**Impact of duration of neoadjuvant aromatase inhibitors on molecular expression profiles in estrogen receptor positive breast cancers**

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**Statement of translational relevance:** Our study shows that neoadjuvant treatment with short and longer-term aromatase inhibitors (AI) in primary estrogen receptor (ER+) positive breast cancer (BC) exerts comparable impact on changes in intrinsic subtypes between baseline and surgery. However, neoadjuvant AI treatment beyond 2 weeks leads more changes in molecular characteristics at a transcriptional level, such as genes involved in pathways like MAPK and PI3K/AKT/mTOR and characteristics for immune response landscape, including those covering immune-checkpoint component. These findings provide rationale for considering neoadjuvant AI therapy beyond 2 weeks in high-risk ER+ BC tumours. The role of immune-checkpoint component inhibition for endocrine therapy resistant ER+ tumours in this setting warrants careful investigation.

**ABSTRACT**:

Background: Aromatase inhibitors (AI) treatment is the standard of care for post-menopausal women with primary estrogen receptor positive breast cancer (BC). The impact of duration of neoadjuvant endocrine therapy (NET) on molecular characteristics is still unknown. We evaluated and compared changes of gene expression profiles under short-term (2-week) versus longer-term neoadjuvant AI.

Methods:

Global gene expression profiles from POETIC trial (137 received 2 weeks of AI and 47 no-treatment) and targeted gene expression from 80 BC patients treated with NET for more than one-month (NeoAI) were assessed. Intrinsic subtyping, module-scores covering different cancer-pathways and immune-related genes were calculated for pre- and post-treated tumours.

Results: The differences in intrinsic subtypes after NET were comparable between the two cohorts, with most Luminal B (90.0% in POETIC and 76.3% in NeoAI) and 50.0% of HER2-enriched at baseline re-classified as Luminal A or Normal-like after NET. Downregulation of proliferative-related pathways was observed after two-weeks of AI. However, more changes in genes from cancer-signaling pathways such as MAPK and PI3K/AKT/mTOR and immune response/immune-checkpoint components that were associated with AI resistant tumours and differential outcome were observed in the NeoAI study.

Conclusions:

Tumour transcriptional profiles undergo bigger changes in response to longer NET. Changes in HER2-Enriched and Luminal B subtypes are similar between the two cohorts, thus AI sensitive intrinsic subtype tumours associated with good survival might be identified after 2 weeks of AI. The changes of immune-checkpoint component expression in early AI resistance and its impact on survival outcome warrants careful investigation in clinical trials.

Introduction

Breast cancer (BC) is molecularly and clinically heterogeneous, with approximately 60-80% of cases being estrogen receptor positive (ER+). The standard of care for postmenopausal women with ER+BC includes aromatase inhibitors (AI) over a 5 to 10 year-period. However, 20-25% of ER+BC patients will eventually relapse and additional biomarkers to identify resistance mechanisms to AI are warranted [1-4].

Global gene expression analyses in BC have shown molecular heterogeneity with a far more complex portrait beyond clinicopathological classification [5-7]. The elucidation of the molecular intrinsic subtypes has led to the categorisation of BC tumours into clinically relevant but molecular distinct subgroups that can be optimally defined by the 50-genes based PAM50 classifier [8-10]. These molecular subtypes are associated with different incidence and racial disparity, response to treatment and prognosis [8]. However, there is still insufficient data about changes of those molecular characteristics under different lengths of AI treatment and whether pre- or post-treatment characteristics are better predictors of prognosis [11,12].

Preoperative and neoadjuvant trials involving the collection of viable paired biopsies at diagnosis and at surgery provide a valuable source to understand genes and pathways involved in resistance to therapy, with the possibility to use, for example, Ki67 proliferation markers as a valuable endpoint associated with prognosis [13,14]. Our group previously suggested that reduced ER-dependence and E2F-signalling activation after short- and long-term neoadjuvant AI are associated with poor response [15,16]. However, we also reported the enrichment of *ESR1* mutation with long-term neoadjuvant AI in primary BC using a real-world cohort of patients treated in the Royal Marsden Hospital, UK [16]. Therefore, the comparison of the effect of different lengths of neoadjuvant AI therapy in molecular features might be necessary to elucidate the full impact on molecular alterations that might limit response and lead to clinical resistance.

In this study, the impact of short- and long-term neoadjuvant AI therapy on molecular changes, including intrinsic subtypes and signalling pathways was comprehensively evaluated. Gene expression profiles from two cohorts of early primary ER+ BC patients were analysed: 1) POETIC trial, in which patients were treated for 2-weeks [15]; 2) patients treated for more than one month, named in the present study as NeoAI [16].

Methods

Patients’ populations

Data from two different cohorts of post-menopausal women with primary ER+ BC treated with different lengths of neoadjuvant AI was analysed (**Supplementary Figure 1**).

