$) in the NACT cohort, while *MMP11* messenger RNA levels were the only independent and negative predictor ($P = 0.014$) in the NET cohort. Both treatments shifted the tumor types toward less aggressive forms (i.e. PAM50 luminal A/normal-like), lowered the risk of recurrence in terms of ROR-P, up-regulated selected immune genes and PAM50 basal-like-related genes/signature and significantly downregulated proliferation-/luminal-/HER2-related genes/signatures, though NACT more than NET. Molecular findings were confirmed after PSM. A net reduction in proliferation-related genes and ROR-P was confirmed in cell lines with chemotherapy and endocrine therapy. Different baseline molecular features associated with diverse kind of responses (ROR-P downstaging, Ki67 reduction or pathological responses) with NACT and NET. Decreasing ROR-P and transitioning the tumor subtype to resemble normal tissue (i.e. PAM50 normal-like) suggested improved EFS.

Conclusions: NACT was more effective in the molecular and dimensional tumor 'downstaging' than NET but baseline molecular features associated with differential responses according to treatment strategy. Examining baseline and post-treatment GE might help tailor more personalized and effective care.

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Key words: breast cancer, neoadjuvant chemotherapy, neoadjuvant endocrine therapy, intrinsic subtypes, PAM50, molecular downstaging

INTRODUCTION

Hormone receptor-positive (HoR+)/human epidermal growth factor receptor 2 (HER2)-negative breast cancer (BC) constitutes ∼70% of all breast malignancies.[1] In the context of early-stage disease, the foundational systemic treatment encompasses adjuvant endocrine therapy (ET) over 5-10 years. For patients at an elevated risk of relapse and/or those in the premenopausal phase, 4-6 months of chemotherapy (CT) is also considered.[2,3] Neoadjuvant endocrine therapy (NET) or chemotherapy (NACT) is occasionally administered to facilitate tumor downstaging for less invasive surgical approaches or to transform locally advanced inoperable tumors upon diagnosis into operable.[4] Additionally, patients with HoR+/HER2-negative disease who achieve a residual cancer burden (RCB) of 0 [i.e. pathological complete response (pCR)] or RCB-I have demonstrated improved prognosis compared with those with minimal tumor downstaging,[5,6] which has led to the expanded adoption of NACT, particularly for larger tumors or with axillary involvement.

From a molecular perspective, HoR+/HER2-negative BC has been segmented into a minimum of four distinct molecular subtypes, referred to as intrinsic subtypes (IS): luminal A, luminal B, HER2-enriched (HER2-E), basal-like and a normal-like group.[7-10] These entities are detectable through the PAM50 gene expression (GE) assay.[8,10] Although the most prevalent subtypes are the luminal A and B,[9,11] non-luminal subtypes represent 5%-10% of cases in the early-stage setting, and up to 30%-44% in the metastatic setting.[12,13] These non-luminal subtypes tend to be associated with less favorable outcomes, decreased sensitivity to ET and heightened sensitivity to CT, particularly when compared with the more favorable luminal A subtype.[9,12,14,15]

Over the years, our group and other researchers have demonstrated that systemic treatments can induce molecular subtype switches and molecular 'downstaging' [i.e. decrease in PAM50 molecular risk-of-relapse (ROR) score] in HoR+/HER2-negative disease.[15-19] However, the prognostic and therapeutic implications of these molecular changes are currently unknown. Additionally, a comprehensive comparison of induced changes between standard NACT and NET, extending beyond ROR, is absent.

The aim of this study was to comprehensively describe research-based PAM50 IS,[10] ROR and genomic changes induced by standard NACT and NET, assess their potential prognostic implications, compare treatment-related pathological and survival outcomes and assess baseline molecular and clinicopathological factors associated with treatment response.

PATIENTS AND METHODS

Study population

We included a consecutive cohort of patients with HoR+/HER2-negative early-stage breast cancer (EBC) of stage I-IIIB treated as per standard of care with NACT or NET at the Breast Cancer Unit of the Hospital Clinic of Barcelona (HCB) between 2014 and 2018. The study was approved by the HCB Ethics Committee (institutional review board No. HCB/2021/0007). Written informed consent for participation was obtained from all patients. Full inclusion criteria and variables of interest are detailed in Supplementary Methods, available at https://doi.org/10.1016/j.esmoop.2024.103989. NACT consisted of standard anthracycline and/or taxane-based regimens. NET consisted of 3-6 months of an aromatase inhibitor (AI) or tamoxifen. The medical records were retrospectively reviewed to obtain the relevant clinicopathological information.

Objectives

The primary objectives were to compare the two types of neoadjuvant approaches (i.e. NACT and NET) in terms of molecular changes induced from baseline to surgery, as well as surgical and survival outcomes between NACT and NET. The secondary objectives were (i) to explore the association of baseline and surgical clinicopathological/molecular features with survival outcomes; (ii) to identify potential baseline predictive factors of response to each neoadjuvant approach; (iii) to study the molecular changes induced by each therapy approach within each of the PAM50 IS.

PAM50 GE analysis

RNA was purified from available archival formalin-fixed paraffin-embedded tumor tissues from pre-treatment baseline diagnostic biopsies and surgical specimens and analyzed at the nCounter platform (NanoString Technologies Inc., Seattle, WA) using a research-based PAM50 assay.[8] All tumors were assigned to a PAM50 IS or to the normal-like group. In addition, we evaluated the correlation to each IS centroid, four gene signatures derived from the PAM50's luminal/hormone-regulated pathway-associated genes, basal-related genes, proliferation/cell-cycle-related genes and HER2 cluster genes (henceforth luminal, proliferation, basal and HER2 signatures) and the PAM50 ROR score based on subtype and proliferation (ROR-P).[8,14] More details are reported in Supplementary Methods, available at https://doi.org/10.1016/j.esmoop.2024.103989.

Cell line experiments

PAM50 GE changes induced by ET (i.e. fulvestrant) and CT (i.e. paclitaxel) were evaluated in hormone-dependent HER2-negative BC cell lines T47D and MCF7.[20] Messenger RNA was extracted using QIAGEN's RNeasy extraction kit (QIAGEN, Hilden, Germany). All experiments were replicated three times in different days. GE analysis was carried out on an nCounter platform as previously described. More details are provided in Supplementary Methods, available at https://doi.org/10.1016/j.esmoop.2024.103989.

