UNIVERSITAT DE BARCELONA

Unravelling the molecular complexities of early-stage oestrogen receptor positive breast cancer to identify biomarkers of resistance to endocrine therapy

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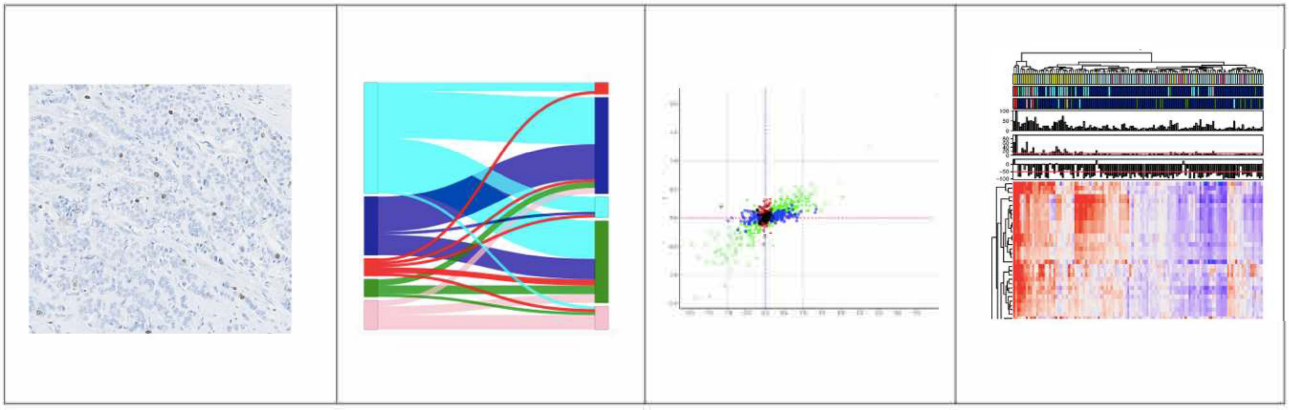
TESIS DOCTORAL

UNRAVELLING THE MOLECULAR COMPLEXITIES OF EARLY-STAGE OESTROGEN RECEPTOR POSITIVE BREAST CANCER TO IDENTIFY BIOMARKERS OF RESISTANCE TO ENDOCRINE THERAPY



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Dra. Sonia Pernas and Dr. Maggie C.U. Cheang agree with the presentation of Milana Bergamino Sirvén's thesis with the title: "Unravelling the molecular complexities of early-stage oestrogen receptor positive breast cancer to identify biomarkers of resistance to endocrine therapy".

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Dr. Sonia Pernas

Dr. Maggie C.U. Cheang

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Glossary/Glosario

English abbreviations:

AIs: Aromatase inhibitors

APM: Antigen Processing Machinery

AR: Androgen Receptor

BC: BC

BCR: Biospecimen Core Resource

BRCAness: BC Gene deficiency

C: Cluster

CD8 T cells: Cytotoxic T lymphocytes

CDK4: Cyclin-dependant kinase 4

CDK6: Cyclin-dependant kinase 6

CES: Chemo-endocrine score

CI: Confidence Interval

EMC: Extracellular matrix

ER: Oestrogen receptor

ERBB2: HER2 gene

ESR1: Oestrogen Receptor 1 gene

ET: Endocrine Therapy

FDR: False discovery rate

FFPE: Formalin-fixed paraffin-embedded

FGFR1: Fibroblast growth factor receptor

FOXA1: Forkhead BoxA1

GFRs: Growth factor receptors

GR: Good responders

H-H: $High_{baseline}$-$High_{surgery}$

H-L: $High_{baseline}$-$Low_{surgery}$

Her2-E: Her2- enriched

HER2+: Human epidermal growth factor receptor positive

HR: Hazard Ratio

HRD: Homologous Recombination Deficiency

IDO1: Indoleamine 2,3-dioxygenase gene

IFN: Gamma, Interferon Gamma

IGF1R: Insulin-like growth factor 1 receptor

IHC: Immunohistochemical/immunohistochemistry

IO: Immuno-oncology

IR: Intermediate responders

iRECIST: Immune-response evaluation criteria in solid tumours

$Ki67_{2w}$: Ki67 at the two-week time point

L-H: $Low_{baseline}$-$High_{surgery}$

L-L: $Low_{baseline}$-$Low_{surgery}$

log2 FC: log2 fold change

LumA: Luminal A

LumB: Luminal B

MHC2: Major histocompatibility complex 2

NET: Neoadjuvant ET

NSCLC: Non-small cell lung cancer

OR: Odds Ratio

ORR: Objective response rate

OS: Overall survival

PD-L1: Programmed death-ligand 1

PD-L2: Programmed death-ligand 2

PD1: Programmed cell death protein 1

PGR: Progesterone Receptor

PFS: Progression-free survival

PR: Poor responders

PTEN: Phosphatase and tensin homolog

RECIST: Response evaluation criteria in solid tumours

Rb1: Retinoblastoma

ROR score: Risk of recurrence score

SAM analysis: Significant analysis of microarrays.

SOX2: SRY(Sex determining region Y)-box2

TGFB: Transforming growth Factor Beta

TCGA: The Cancer Genome Atlas

TIGIT: T cell immunoreceptor with Ig and ITIM domain

TILs: Tumour-infiltrating lymphocytes

TIS: Tumour Inflammation Signature

Treg: Regulatory T cells

TTR: Time to recurrence

Spanish abbreviations:

CM: Cáncer de mama

RE: Receptores de estrógenos

HER2: Receptor 2 del factor de crecimiento

IHC: Inmunohistoquímica

HT: Hormonoterapia

TTR: tiempo hasta la recurrencia

SAM: Análisis de Significación de Microarrays

HER2-E: HER2-enriched

Enumeration of the articles included in this thesis/ Enumeración de los artículos incluidos en la tesis.

Esta tesis está en formato de compendio de artículos. Consta de 9 objetivos, dos artículos científicos y una revisión:

Manuscript 1. Lights and Shadows in Immuno-Oncology Drug Development
Authors: **Milana Bergamino**, Sonia Pernas, Maggie Cheang.
IF: 6.63
Quartile and knowledge area (following Journal Citation Report, JCR, o Scimago Journal Rank)
Cancers, Q1, Medicine: Oncology.

Apart from the supplementary materials from the manuscripts, which are included at the end of this thesis, it also contains two additional data sets that have not been included in each of the main manuscripts:

Manuscript 2.
Title: Impact of duration of neoadjuvant aromatase inhibitors on molecular expression profiles in oestrogen receptor positive BCs.
Authors: **Milana Bergamino**, Gabriele Morani, Joel Parker, Eugene F Schuster, Mariana F Leal, Elena López-Knowles, Holly Tovey, Judith M. Bliss, John F.R Robertson, Ian E. Smith, Mitch Dowsett and Maggie, C.U. Cheang.
IF: 12.53
Quartile and knowledge area (following Journal Citation Report, JCR, o Scimago Journal Rank): Clinical Cancer Research, Q1, D1, Molecular biology: cancer research; Medicine: oncology

Manuscript 3.
Title: HER2-enriched subtype and novel molecular subgroups drive aromatase inhibitor resistance and an increased risk of relapse in early ER+/HER2+ BC.
Authors: **Milana Bergamino**, Elena López-Knowles, Gabriele Morani, Holly Tovey, Lucy Kilburn, Eugene F Schuster, Anastasia Alataki, Margaret Hills, Chris Holcombe, Anthony Skene, John Robertson, Hui Xiao, Ian Smith, Judith Bliss, Mitch Dowsett and Maggie, C.U. Cheang on behalf of the POETIC investigators.

IF: 11.205

Quartile and knowledge area (following Journal Citation Report, JCR, o Scimago Journal Rank) *ebiomedicine*, Q1, Medicine: oncology

Additional data set manuscript 2. **Additional information on baseline gene expression in ER+/HER2- breast cancer and its predictive value.**

Additional data set manuscript 3. **Additional information on new molecular subgroups based on BC360 signature expression**

Summary of the thesis in Spanish/ Resumen de la tesis en castellano

Directoras de tesis: Dra Sonia Pernas y Dra Maggie Cheang

Título: Descifrando las complejidades moleculares del cáncer de mama precoz con receptores de estrógeno positivos para identificar biomarcadores de resistencia a la hormonoterapia.