POETIC subset: POETIC was a phase III, randomized study of 4,486 ER+ BC post-menopausal patients. Patients were randomized 2:1 to receive 2 weeks of pre-operative AI vs no treatment to determine whether peri-operative aromatase inhibitors followed by standard adjuvant therapy would improve survival [15]. The subset used in this study comprised 184 patients with paired samples:137 tumours treated with AI (86.1% (118) were human epidermal growth factors receptor not amplified or overexpressed (HER2-) and 13.9% (19) HER2+) and 47 patients who did not receive peri operative AI as a control group.

NeoAI study: This was a retrospective cohort of patients treated with neoadjuvant AI for at least one month (mean 6.24 months ± Standard Deviation 3.9) at the Royal Marsden Hospital (RMH) between 2003 and 2016 [16]. Data from 80 patients from this study was analysed: 93.8% (75) were HER2– and 6.2% (5) HER2+. Seven patients with baseline Ki67%<5% or lack of clinical data or gene expression were excluded. In order to provide a view of real-world AI resistance mechanisms in ER+ BC, both HER2+ and HER2- were included in this study, with subsequent subgroup analyses focused on ER+HER2- tumours.

Gene expression profiles

In the POETIC subset, gene expression data from microarray was obtained as previously described [15]. Probes targeting 16,528expressed genes (detection p-value <0 .01 in at least 20% of samples) were included in this analysis. Expression data was then log2-transformed and quantile-normalised for downstream analysis, and probes were collapsed to gene-level expression based on highest standard deviation across samples. Expression levels of 649 published modules covering different cancer, immune response and proliferation related pathways were generated by taking the median of the genes available within the normalized microarray data [17].

In the NeoAI study,normalized log2 expression of 744 different genes covering the most important aspects of BC – such as proliferation, invasion, PI3K-AKT-mTOR pathways, MAPK signalling, inflammation and the PAM50 gene set – previously analysed using NanoString technology, were included [16,18]. We also explored the changes in two immune-related pathway module scores that had previously been reported to be associated with AI resistance and to predict benefit from immunotherapy [19,20].

PAM50 intrinsic subtypes

In the POETIC subset, each tumour sample was classified into one of the five intrinsic subtypes namely Luminal A, Luminal B, Her2 enriched (Her-E), Basal-like and Normal-like using the 50-gene PAM50 classifier after subgroup-specific centering as reported [7], [15].

In the NeoAI study,the 46 genes raw expression values used in Prosigna were first normalised to eight housekeeping genes (*ACTB, GUS, MRPL19, PSMC4, PUM1, RPLP0, SF3A1* and *TFRC*) and then normalised to a cohort of 229 sample ER+/HER2- tumours previously subjected to the Prosigna assay for subgroup-median centering. Samples were finally classified using the PAM50 classifier applying the proper technical calibration factor as reported [21].

Biomarker analysis

ER status was measured locally and centrally reviewed by IHC. HER2 status was measured locally using immunohistochemistry (ICH) and/or in situ hybridisation. Ki67 proliferation rate was obtained by immunohistochemistry from staining on formalin-fixed samples using anti-MIB-1 (M7240, DAKO UK). Ki67 rate was categorised into High (≥ 10%) and Low (<10%) at baseline and surgery. Tumours were also classified into 4 classes according to Ki67 changes between the two timepoints: Highbaseline-Highsurgery (H-H), Highbaseline-Lowsurgery (H-L), Lowbaseline-Lowsurgery (L-L) and Lowbaseline-Highsurgery (L-H) as reported previously [22].

Statistical and data analysis

Statistical analysis was performed with R version 3.6.3 software. A two-tailed p-value of less than 0.05 was considered statistically significant. T-tests were applied in all unpaired comparisons. Paired t-tests followed by Benjami-Hochberg corrections for multiple comparisons were carried out to compare the changes of PAM50 intrinsic subtype’s correlation scores between baseline and surgery biopsies. For module score, a combined threshold of significance was defined as adjusted p-value < 0.05 and log2 fold-change (FC) > |0.3785116|. For the single gene analysis, a more restrictive fold-change threshold was applied (*Log*2FC > |1|). Spearman Rank correlation was used to explore the correlation of changes in intrinsic subtype classification, expression of some particular genes and/or module scores with duration of AI treatment in the NeoAI study. Significance Analysis of Microarrays (SAM analysis) was used to select key gene module scores associated with early AI resistance to evaluate the impact of AI on changes in their expression [23, 24]. Survival analyses of Time to Recurrence (TTR) and Overall Survival (OS) in the POETIC and of OS in the NeoAI were performed respectively. Due to the lack of data of recurrence, within the NeoAI study, we also determined the association of changes in gene expression with risk of recurrence score (ROR score) at surgery as a surrogate biomarker of relapse. To do so, we previously assessed the correlation between ROR score at surgery and TTR in the POETIC subset (p-value 0.03). Multivariable Cox regression models adjusted for standard clinicopathological variables including PR, HER2 status, tumour grade, pathological tumour size, histological type, nodal status and vascular invasion were performed to assess the independent prognostic value of changes in gene-expression and intrinsic subtypes.