Statistical analysis

Paired and unpaired Student's *t*-tests were used to compare continuous variables between groups of interest, while chi-square or Fisher's exact test was used to compare categorical variables. McNemar's or Bhapkar's test was used to compare pre-/post-treatment categorical variables within the same patient group. Survival curves were estimated by the Kaplan−Meier method and differences were assessed with the log-rank test. The study of association among clinical, pathological and molecular features with event-free survival (EFS) was conducted with Cox regression models to estimate hazard ratios (HRs) with their 95% confidence intervals (CIs). Associations with RCB and pCR were determined with logistic regressions to estimate odds ratios (ORs) with their respective 95% CIs. We carried out a sensitivity analysis to control potential confounders and selection bias using a propensity score matching (PSM). We implemented a 1 : 1 nearest neighbor PSM without replacement, with a caliper of 0.5. Propensity scores were estimated using logistic regression of the neoadjuvant treatment on the covariates of interest. For all analyses, significance was set at $P < 0.05$. Multiclass and two-class, paired or unpaired, Significance Analyses of Microarrays (SAM) were used to assess GE changes between timepoints (paired) or groups (unpaired). A false discovery rate (FDR) $\leq$5% was considered for significance. More details are reported in Supplementary Methods, available at https://doi.org/10.1016/j.esmoop.2024.103989.

RESULTS

Baseline pre-treatment clinicopathological and molecular features

We enrolled 186 consecutive patients diagnosed with HoR+/HER2-negative EBC at the HCB and treated with neoadjuvant therapy (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2024.103989). Of these, 97 patients (52.2%) underwent NACT, while 89 patients (47.8%) received NET. Women in the NACT cohort were younger and showed a higher proportion of axillary lymph node-positive (cN+) and stage III tumors, along with less lobular histology ($P = 0.035$), lower progesterone receptor (PgR) immunohistochemistry (IHC) levels, higher Ki67 and stromal tumor-infiltrating lymphocyte (sTIL) levels and more histological grade (G)3 tumors than those treated with NET (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2024.103989). Baseline tumor samples from core needle biopsies were available for 70 (72.2%) and 67 (75.3%) patients, respectively. At the GE level, tumors in the NACT cohort displayed a higher prevalence of luminal B, HER2-E and basal-like subtypes, along with a lower incidence of luminal A subtype compared with NET tumors ($P < 0.001$). Furthermore, the former group exhibited a higher mean ROR-P score and a greater representation in the intermediate-/high-risk groups than tumors in the NET cohort (all $P < 0.001$). In contrast, tumors in the NET cohort exhibited significantly higher baseline expression levels of luminal-related genes/signatures, HER2 cluster genes (*ERBB2* and *GRB7*), luminal A and normal-like scores. They also showed reduction of expression of proliferation-related genes/signature, basal-like and luminal B scores, as well as the programmed cell death protein 1 (PD-1) gene (*PDCD1*) (FDR <5% for each significant gene/score/signature) (Figure 1A).

After PSM, a total of 28 patients with NACT and 28 with NET could be compared. No significant baseline clinicopathological differences were observed (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2024.103989). Consistently, only minimal differences at the GE level were observed (Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2024.103989).

Surgical and survival outcomes

Neo/adjuvant treatments (systemic and locoregional) are detailed in Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2024.103989. Patients treated with NACT showed significantly higher rates of pCR and RCB-0/I, with 18.6% achieving a pCR and 38.2% reaching RCB-0/I. In contrast, only 3.4% of patients in the NET group achieved pCR, and 13.5% reached RCB-0/I ($P = 0.001$ and $P < 0.001$, respectively). When restricting the analysis to a more balanced subcohort of patients after PSM, still a higher proportion of RCB-0/I was observed in patients undergoing NACT, as compared with NET ($P = 0.036$). Furthermore, in this matched population, we carried out a multivariable logistic regression that confirmed an independent and significant association with RCB-0/I for NACT versus NET [adjusted OR (aOR) 6.6, 95% CI 1.1-39.8, $P = 0.039$] regardless of TNM stage, G, Ki67 and PgR. Breast conservative surgery rates did not differ significantly between NET and NACT (54.6% versus 42.7%, $P = 0.104$) (Figure 1B). Patients treated with NET received significantly less axillary lymph node dissections (ALND) than patients treated with NACT (30.3% versus 51.5%, $P = 0.005$). However, the latter had a higher prevalence of nodal involvement at baseline. In fact, after PSM, NACT and NET cohorts showed similar rates of ALND (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2024.103989).

At a median follow-up of 64.0 months (95% CI 62.4-66.7 months), median EFS and overall survival (OS) were not reached (summary of events in Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2024.103989). The 5-year EFS was 90.4% (95% CI 86.2% to