Introducción:

El 80% del cáncer de mama (CM) expresa receptores de estrógenos positivos (RE+) y hasta un 15% de ellos también sobre-expresa o amplifica el receptor 2 del factor de crecimiento epidérmico humano (HER2), lo que confiere una biología molecular y comportamiento distintos en el CM precoz con RE+. Los mecanismos de sensibilidad y resistencia a los distintos tratamientos en CM aún se desconocen en su totalidad, sin embargo, se sabe que los biomarcadores derivados de la expresión proteica determinada únicamente por inmunohistoquímica (IHC) no siempre predicen de manera adecuada la respuesta a los diferentes tratamientos en CM. Por ejemplo, la sobreexpresión HER2+ o los RE+ no son predictores obligados de respuesta a tratamientos con terapia anti-HER2 u hormonoterapia (HT), respectivamente. En el año 2000 se descubrió la actual clasificación molecular en CM que clasificó a los tumores en subtipos de acuerdo con diferencias en sus patrones de expresión génica (Luminal A y B, Basal like, Normal like y Her2-enriquecido (HER2-E)). Estos subtipos moleculares revelaron diferencias críticas en incidencia, supervivencia y respuesta a los distintos tratamientos en CM y en consecuencia, han permitido el avance hacia una medicina más personalizada. Los subtipos intrínsecos moleculares se encuentran representados en todos los subtipos histológicos, pero en distinta distribución: entre los tumores con RE+/HER2-, hasta un 80-85% son Luminal A o B con solo un 5-10% de HER2-E y un 5% de basal-like, mientras que entre los tumores RE+/HER2+, aproximadamente un 40% son HER2-E, un 55% Luminal A o B y hasta un 5% basal-like.

A pesar de los avances médicos experimentados en la ultima década, una alta proporción de pacientes con CM precoz con RE+ (alrededor del 20%) desarrollará resistencias a la HT con un consecuente aumento en su riesgo de recaída. Se han descrito múltiples mecanismos de resistencia a la HT y se conoce que pueden variar dependiendo del subtipo tumoral. Algunos de ellos incluyen: mutaciones somáticas, la intercomunicación entre factores de crecimiento

HER2 y RE o alteraciones en la señalización. Los subtipos intrínsecos definidos en base a la distinta expresión génica también influyen en la heterogeneidad molecular del CM, confiriendo distinto pronóstico y respuesta a los tratamientos. Por ejemplo, se sabe que los tumores HER2-E predicen mayor sensibilidad a tratamiento anti-HER2, pero peor pronóstico en comparación a los tumores luminales.

Existe también cierta evidencia que apunta a un papel relevante de la inmunidad tumoral en la respuesta a la HT tanto en CM precoz que sobre-expresa HER2 como en tumores HER2-negativo (HER2-). Por tanto, es imprescindible mejorar el conocimiento sobre la biología molecular en CM precoz con RE+ para la optimización de los distintos tratamientos disponibles y su consecuente mejora en el pronóstico. Sin embargo, definir los mecanismos de resistencia a la HT en CM RE+ es difícil ya que las pacientes permanecen libres de enfermedad durante largos períodos de tiempo. Además, existe evidencia que indica que puede haber diferencias moleculares significativas entre las muestras tumorales de la biopsia y la cirugía, conocido como un efecto artefactual, probablemente en relación con la manipulación/estrés que sufren las muestras y que no al efecto directo de la HT. Por ello, los ensayos clínicos pre-quirúrgicos o "window off opportunity" o "de ventana", es decir aquellos que evalúan un fármaco durante un corto periodo de tiempo (generalmente durante 2-4 semanas) antes de realizar el tratamiento estándar, son clave para investigar y entender el efecto que el propio tratamiento ejerce sobre el tumor y microambiente tumoral ya que tanto las características basales como los cambios que se producen por el tratamiento, se pueden correlacionar con la respuesta tumoral. Un ejemplo de ello, son los cambios en proliferación celular tales como el Ki67.

En esta tesis doctoral, utilizamos muestras biológicas del ensayo clínico fase III POETIC, el estudio "window of opportunity" más grande del mundo que aleatorizó a casi 4500 pacientes a recibir inhibidores de la aromatasa (IA) de forma pre-operatoria por dos semanas durante el tiempo de espera hasta la cirugía (rama de tratamiento) o bien a no tratamiento (rama control). Utilizamos datos de expresión génica tanto de pacientes con tumores RE+/HER2- como con RE+/HER2+ incluidos también en el estudio POETIC. Asimismo, analizamos los cambios moleculares que se produjeron en una cohorte independiente de pacientes con CM precoz RE+/HER2- del Hospital Royal Marsden que recibieron HT neoadyuvante durante más de 1 mes (hasta 2 años) para compararlos con los resultados del estudio POETIC, tras un período corto de tratamiento con HT.