Ethics statement:

Ethical approval for POETIC (Trial Number CRUK/07/ 015) was provided by NRES Committee London–South East. For the NeoAI study ethical approval was received from an NHS research ethics committee (reference 17/EM/0145). Patients from both studies consented to molecular analysis of their samples for research purposes.

Results

1. **Changes of intrinsic subtypes induced by short- and longer-term neoadjuvant AI therapy.**

The demographics and molecular characteristics of the patients of the two cohorts are shown in **Supplementary Table 1.** Baseline molecular characteristics were different between POETIC and NeoAI cohorts with the majority of samples being Luminal A 64.2% (88/137) in the POETIC treated samples subset and Luminal B (38/121; 47.5%) in NeoAI. The rest of baseline clinicopathological characteristics were similar amongst the two subsets.

The differences of intrinsic subtype between baseline and surgery were more frequent in the POETIC treatment group than in controls (38% vs 23.4%).In the treated group, most Luminal B tumours at baseline (90.0%, 27/30) and 50.0% (6/12) of Her2-E were re-designated as Luminal A or Normal-like subtypes (**Figure1A and B**), while 41.7% of Her2-E and most Luminal A, 85.2% (75/88) and basal-like 66.7% (2/3) tumours remained unchanged after 2 weeks of AI. **Figure 1B** illustrates the changes within Luminal B tumours at baseline after 2wk of AI, and that the tumours were increasingly more similar to prototypical Luminal A and Normal-like tumours at 2wk timepoint. In the control group, although 33.3% (4/12) of Luminal B tumours were re-classified into Luminal A and the majority did not change (**Figure 1C),** the difference in intrinsic subtypes after 2wk (untreated) were likely due to cases that had close similarity with more than one subtype. In particular, baseline Luminal B that were re-classified into Luminal A tumours had close proximity to prototypical Luminal A tumours as illustrated in **Figure 1D,** in contrast to the clear shift from Luminal B to Luminal A seen in the treatment arm. In addition, all PAM50 intrinsic subtypes scores, defined as the correlation coefficient scores to each prototypical intrinsic subtype average gene expression profile (i.e. centroid), changed significantly after 2-weeks of AI, while there were no significant changes in the controls (**Supplementary figure 2A**).

Similar to POETIC, in the NeoAI study, most Luminal B tumours (76.3%, 29/38) were re-designated as Luminal A or Normal-like; only 13.2% remained unchanged, while10.5% as Basal-like or Her2-E. Fifty precent (5/10) of Her2-E tumours remained as HER2-E while 20.0% (2/10) re-designated to Luminal A and 30.0% (3/10) to Normal-like (**Figure 1E and 1F**). In this cohort, the changes of the correlation coefficient between baseline and surgery in all intrinsic subtypes were also significant except to basal-like, probably due to the low number of samples in that subtype (**Supplementary figure 2B**).

To further investigate the impact of AI duration on intrinsic subtypes, we tested the correlation of duration of AI with changes of intrinsic subtype and there was no statistically significant observed relationship (p=0.19; **Supplementary figure 3**). Overall, the differences in intrinsic subtype classifications were comparable after neoadjuvant endocrine therapy regardless of the duration of treatment, although the total numerical changes in the NeoAI study appeared higher compared to the treated samples in POETIC (67.5% vs 38.0%), likely due to a higher proportion of Luminal B tumours at baseline in the NeoAI study.

1. **Changes of gene expression profiles by short- and long-term neoadjuvant AI treatment**

Gene expression data in the POETIC subset was computed in module scores according to annotated pathways, immune-response, and selected drug-target response signatures. Two module scores (FOS-JUN modules) were significantly up-regulated and eleven significantly down-regulated post short-term AI therapy, and these modules included pro-tumorigenic signalling modules associated with proliferation, retinoblastoma (RB) loss and chromosome instability (CIN) (**Figure 2A**). Within Luminal A samples, twelve of them also showed a significant change including the upregulation FOS and JUN. Within Luminal B tumours, eight modules’ scores increased, and nineteen decreased significantly post-treatment (**Figure 2B**). As expected for a highly proliferative ER-dependent intrinsic subtype, Luminal B tumours showed a remarkable downregulation of module scores involving proliferation, RB-loss, p53 status, B cell pathways and the Chemo-Endocrine Score (CES). Significant upregulation of FOS and JUN module scores was also observed in this subset.