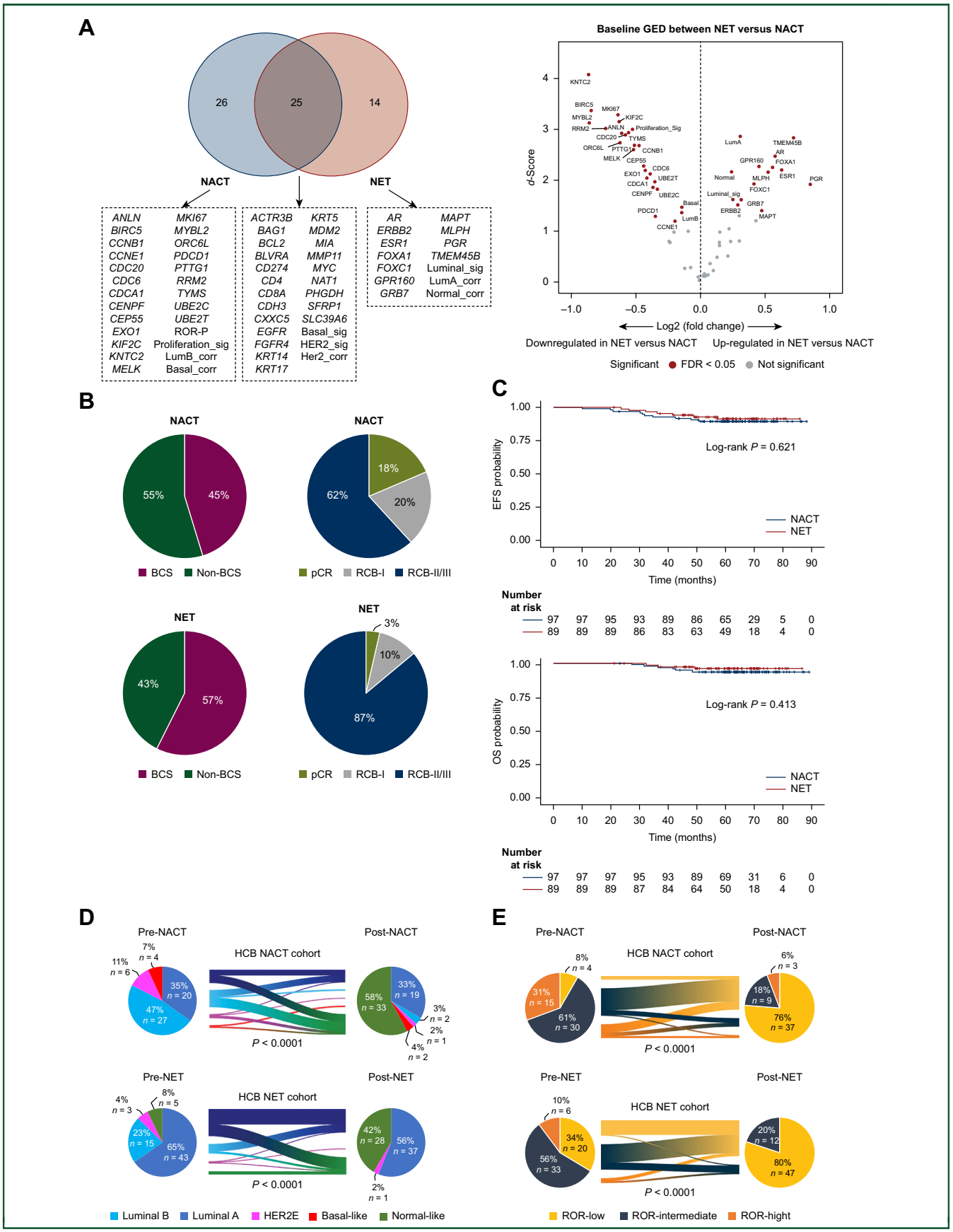

Figure 1. Baseline GEDs, main surgical and long-term outcomes and treatment-induced PAM50 IS and ROR-P changes according to NET and NACT. (A) Venn diagram reporting differentially expressed and similarly expressed (in the middle) genes, PAM50 IS centroid correlations and signatures between the two treatment cohorts at baseline and companion volcano plot representing baseline GEDs between the two treatment cohorts. (B) Conservative and non-conservative breast surgery rates and pathological response type according to neoadjuvant strategy. (C) Kaplan–Meier curves of EFS and OS according to neoadjuvant therapy. (D) PAM50

Table 1. Treatment-induced main pathological changes

Pathological features	NACT cohort					NET cohort					Overall population				
	Baseline		Post-surgery		P*	Baseline		Post-surgery		P*	Baseline		Post-surgery		P*
	n	%	n	%		n	%	n	%		n	%	n	%	
	97	52.2	97	52.2		89	47.8	89	47.8		186	100.0	186	100.0	
sTILs (%)					0.505					0.401					0.994
Mean	9.4	—	8.5	—		5.0	—	4.3	—		7.3	—	6.3	—	
SD	±16.1	—	±13.5	—		±9.6	—	±10.5	—		±13.6	—	±12.1	—	
Overall	95	97.9	78	80.4		86	96.6	89	100.0		181	97.3	167	89.8	
PgR (%)															
Mean	42.0	—	20.7	—	*<0.001*	53.9	—	14.0	—	*<0.001*	47.7	—	17.4	—	*<0.001*
SD	±36.6	—	±29.2	—		±37.4	—	±26.6	—		±37.4	—	±28.0	—	
Overall	97	100.0	48	49.5		89	100.0	48	53.9		186	100.0	96	51.6	
Ki67 (%)					*<0.001*					*<0.001*					*<0.001*
Mean	30.9	—	12.1	—		15.0	—	5.5	—		23.3	—	8.5	—	
SD	±18.1	—	±16.8	—		±12.9	—	±8.7	—		±17.7	—	±13.4	—	
Overall	97	100.0	69	71.1		89	100.0	84	94.4		186	100.0	153	82.3	

NACT, neoadjuvant chemotherapy; NET, neoadjuvant endocrine therapy; PgR, progesterone receptor; SD, standard deviation; sTILs, stromal tumor-infiltrating lymphocytes.
*P values for paired Student's *t*-tests for paired samples. Significant *P* values are reported in italics.

94.9%) and the 5-year OS was 95.0% (95% CI 91.8% to 98.2%). No significant difference was observed according to the neoadjuvant treatment strategy (Figure 1C). Of note, 28.1% of patients in the NET cohort received adjuvant CT. The result was confirmed also after PSM ($P = 0.326$ for both EFS and OS). Although no significant difference in EFS and OS was observed according to pCR or RCB status (all $P > 0.05$), all EFS and all OS events occurred in the non-pCR cohort, with only two events in the RCB-I group.

Treatment-induced pathological and subtype changes

We explored the effect of neoadjuvant therapy on main pathological and molecular features. Firstly, we observed a significant reduction in PgR levels and Ki67 levels by IHC. Conversely, no significant modifications in sTIL levels were observed. The effects were consistent with both neoadjuvant strategies adopted (Table 1), though mean Ki67 reduction was lower with NET than with NACT (difference in mean Ki67 reduction: 7.3, 95% CI 2.5-12.0, $P = 0.003$). HER2 dynamics were explored and reported elsewhere.[21] Similar results were observed after PSM, though PgR reduction did not reach statistical significance in the NACT cohort (Supplementary Table S5, available at https://doi.org/10.1016/j.esmoop.2024.103989).

Molecularly, a significant shift toward less aggressive research-based PAM50 IS was observed with NACT and NET ($P < 0.001$ both), with almost all post-surgical IS becoming or remaining normal-like or luminal A in the overall population (Figure 1D), as well as after PSM (Supplementary Table S5, available at https://doi.org/10.1016/j.esmoop.2024.103989). Post-surgical NACT versus NET PAM50 IS distribution slightly differed ($P = 0.048$), with the absence of post-surgical luminal B and basal-like tumors, lower proportion of normal-like and higher rate of luminal A in the NET cohort (Figure 1D).

Treatment-induced changes in individual genes, ROR-P and other PAM50 signatures

Consistent with PAM50 IS changes, a mean reduction in ROR-P continuous score was observed in the overall population with both NACT and NET ($P < 0.001$ all), accompanied by a significant shift from ROR-high/-intermediate to -low category (all $P < 0.001$) (Figure 1E). Mean ROR-P reduction was lower with NET than with NACT (difference in mean ROR-P reduction: 14.1, 95% CI 6.0-22.2, $P < 0.001$). Consistent ROR-P shifts were observed also after PSM (Supplementary Table S5, available at https://doi.org/10.1016/j.esmoop.2024.103989). There was no significant difference in post-neoadjuvant ROR categories between the two cohorts ($P = 0.155$).

Overall, 118 (64.4%) paired pre-/post-neoadjuvant samples assessable for GE were available, 57 (48.3%) for the NACT and 61 (51.7%) for the NET cohort, respectively. In general, NACT and NET induced a significant up-regulation of assessable immune genes *PDCD1*, *CD274*, *CD8A*, *CD4* (only NACT) and basal-like-related genes/signature, while a significant reduction of expression was observed for ROR-P, proliferation-related genes and signature, as well as HER2-related and luminal-related genes and signatures, though less profoundly (all FDR < 5%) (Figure 2). To note, mean reduction in the proliferation signature levels was not significantly different between NET and NACT ($P = 0.060$). NET was associated with less significant GE changes than NACT. Coherently, when comparing the post-surgical GE

IS changes induced by neoadjuvant therapy in the HCB cohort; (E) PAM50 ROR-P changes induced by neoadjuvant therapy in the HCB cohort. In the volcano plot, gray dots represent genes not differentially expressed, while red dots identify significantly differentially expressed genes for an FDR <5%. In Sankey plots, *P* values are referred to Bhapkar's tests. Significant if $P < 0.05$.
BCS, breast conservative surgery; EFS, event-free survival; FDR, false discovery rate; GEDs, gene expression differences; HCB, Hospital Clinic of Barcelona; HER2-E, HER2-enriched; IS, intrinsic subtypes; NACT, neoadjuvant chemotherapy; NET, neoadjuvant endocrine therapy; OS, overall survival; pCR, pathological complete response; RCB, residual cancer burden; ROR-P, risk-of-relapse score based on subtypes and proliferation.

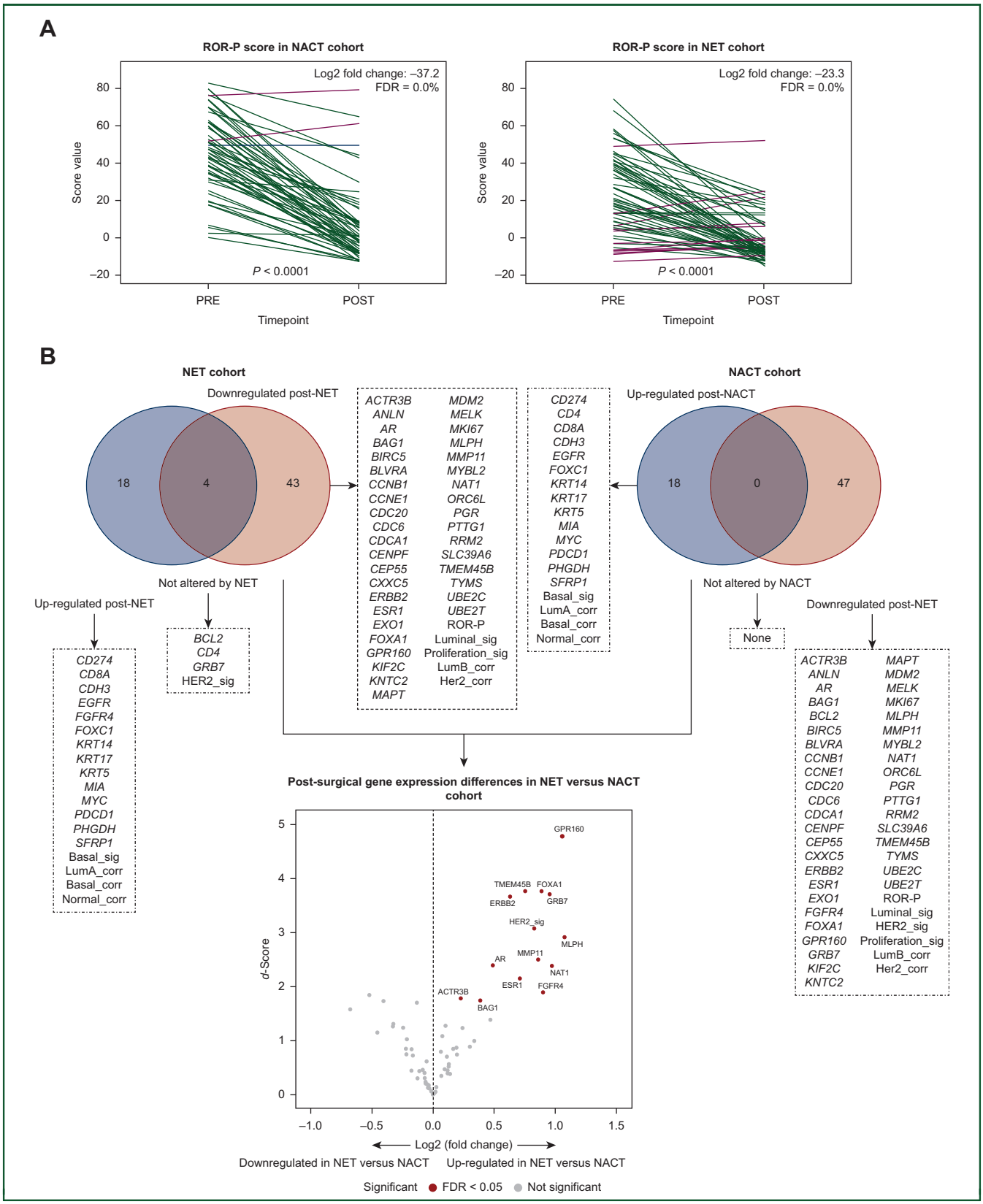

Figure 2. Differentially expressed genes, PAM50 signatures/scores and ROR-P after surgery and treatment-induced changes.
(A) PAM50 ROR-P score reduction after NET and NACT. Green lines highlight a numerical reduction in mRNA levels after treatment, while burgundy lines represent a numerical increase and blue lines represent stability. Reported *P* values are referred to paired Student's *t*-tests, while the log2 fold changes and FDR are referred to paired pre-/post-SAM analyses. (B) Venn diagrams reporting genes, signatures and PAM50 IS centroid correlations significantly up- and downregulated by NET and NACT and volcano plot of post-surgical gene expression differences between NET versus NACT cohort. Gray dots represent genes not differentially expressed, while red dots identify significantly differentially expressed genes for an FDR <5%.
corr, correlation coefficient; FDR, false discovery rate; NACT, neoadjuvant chemotherapy; NET, neoadjuvant endocrine therapy; sig, signature.

profile of the NET versus NACT cohort, the two subpopulations did not differ significantly for most genes, but the NET cohort showed slightly more up-regulated luminal-related (e.g. *ESR1*, *TMEM45B*, *FOXA1*) and HER2 cluster-related genes (*ERBB2*, *GRB7*, *FGFR4*) than the NACT subpopulation (FDR $<$ 5% each) (Figure 2). The same result was obtained after PSM (Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2024.103989). Furthermore, treatment-induced GE changes remained consistent after PSM (Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2024.103989). In this analysis, matched pre/post samples were available for 18 and 17 patients in the NACT and NET cohorts, respectively.