As expected, there were only three module scores significantly different between baseline and surgery in POETIC controls, including the upregulation of FOS and JUN modules (**Figure 2C**).

Our group had previously identified the upregulation of *FOS* and *JUN* expression in both treated and control samples as an artefactual effect resulted from pre-analytical sample processing due to handling methodology [25][26]. In this study, the expression of the seventeen genes from FOS and JUN module scores was explored. The expression of all those genes was strongly correlated at surgery in both POETIC treated and control samples. Six genes – *JUN*, *FOS*, *FOSB*, *EGR1*, *ZFP36* and *DUSP1* – showed significantly higher expression in surgical samples in relation to the paired baseline samples in both treated (p-value all genes < 0.0001, Log2 FC = 0.5-1.8) and controls (p-value all genes < 0.0001, Log2 FC = 0.5-2.2; **Figure 3).**

Looking at the changes of expression profiles at a single gene level from baseline to surgery in the two studies (**Supplementary Table 2**), a higher number of genes involving proliferation, keratin expression and endocrine related pathways like *PGR*, were down-regulated in NeoAI when compared to POETIC. More genes from key pathways in BC such as MAPK and PI3K-AKT (i.e *IGF1, NR4A1 and NGFR*) or mTOR (*BTG2*) [15] were up-regulated in the NeoAI study (**Supplementary Table 3)**.

The differential expression of genes in common between both datasets are shown in **Figure 4A and 4B**. Genes from FOS-JUN modules *(FOS, JUN and ERG1*), MAPK/ERK, PI3K-AKT and JAK/STAT pathways, and *IGF1* involved in tumour growth and resistance to AI, were up-regulated in both studies. Consistent to the mechanisms of endocrine therapy, most of the downregulated genes in common were involved in cell-cycle regulation and proliferation. A higher number of genes changed significantly from baseline to surgery after longer term treatment compared to shorter AI therapy in the overall populations (**Figure 4A)** and in Luminal B tumours only (**Figure 4B)**. In addition, although fold changes were highly correlated between the two datasets, the magnitude of changes for individual genes in POETIC microarray data matrix was smaller compared to the NeoAI nanostring data matrix.

In order to investigate gene expression changes under AI treatment relating to the biology of intrinsic subtyping and artefact effect, the list of genes were compared among the following subgroups: (1) All POETIC treated (2) POETIC Luminal A treated, (3) POETIC Luminal B treated, (4) POETIC controls, (5) all NeoAI, (6) NeoAI Luminal A and (7) NeoAI Luminal B. **Figure 4C** shows the exclusive genes that were found significantly differently expressed between baseline and surgery in each particular subgroup and the genes in common with the other subgroups, namely intersections. The four common genes that changed significantly in all categories of patients treated with 2wk of AI or longer-term AI were *CD20*, *EGR1*, *TOP2A* and *UBE2C*, all being involved in cell-cycle regulation and proliferation. Noteworthy, only *FOS* was common for all patients including treated and controls.

In a separate analysis, gene expression levels in non-Luminal tumours of NeoAI study were also significantly affected by AI treatment despite being thought to be associated with non-response to endocrine therapy. Those changes include the upregulation of *FOS* and *JUN* and the downregulation of some proliferation and endocrine-related genes including *BIRC5*, *MKI67* and *PGR*.

Finally, to understand the impact of duration of AI on gene expression, multiple t-tests comparing the changes in gene expression between patients receiving shorter (1-2 months) vs longer (>2 months) AI treatment in the NeoAI dataset and Kruskall Wallis tests to compare patients grouped in 1-2 months vs 2-6 months vs >6 months, respectively. There were not significant differential changes in gene expression amongst those categories. We also assessed whether there was positive correlations between the length of AI treatment with the changes in expression level of those genes associated with an artefactual effect in POETIC control samples. We explored the expression of the significant genes within FOS and JUN modules, namely *FOS*, *JUN* and *EGR1*, in the NeoAI study. No correlation of gene expression changes (Log2FC) with length of AI treatment was observed (p-value range=0.68-0.90) (**Supplementary figure 4**).

1. **Impact of longer neoadjuvant endocrine therapy on gene module scores associated with early aromatase resistance between baseline and surgery**

Next, we explored whether there was an association between changes of intrinsic subtypes (i.e. from high-risk subtype to lower-risk) with classes of Ki67-level changes (H-H/H-L).  All intrinsic subtypes with the capacity of lowering the risk (all except LumA and normal) were classfied into “changes” if they turned into a lower-risk intrinsic subtype or “not changes” if they remained the same subtype or turned into a higher risk one. There was a statistical significant association between “No-changes or changes to a higher risk intrinsic subtype” with H-H Ki67 response category in both subsets (POETIC treated cohort: 100% of no-changes were classified as H-H tumours and 48.5% of changes being H-H and 51.5% being H-L; p=0.0013; NeoAI study: 58.8% of no-changes were in the H-H group and 41.2% in H-L and 100% of changes in H-L; Fisher’s exact test p<0.0001).

Most treated Luminal B tumours in the POETIC subset (17/27; 63%) and all Luminal B tumors in the NeoAI study (33/33; 100%) that were re-classified as Luminal A or Normal-like changed from high Ki67 at baseline to low Ki67 at surgery (H-L). These data support that these reclassified Luminal B tumours were AI sensitive ( Fisher’s exact test p < 0.0001).

Using SAM analysis, we selected 103 candidate gene modules at baseline that were associated with response to early neoadjuvant AI amongst 105 ER+HER2 negative tumours in the POETIC treated group. As expected, baseline Ki67 was remarkably higher within Luminal B intrinsic subtype samples with a trend on retaining high Ki67 after 2 weeks of AI compared to Luminal A tumours (**Supplementary Figure 5A**). There were twenty-four immune-related gene modules covering immune-cell pathways, immune-checkpoint component and IFN gamma biology high-expressed at baseline that were associated with early AI resistance **(Supplementary Figure 5A**). These gene modules include some genes that have been previously associated with Luminal B resistant tumours such as *IFNG, STAT1, IDO1, LAG 3* and *CTLA4* [19]. Visualising the gene expression changes in paired baseline-surgery samples following short AI treatment, there was a general trend observed for downregulation of proliferation-related module scores but otherwise, no changes on expression of the selected modules, including the 24 immune-related gene modules, were associated with differential response to AI (**Figure 5**). To assess the differential gene expression changes between responders and non-responders tumours (H-H vs H-L), SAM analysis based on changes of the module scores from baseline to surgery was performed in the POETIC subset. Changes in five module scores covering ER signaling, proliferation and cell-cycle were significant **(Supplementary Figure 5B**). Supplementary figure 6 demonstrates the overview of differential changes in gene expression levels (i.e. expression level at surgery minus expression level at baseline) selected by SAM on Ki67 response categories (H-H vs H-L, n=74) within the NeoAI dataset. Ninety-nine differentially expressed gene changes were selected by SAM (FDR<0.001, (**Supplementary figure 6**). GO enrichment analysis showed that genes related to proliferative and cell cycle pathways were upregulated in the H-H group compared to H-L.

To investigate further the changes of immune-related features associated with resistance to AI by duration of NET, two immune-related module scores were calculated: (1) The “Durvalumab signature” (median of *PD-L1*, *LAG3*, *CXCL9*), previously reported to predict response to immunotherapy in melanoma [20] and (2) the¨Immune-tolerance signature” (median of *PD-L1*, *LAG3*, *IDO1*), a module score reported by *M.Ellis* group as associated with resistance to AI in Luminal B tumours in the neoadjuvant setting [19].

In the POETIC subset, the higher expression of “Durvalumab signature” and ¨Immune-tolerance signature” was associated with H-H tumours (**Supplementary figure 7A**). In that setting no significant changes of the signature expression from baseline to surgery were seen in either H-H or H-L categories (**Figure 6A and supplementary figure 7A**). On the other hand, in the NeoAI cohort, there was also a higher expression of immune-related signatures in H-H tumours compared to H-L at baseline (**Supplementary figure 7B)** with a differential increase in the expression of “Durvalumab signature” (p=0.053) and “Immune-tolerance signature” (p=0.022) from baseline to surgery in H-L tumours compared to H-H tumours (**Figure 6B**). Although the expression of both immune signatures at surgery remained significantly higher in H-H tumours compared to H-L in the POETIC subset (**Figure 6C**), it was not significantly different after long-term AI therapy (**Figure 6D**). The association of the changes of the individual genes included in the two immune-related module scores with resistance to AI, was also assessed. Our results suggest that the enrichment on *PDL1* after longer AI might be the key driving the differences between responders and non-responders in the NeoAI study (**Supplementary figure 7C**).

Finally, to explore the impact of AI duration on the expression of these two early endocrine-resistance immunes-module scores, we looked at the correlation of their expression with time under AI in the NeoAI study. There was no correlation between the changes on their expression from baseline to surgery (Log2FC) with duration of AI (**Supplementary figure 8)**.

**4. Impact of the significant molecular changes under neoadjuvant aromatase inhibitor treatment on survival**

To assess the clinical impact of the observed findings, we tested the association of the significant features described above with patient survival data, in each of the two datasets as follows: 1. changes in the correlation coefficient scores to prototypical intrinsic subtype centroids from baseline to surgery, 2. significant changes that associated with resistance to AI, 3. significant changes from baseline to surgery in all tumours, 4. significant changes from baseline to surgery in Lumina B tumours.