Treatment-induced molecular changes according to menopausal status were consistent with main results (not shown) (Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2024.103989).

Treatment-induced GE changes according to baseline IS mostly resembled those observed in the overall population (Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2024.103989). To note, NACT induced more profound changes than NET (Supplementary Table S6, available at https://doi.org/10.1016/j.esmoop.2024.103989).

Baseline GE profiles according to response type and neoadjuvant strategy

We sorted patients undergoing NACT and NET into groups based on treatment response. We defined a molecular response in terms of ROR-P (i.e. ROR-low versus ROR-intermediate/-high after treatment)[17] or Ki67 reduction [i.e. low ($\leq$10%) versus high ($>$10%) Ki67 after treatment],[22] and a pathological response (i.e. RCB-0/I versus RCB-II/III).[5] We used multiclass SAM to examine differential GE at baseline associated with treatment response to find clues that might predict different responses to NACT and NET. We found that those experiencing the best molecular responses (i.e. ROR-low and Ki67 $\leq$10%) to both NACT and NET had lower initial levels of ROR-P and proliferation-related genes/signature and higher luminal-related genes/signature compared with non-responders. Nonetheless, molecular responders with NACT showed a significant up-regulation of proliferation-related genes/signature and reduction of expression of luminal-related genes/signature than NET responders (Figure 3). Pathological responses (RCB-0/I) to NACT were obtained especially in those with the highest baseline ROR-P and proliferation-related genes/signature and lowest luminal-related genes/signatures, while the best pathological responses to NET were observed in those with the highest expression of luminal-related genes/signature, without notable differences in the expression of proliferation-related genes between NET responders and non-responders (Figure 3). Further details are reported in Supplementary Results, available at https://doi.org/10.1016/j.esmoop.2024.103989. After PSM, baseline GE could be compared according to response type to NACT and NET only for 64.0% of matched patients. No significant differences could be observed (not shown), most likely due to insufficient statistical power.

Validation in cell lines

In HoR+/HER2-negative T47D and MCF7 cell lines treated with fulvestrant or paclitaxel, most proliferation-related genes and signature, along with ROR-P, were significantly more up-regulated in untreated versus treated cell lines, regardless of the administered drug. Still, CT induced a broader downregulatory effect than ET. Opposite effects with CT and ET were observed regarding luminal-related genes/signatures. CT showed a more pronounced effect than ET on basal-like-related genes, signatures and score. However, the basal-related gene epidermal growth factor receptor (*EGFR*) was especially up-regulated by ET and, less profoundly, by CT, as compared with control (Figure 4A, Supplementary Table S7 and Results, available at https://doi.org/10.1016/j.esmoop.2024.103989).

Clinicopathological and molecular features of tumors achieving different pathological responses based on the neoadjuvant cohort

In the NACT cohort, there were no baseline GE differences among patients achieving pCR, RCB-I or RCB-II/III (all genes/signatures FDR $>$5%). In the NET cohort only the luminal-related *MMP11* gene was differentially expressed, being downregulated in patients achieving pCR (*d*-score: $-$2.21) or RCB-I (*d*-score: $-$0.83) and up-regulated in those achieving RCB-II/III (*d*-score: 0.18) (FDR = 0.0%). In the NACT cohort, non-luminal IS (versus luminal A + B) was the only feature independently associated with RCB-0/I when adjusting for significant factors at univariate analysis, namely, tumor size (cT), cN and G (aOR 6.13, 95% CI 1.27-29.52, $P = 0.024$). In the NET cohort, only *MMP11* showed significant (and negative) association with RCB-0/I, when adjusting for tumor dimension (aOR 0.55, 95% CI 0.34-0.88, $P = 0.014$).

Associations of baseline and post-surgical clinicopathological/molecular factors and molecular downstaging with survival outcomes in the entire study cohort

Considering the paucity of OS events, we explored potential associations of clinicopathological and molecular pre-/post-neoadjuvant variables only with EFS. Among baseline clinicopathological features, only stage (TNM III versus TNM I-II) and PgR% were associated with EFS (HR 4.26, $P = 0.006$ and HR 0.98, $P = 0.007$, respectively). Regarding post-surgical tumor features, although pCR and RCB-0/I *per se* did not show a significant association with outcomes, a clear trend for better EFS was observed (Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2024.103989). Moreover, ypTis/0/1 and ypN0 statuses, taken separately, were associated with EFS (HR 3.52, $P = 0.010$ and HR 3.32, $P = 0.026$, respectively). Post-surgical Ki67% levels were associated with EFS (HR 1.03, $P = 0.012$), as well.

Molecularly, a significantly higher proportion of baseline luminal A and ROR-low cases, compared with non-luminal A

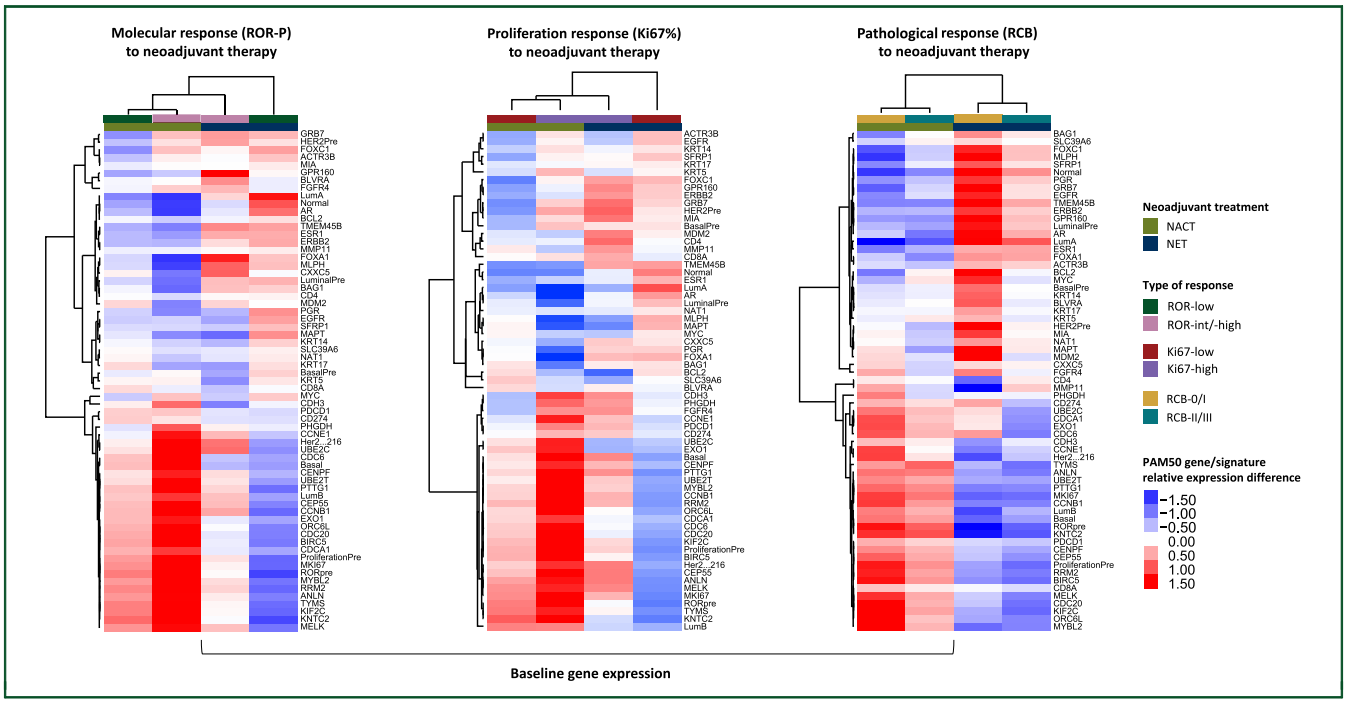

Figure 3. Differential gene expression at baseline in different responder subgroups according to neoadjuvant treatment strategy. Supervised clustering of 55 genes, 4 PAM50 signatures, ROR score and the 5 PAM50 intrinsic subtype correlation coefficients across four tumor classes defined according to different response subgroups, according to neoadjuvant treatment. All samples and gene expression data in each category have been combined into a single group. For each gene in a group, we calculated the standardized mean difference between the gene's expression in that class versus its overall mean expression in the dataset using a four-class Significance Analyses of Microarrays. The red color represents relatively high gene score, blue represents relatively low gene score and white represents median gene score.

Basal, basal-like PAM50 intrinsic subtype correlation score; BasalPre, basal-like-related baseline genomic signature; Her2, HER2-enriched PAM50 intrinsic subtype correlation score; HER2Pre, HER2 amplicon-related baseline genomic signature; Int, intermediate; LumA, luminal A PAM50 intrinsic subtype correlation score; LumB, luminal B PAM50 intrinsic subtype correlation score; LuminalPre, luminal-related baseline genomic signature; NACT, neoadjuvant chemotherapy; NET, neoadjuvant endocrine therapy; Normal, normal-like PAM50 intrinsic subtype correlation score; ProliferationPre, proliferation-related baseline genomic signatures; RCB, residual cancer burden; RCB-0, pathological complete response

($P = 0.050$) and ROR-intermediate/-high cases at baseline, were observed ($P = 0.021$) in the cohort of patients without relapses and deaths (Figure 4B), which translated into a trend toward better EFS at univariate analysis (Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2024.103989). After surgery, there was no significantly different distribution of PAM50 IS ($P = 0.448$) and ROR-P ($P = 0.777$) classes in patients with and without events (Figure 4B). The baseline PAM50 proliferation signature was associated with EFS (HR 3.50, $P = 0.013$), while all other PAM50 signatures and baseline immune genes' levels were not. Similarly, post-treatment ROR-P, PAM50 IS, immune genes and PAM50 signatures did not show any association with EFS.

We also explored the impact on EFS of molecular downstaging. Patients with a tumor IS switch to normal-like disease or remaining normal-like and luminal A from baseline showed a better EFS (HR 3.13, $P = 0.048$) than all other cases (Supplementary Figure S4 and Table S8, available at https://doi.org/10.1016/j.esmoop.2024.103989). Tumors with stable ROR-low from baseline to post-surgery showed numerically better EFS compared with all other ROR-P dynamics, without relapses or deaths (Supplementary Figure S4 and Table S8, available at https://doi.org/10.1016/j.esmoop.2024.103989). We then subdivided the PAM50 proliferation signature and ROR-P score treatment-induced numerical reductions in tertiles. Patients in the lowest tertiles (T1, highest biomarker reduction) experienced better outcomes compared with patients in the upper tertiles (T2-3) (Supplementary Results, available at https://doi.org/10.1016/j.esmoop.2024.103989). Other PAM50 signatures' dynamics, as well as changes in immune genes, did not show any potential association with EFS. An exploratory multivariable analysis without molecular data showed that only baseline cT (HR 3.52, $P = 0.044$) and PgR% at IHC (HR 0.98, $P = 0.046$) were significantly associated with EFS, irrespective of baseline N, ypT, ypN and post-surgical Ki67% (Supplementary Results, available at https://doi.org/10.1016/j.esmoop.2024.103989).