Results are shown in **supplementary** **table 4** (POETIC) and **5** (NeoAI). First, the increase of correlation score to Luminal B centroid was associated with worse survival in both datasets, while the increase of the correlation scores to Luminal A and Normal centroids were associated with better survival. These findings are in line with the observed association of changes in intrinsic subtype with response to AI. Meanwhile, most of the changes associated with resistance to AI (H-H tumours) and a subset of the reported significant changes from baseline to surgery found in both datasets were associated with differential survival. There was no statistically significant association of the immune features such as the increase of *LAG3*, and the increase of “Durvalumab” signature expression with differential outcome observed.

Our results suggested that some of the molecular changes that were associated with resistance to AI, particularly those associated with significant patient survival may be evaluated further as predictive and prognostic biomarkers.

Discussion:

Although AI treatment is the standard of care and most effective therapy for post-menopausal women with early ER+ BC, recurrence to AIs is still a main issue. Molecular characterization of gene expression profiles that occur in response to neoadjuvant AIs is necessary to identify mechanisms of resistance**.** This study was designed to understand the complexities of RNA-based expression changes under short time exposure to ET and to compare them with those that occur under longer-term AI therapy. The main observations from this study are: (i) Most AI sensitive Luminal B and HER2-Enriched tumours change their intrinsic subtype within just two weeks of treatment, mainly from Luminal B towards Luminal A or Normal-like; these changes are associated with differential response to AI and outcome; (ii) by contrast, longer AI treatment may induce additional and greater gene expression changes than 2wk alone (iii) confirmation that FOS and JUN related gene modules and single gene expression upregulation might be explained by sampling manipulation and not just by AI treatment; (iv) BC tumours showing early resistance to AI are characterised by a greater expression of immune-checkpoint component, immune-cells enrichment and proliferation, and these signatures were more impacted by longer AI treatment.

ER+/HER2- tumours should not be considered and treated as a homogeneous disease, thus the analysis of intrinsic subtypes may help to predict response to therapy even in early stage BC [27-29]. Previous data has shown that exposure to ET might lead to profound changes on intrinsic subtypes, mainly from luminal B or Her2-E to Luminal A [30]. However, most of those studies used long-term treatment and included a low proportion of “high-risk” tumours, the majority of them being Luminal A at baseline. From a biological perspective, our study also shows that most Luminal B or HER2-E tumours with the potential of lowering their proliferative biology will change their intrinsic subtype to Luminal A or Normal-like within just 2 weeks of treatment, but more ‘endocrine-resistant’ BC such as basal-like and some Luminal B and Her2-E will not change despite prolonged AI treatment. Based on prior studies, Luminal A and Luminal B baseline tumours are more likely to respond to endocrine therapy than other intrinsic subtypes [30,31], however our study also suggests that changes towards a lower-risk subtype correlate with sensitivity to AI treatment and better survival, beyond baseline intrinsic subtypes. Thus, an early re-assessment of the intrinsic subtype at two-weeks timepoint could be essential to distinguish those “sensitive” tumours from the “resistant” to optimize clinical management following surgery.

Clustering gene expression into signatures/modules catches the biology of main cancer pathways and can be more easily associated with clinical outcome [29][32-35]. In our study, gene expression changes were far more discrete after short-term AI compared to longer AI treatment. As expected, most of the downregulated module scores in the POETIC subset involved a decrease of the “high-risk” characteristics towards a “lower-risk” profile. The general transition to a ‘lower proliferative’ phenotype seen in the POETIC cohort with slight changes on the rest of the genes might be explained by the dominant impact of AI on proliferation and cell-cycle pathways, also in agreement with the rapid changes observed in intrinsic subtypes. For example, RB protein – a critical protein in cell cycle regulation, by preventing unscheduled entry into the mitotic cell cycle. RB-loss would impede the antiproliferative effect of AI treatment and consequently, the downregulation seen under AI would revert this negative feedback [36, 37]. Secondly, a PAM50-based CES in ER+/HER2− early disease is capable of predicting response to ET in comparison to chemotherapy [38]. Higher CES values are associated with endocrine sensitivity and chemo-resistance, hence, the oestrogen deprivation occurring under AI treatment would lead to a drop on this score.

The common differential genes observed for both short- and long-term AI treated cohorts were also involved mainly in cell-cycle, de-differentiation and proliferation pathways, reflecting the main molecular features that would be affected by hormone deprivation regardless duration of treatment. In agreement to previous studies, the effect of longer AI treatment was also seen as a general but deeper downregulation of proliferation and endocrine-related genes, [15][20][24][35]. Noteworthy, only after longer-term neoadjuvant AI, some genes involved in key signalling pathways associated with AI resistance, such as MAPK and PI3K/AKT/mTOR, showed increased expression, and thus a possible mechanism of ER activation in a ligand-independent manner [39-42]. Prior studies have also suggested that ET could have an immune effect leading to an enrichment of tumour infiltrating cells (TILs) and immune-related characteristics [43-45] as well as the importance of TME in cancer progression and therapeutic responses [46]. Here, some stromal-related module scores and single genes within Luminal B samples increased their expression significantly at surgery in both datasets while only long-term AI had an immune-enrichment effect with a significant upregulation of genes involving inflammatory chemokines or immune pathways such as SOCS3, JAK/STAT signalling and other chemokines and interleukins, as well as the two immune-related gene modules in H-L tumours. In this manuscript we have also reported the prognostic value of some of those gene expression changes induced by two-weeks and longer AI treatment respectively, suggesting that the clinical utility of these molecular changes as prognostic or predictive biomarkers to treatment should be studied further. The sample size was small and thus a bigger study is warranted.

Furthermore, our group had previously characterized for the first time the increase of expression of some genes, such as *FOS* and *JUN* as an artefactual effect resulted from pre-analytical sample processing [24]. In the present study, we demonstrate that FOS- and JUN-related module scores increase significantly from baseline to surgery in both NeoAI and POETIC treated cohorts, as well as in surgical samples from POETIC non-treated patients. This confirms that the upregulation of the expression of several genes included in those module scores is induced by sample manipulation rather than only by AI treatment. In the absence of a control group, these artefactual changes would likely be considered as an exclusive effect of AI what might be relevant to all archival collections of ER+BC.

Finally, POETIC trial has previously validated Ki67 as a prognostic marker showing that patients whose Ki67 remains “HIGH” (≥ 10%) after 2 weeks of AI treatment have substantially poorer prognosis than those with a “HIGH” baseline Ki67 which is markedly reduced to “LOW” (<10%) [15,20]. Thus, differential gene expression between H-H and H-L response groups is essential to distinguish those patients who might benefit the most from AI treatment from those who would not. Most of the H-H tumors in our POETIC cohort were Luminal B at baseline and in the NeoAI being Luminal B, basal-like and Her2-E. As expected, the upregulation of cell-cycle and proliferation related genes and modules from baseline to surgery was associated with resistance to AI as measured by changes in Ki67 value and worst patient survival outcome.

Luminal tumours are usually known to be less immunogenic than HER2-E and Basal-like subtypes [46], but those with higher immunogenicity have been correlated with poor prognosis or response to ET therapy [3][19]. Anurag et al have recently demonstrated that immune-checkpoint related genes are upregulated in most Luminal B tumours that show poor response to ET as measured by higher Ki67 [19]. Another study has shown association of AI treatment with a variety of autoimmune disorders in some patients, suggesting a clear effect on immune cells and tumour immunity of AI therapy [47]. However, the magnitude of that effect is still unknown and a clinical study comparing changes on immune-related features after different length of AI to understand the real impact of AI treatment could be important for clinical management. In our study, we looked at both baseline characteristics and changes on immune-related features under different lengths of AI and observed an association of high expression of immune-related module scores measured at 2wk of AI with non-responder tumours in POETIC but not at the surgical timepoint after longer term AI in the NeoAI, probably due to the significant upregulation observed on the expression of those signatures after longer treatment. There was no statistically significant association of the increase in some of those immune related features with survival in the NeoAI cohort but a larger study would be needed to properly refute the hypothesis. Taking together our results and those from literature, a small subgroup of ER+/HER2- BC could potentially benefit from immunotherapy, currently approved for metastatic triple-negative BC and having been tested in other subsets [48-50]. Although the assessment of immune characteristics at baseline could be informative to detect mechanism of resistance to AI, further investigation is still necessary to understand the utility of the analysis of immune-related module scores in surgical samples of patients treated with long-term AI and whether the enrichment of some immune-related signatures in H-L tumours after longer AI treatment has a role in acquired therapy resistance and survival.

Our study has some limitations and strengths. First, we analysed data from two subsets with very different backgrounds, data collection and analytical methodology. However, our targeted analyses were focused on pathways/modules, facilitating the evaluation of changes under AI treatment and comparison amongst datasets. Moreover, we included a control group that enables the distinction between real impact of AI therapy and artefactual effect derived from sample manipulation. Second, although we aimed to compare short- versus long-term AI treatment, the NeoAI dataset includes patients treated with a huge range of AI therapy duration in a pre-surgical setting. Additional whole transcriptome work in a much larger subset of the POETIC treatment arm is on-going to better understand the diversity of intrinsic resistance mechanisms to AI treatment and to increase the power of our survival analyses. This work will also include genomic analysis to determine if there is subset of resistant Luminal patients with immune tolerance and high antigenicity that could benefit from immunotherapy. However, this is a modest but real-world cohort and has a unique value to assess global gene expression data from both pre- and post-AI treatment as defined in the clinical practice. Last but not least, this is the first study to our knowledge to investigate and compare the molecular changes from short- and long-term AI treatment.