DISCUSSION

In our clinical practice cohort of patients with HoR+/HER2- negative BC, we observed that NACT or NET significantly contributed to pathological and molecular downstaging. Still, despite higher rates of stage II-III BC at baseline, NACT yielded higher rates of pCR and RCB-0/I than NET (18.6% and 38.2% versus 3.4% and 13.5%, respectively), consistent with previous literature.[5,6] Moreover, when comparing the two treatment cohorts after PSM, a clear association of RCB-0/I with NACT was further confirmed, regardless of tumor stage, G, Ki67 and PgR levels. In apparent

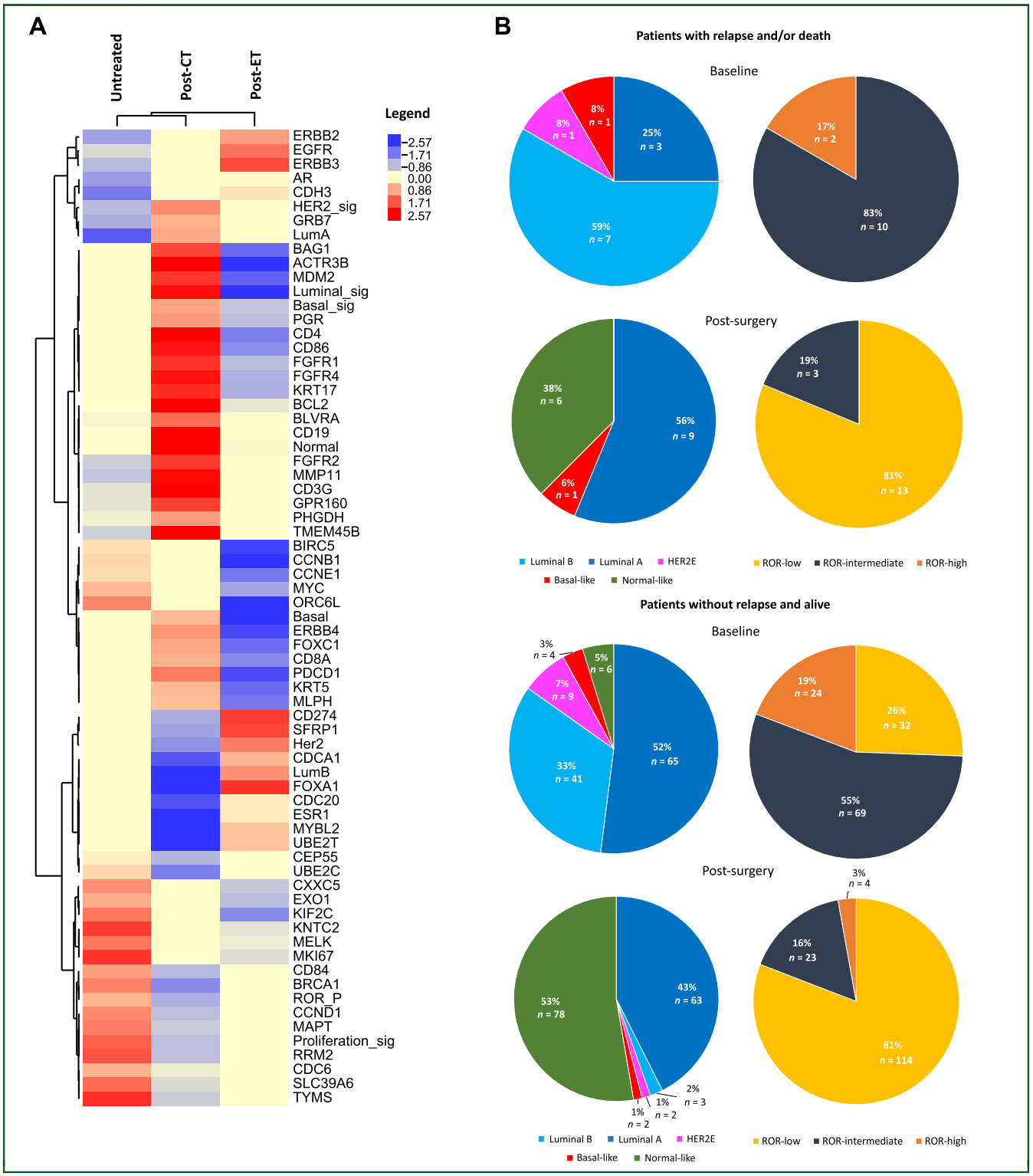

Figure 4. GEDs in cell lines and baseline and post-surgical PAM50 IS and ROR-P according to survival outcomes. (A) Supervised clustering of 66 genes, 4 PAM50 signatures, ROR-P score and the 5 PAM50 intrinsic subtype correlation coefficients across three treatment groups including two different HoR+/HER2-negative BC cell lines (i.e. T47D and MCF7). All samples and gene expression data in each category have been combined into a single group. For each gene in a group, we calculated the standardized mean difference between the gene's expression in that class versus its overall mean expression in the dataset using a three-class Significance Analyses of Microarrays. The red color represents relatively high gene score, blue represents relatively low gene score and yellow represents median gene score. (B) Baseline and post-surgical PAM50 intrinsic subtype and ROR categories distribution based on event-free survival events.

Basal, basal-like PAM50 intrinsic subtype correlation score; CT, chemotherapy (paclitaxel); ET, endocrine therapy (fulvestrant); GEDs, gene expression differences; Her2, HER2-enriched PAM50 intrinsic subtype correlation score; HER2E, HER2-enriched; HoR+, hormone receptor positive; LumA, luminal A PAM50 intrinsic subtype correlation score; LumB, luminal B PAM50 intrinsic subtype correlation score; Normal, normal-like PAM50 intrinsic subtype correlation score; pCR, pathological complete response; RCB, residual cancer burden; ROR, PAM50 risk of relapse with proliferation and subtype score; sig, gene expression signature.

https://doi.org/10.1016/j.esmoop.2024.103989

contradiction, in the overall study cohort, patients treated with NET ultimately received less ALND than patients treated with NACT. Nevertheless, a significantly higher frequency of baseline nodal involvement in this cohort was observed. This likely favored a more aggressive surgical attitude *per se*, since additional axillary involvement after NACT has been reported in up to 60% of cases.[23] In support to this hypothesis, ALND rates did not differ between the two treatment cohorts after PSM. Noteworthy, better pathological responses trended toward improved long-term outcomes, aligning with existing research.[5,6] Molecularly, the two strategies significantly reduced Ki67 and PgR protein levels and downregulated related genes, including those associated with tumor proliferation, luminal biology and HER2 characteristics. Notably, a substantial shift toward luminal A/normal-like and lower post-treatment ROR-P scores was observed with both neoadjuvant approaches. Also, NACT and NET up-regulated basal-like and selected immune genes, though this did not notably alter the levels of sTILs.

When dissecting different types of therapeutic response (i.e. molecular or pathological) according to neoadjuvant strategy, we observed that baseline luminal biology-related genes were more up-regulated in tumors achieving molecular response with both NET and NACT, in comparison to non-responders. However, NET molecular responders showed a more pronounced expression of luminal-related genes than NACT molecular responders, which in turn had higher expression of proliferation-related genes. At the same time, a marked expression of proliferation-related genes seemed to impair molecular responses under both neoadjuvant strategies, as well as pathological responses with NET, but seemed to favor the achievement of pathological responses with NACT. This is consistent with non-luminal PAM50 IS being the only baseline feature to be independently associated with pCR with NACT in our cohort. Tumors achieving minimal residual cancer with NET showed a marked up-regulation of luminal-related genes.

Overall, these findings suggest that tumor biology might help in predicting different kinds of responses and plan treatment strategies accordingly. For example, if a tumor dimensional downstaging is required, highly proliferative or non-luminal tumors should receive NACT-based treatments, while very luminal non-proliferative cancers might benefit from NET. If molecular downstaging is pursued, both NACT and NET could be viable approaches. Still, a molecular response in very proliferative tumors seems to be unlikely in either case. However, therapeutic strategies targeting proliferation, like cyclin-dependent kinase 4 and 6 inhibition, could be envisioned. In fact, in the SOLTI-CORALEEN randomized phase II trial of neoadjuvant ribociclib + letrozole versus anthracycline—taxane CT in PAM50 luminal B HoR+/HER2-negative BC, a more profound reduction of expression of the PAM50 proliferation signature was obtained with the former regimen.[17,24]

Unexpectedly, *MMP11* expression levels in the NET group was the only baseline feature independently associated with pCR. *MMP11* is a matrix metalloproteinase mediating matrix degradation, tissue remodeling, inflammation and tumor metastasis, including brain metastases development in BC.[25] Its expression correlates with aggressiveness and poorer prognosis in BC and other solid tumors.[26,27] Potential interactions with NET are unknown and should be further elucidated.

With ∼5 years of median follow-up, outcomes were excellent, with 90.4% of patients disease-free and 95.0% alive at 5 years. Still, a clear trend for better outcomes was observed for baseline luminal A and ROR-low tumors retaining the same baseline subtypes or ROR-P category, as well as for tumors molecularly shifting to the normal-like group after NACT or NET. To note, NACT seemed to reduce more efficiently Ki67 and ROR-P than standard NET. Also in cell lines, while a net antiproliferative effect was observed with both CT and ET, the downregulatory effect was more potent with the former. Notably, the genomic effects induced by NACT in our institutional cohort resembled those observed in the SOLTI-CORALLEEN in terms of molecular downstaging, subtype switching, Ki67 reduction and sTIL stability.[17,24] Differently, when ribociclib was added to letrozole, the degree of Ki67 and ROR-P reductions resembled those obtained with NACT.[17,24] All these findings, while not immediately translatable to the clinical practice, support the development of novel neoadjuvant approaches focused on proliferation reduction and molecular downstaging in HoR+/HER2-negative BC, like the ongoing phase II trial RIBOLARIS (NCT05296746).

Interestingly, we observed in our study an up-regulation of the PAM50 basal-like signature and basal-related genes, like *EGFR*, with both NACT and NET, regardless of the baseline PAM50 IS. In the SOLTI-CORALEEN trial, both NACT and ribociclib + letrozole produced a similar effect in HoR+/HER2-negative luminal B BC.[24] In our cell line models, we observed a more heterogeneous effect, suggesting that the clinical cohorts' up-regulation might reflect an increased proportion of normal breast stromal tissue. Nonetheless, both CT and ET induced a significant up-regulation of the basal gene *EGFR*, suggesting that the clinical cohorts' up-regulation of this gene might reflect a true biological response. A confirmation of this finding might pave the way for the study of EGFR-directed treatments in the post-neoadjuvant scenario of HoR+/HER2-negative disease.[28-30]

Finally, an up-regulation of selected immune genes (e.g. PD-1, programmed death-ligand 1 genes) with both NACT and NET emerged, in line with findings from Bergamino et al. with a short course of preoperatory AIs,[31] as well as with findings with NACT in the SWOG S0800 trial[32] and the recent translational analysis of the SOLTI-CORALEEN study by Pascual et al.[24] The prognostic implications of these findings are yet to be elucidated, as well as potential implication for combinatorial strategies with immune checkpoint inhibitor.

This study has several limitations to consider, including its retrospective nature, a relatively short follow-up for

HoR+/HER2-negative BC and the imbalanced characteristics of patients treated with NACT and NET. However, these differences are coherent to standard-of-care practice and neoadjuvant treatment choice based on clinical characteristics. Furthermore, we used PSM to homogenize the two treatment cohorts, replicated all main comparative analyses between matched NACT and NET cases and re-assessed treatment-induced pathological and molecular changes in the matched subcohorts as sensitivity analyses. Results were largely in line with main findings, supporting their clinical and biological consistency.

In conclusion, validation of our results in diverse patient populations and clinical settings is necessary. Nonetheless, our insights into NACT/NET-induced molecular downstaging, pathological and genomic effects offer promising avenues for future research and clinical trials for the neoadjuvant and post-neoadjuvant scenarios in HoR+/HER2-negative BC.

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DISCLOSURE

FS declares personal fees for educational events and/or materials from Gilead, Daiichi Sankyo and Novartis; travel expenses from Gilead, Daiichi Sankyo and Novartis; advisory fees from Pfizer. AP reports advisory and consulting fees from Roche, Daiichi-Sankyo, AstraZeneca, Pfizer, Novartis, Guardant Health and Peptomyc; lecture fees from Roche, Novartis, AstraZeneca and Daiichi Sankyo; institutional financial interests from Boehringer, Novartis, Roche, AstraZeneca, Daiichi-Sankyo, MedSIR, SL, Celgene, Astellas and Pfizer; stockholder and consultant of Reveal Genomics, SL; a patent PCT/EP2016/080056, the HER2DX patent filed (PCT/EP2022/086493), the DNADX patent filed (EP22382387.3) and the TNBCDX patent filed (EP23382703.9). FBM has the HER2DX patent filed (PCT/EP2022/086493), the DNADX patent filed (EP22382387.3) and the TNBCDX patent filed (EP23382703.9). OMS reports advisory and consulting fees from Roche and Reveal Genomic; lecture fees from Roche, Novartis, Eisai and Daiichi Sankyo; and travel expenses fees from Gilead, Pfizer and MSD. MB has a patent filed for the novel molecular subgroups for HR+/HER2+ early BC at the Institute of Cancer Research, and declares personal fees for educational events and/or materials from AstraZeneca, Novartis and Eisai and advisory fees from Pfizer. All other authors have declared no conflicts of interest.

DATA SHARING

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding authors upon reasonable request.

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