**Conclusion**: Short and longer-term AI treatment have similar effects to the changes of the intrinsic subtype’s classifications. However, longer neoadjuvant AI treatment leads to deeper impact on molecular characteristics (changes in gene expression) beyond intrinsic subtypes, including signatures covering immune-checkpoint component reported previously associated with AI early resistant tumours. Some of the observed changes, such as changes in the intrinsic subtypes or enrichment of immune features, were shown not only associated with response to AI but patient outcome, thus providing a supporting rationale to consider the continuation with ET for higher risk tumours if changes in transcriptional gene expression signatures are desired. Finally, further investigation on use of immune-checkpoint component inhibition in this setting is warranted.

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**Figures legends**

**Figure 1.** Differences in intrinsic subtype classification from baseline to surgery in the POETIC subset and the NeoAI study. Changes of intrinsic subtype classifications in all the POETIC treated samples **(A);** in POETIC Luminal B treated samples **(B);** in POETIC control samples **(C);** in POETIC Luminal B control samples (**D)**;in all the NeoAI study samples **(E)** and in NeoAI Luminal B samples **(F).**

**Abbreviations:** Her2-E: Her2 enriched, LumB: Luminal B, LumA: Luminal A, 2wk: 2 weeks timepoint.

**Figure 2.**  Module scores expression changes in the POETIC cohort **A.** Barplots showing the significant module scores expression changes between baseline and after 2 weeks of AI in the POETIC dataset for all samples **B.** for Luminal B samples only and **C.** for controls. The x axis shows the Log2FC and the y axis the significant module scores that changed. Bars are coloured by the degree of significance of the p-value by paired t-test.

**Abbreviations**: Log2FC: Log2 Fold Change, FDR: False discovery rate.

**Figure 3.** Differential single gene expression changes between baseline and surgery of the seventeen genes included in the FOS and JUN module scores in the POETIC cohort. A Differential expression in the treated samples from POETIC subset and B. in the controls. In red there are the significant genes by p-values by paired t-tests and Log2FC.

**Abbreviations:** Log2 FC: Log2 Fold Change, FDR: False discovery rate, NS: Non-significant, P: Significant by p value paired t-tests, P&Log2FC: Significant by p-value and Log2 Fold Change.

**Figure 4.** Single gene expression changes of genes in common between baseline and surgery in the different cohorts **A.** Scatterplot of differentially expressed genes between baseline and surgery measured by Log2FC amongst the entire short-term and long-term AI cohorts. **B.** Scatterplot of differentially expressed genes between baseline and surgery measured by Log2FC amongst Luminal B treated tumours only in the short-term and long-term AI cohorts **C.** Boxplot showing the intersections of common genes differentially expressed between baseline and surgery amongst different combinations of subgroups of sample patients within the POETIC and NeoAI cohorts: all treated patients in POETIC, only treated Luminal A in POETIC, only treated patients Luminal B in POETIC, all patients in NeoAI, only Luminal A in NeoAI, only Luminal B patients in NeoAI, only controls in POETIC.

**Abbreviations:** Log2 FC: Log2 Fold Change, FDR: False discovery rate.

**Figure 5.** Unsupervised hierarchical clustering showing the difference on gene expression modules scores from baseline to surgery in the POETIC treated subset (gene expression changes: surgery -baseline).The module scores shown in this heatmap are those selected at baseline by two unpaired Significance Analysis of Microarrays (SAM analysis) between Ki67 H-H vs H-L categories in the POETIC subset and annotated by the main categories**.**

**Abbreviations**: H-H: Ki67 Highbaseline- Ki67 Highsurgery, H-L: Ki67 Highbaseline- Ki67 Lowsurgery, Her2-E: Her2 enriched, LumB: Luminal B, LumA: Luminal A, 2wk: 2 weeks timepoint, GE: Gene expression.

**Figure 6.** Boxplots showing changes in gene expression from baseline to surgery of the two immune-related signatures (”Durvalumab” and “Immune-tolerance”) amongst H-H and H-L Ki67 response categories in **A**. the POETIC treated subset and **B.** in the NeoAI study**.** Boxplots showing gene signature expression of the two immune-related signatures at surgery stratified by H-H and H-L tumours in the **C**. POETIC treated subset and **D.** in the NeoAI study.

**Abbreviations:** H-H: Ki67 Highbaseline- Ki67 Highsurgery, H-L: Ki67 Highbaseline- Ki67 Lowsurgery, Her2-E: Her2 enriched, LumB: Luminal B, LumA: Luminal A, 2wk: 2 weeks timepoint.