Hipótesis:

Nuestra hipótesis principal se basa en que los mecanismos de resistencia a la terapia endocrina son consecuencia no sólo de la expresión de ciertas características moleculares basales, sino también de los cambios que el propio tratamiento ejerce sobre el tumor y que varían, en función del subtipo de CM con RE+ (HER2+/HER2-). También creemos que la inmunidad tumoral y algunas características de los puntos de control de la inmunidad están implicados en estos mecanismos de resistencia. La caracterización de estos puede servir para descubrir biomarcadores potenciales de respuesta al tratamiento hormonal, así como de pronóstico. En concreto, en la cohorte de RE+/HER2- creemos que los cambios que la HT produce en el tumor o microambiente tumoral son distintos según la duración del tratamiento, siendo estos mayores a mayor duración del mismo.

Por otro lado, esperamos que dentro del subgrupo de cáncer de mama con RE+/HER2+, el subtipo intrínseco HER2-E, así como otras firmas génicas adicionales (algunas relacionadas con la inmunidad tumoral), jueguen un papel esencial como biomarcadores de resistencia a la HT y de mal pronóstico en CM precoz.

Objetivos:

Nuestro objetivo fprincipal es la búsqueda de biomarcadores pronósticos y de resistencia temprana a la HT en CM precoz con RE+ así como la caracterización de la inmunidad tumoral y su rol en esta resistencia.

1. <u>En la cohorte de CM precoz RE+/HER2- pretendemos:</u>
   1.1. **Objetivo 1:** Evaluar los cambios moleculares a nivel del subtipo intrínseco que se producen tras HT durante 2 semanas pre-operatoria y tras un periodo de tratamiento más largo (>1 mes). Evaluaremos si existe correlación entre la duración del tratamiento con HT y dichos cambios.
   1.2. **Objetivo 2:** Evaluar y comparar los cambios moleculares más allá de los subtipos intrínsecos, a nivel de expresión génica, tanto de genes individuales como en vías moleculares (firmas génicas), entre muestras pareadas, es decir entre la biopsia basal al diagnóstico y a las dos semanas en la cirugía, tras HT durante 2 semanas y tras un periodo de tratamiento más largo (>1 mes).
   1.3. **Objetivo 3:** Caracterizar los cambios/diferencias moleculares que se observan entre muestras pareadas, entre la biopsia basal al diagnóstico y a la cirugía, de pacientes con CM RE+ que no han recibido tratamiento (controles): efecto artefactual.

1.4. **Objetivo 4:** Evaluar el valor predictivo de respuesta a los IA, tanto de la expresión génica basal como de los cambios moleculares producidos tras dos semanas y tras >1 mes de tratamiento con IA, mediante la correlación de dichas características con los cambios de Ki67 entre la biopsia basal y la cirugía (Ki 67 residual ≥ o <10%).

1.5. **Objetivo 5:** Evaluar el valor pronóstico en cuanto a riesgo de recaída y supervivencia global de los cambios moleculares observados tras 2 semanas de tratamiento con IA y tras >1 mes de duración.

2. <u>En la cohorte de CM precoz RE+/HER2+:</u>

2.1. **Objetivo 6**: Evaluar la expresión génica basal, es decir en la biopsia basal pre-tratamiento, del subtipo HER2-E para predecir resistencia precoz a HT, medida por: 1. Reducción del Ki67 (>75%; buenos respondedores, 50-75%; respondedores intermedios o <50%; malos respondedores) y 2. Ki67 residual a las 2 semanas, en la cirugía ≥10% o <10%).

2.2. **Objetivo 7:** Evaluar la capacidad de los genes individuales y firmas génicas basales más allá del subtipo intrínseco para predecir respuesta/resistencia precoz a HT, en base a los cambios de Ki67 y Ki67 residual a las 2 semanas de tratamiento en la cirugía en CM precoz RE+/HER2+.

2.3. **Objetivo 8:** Identificar subgrupos moleculares adicionales más allá de los subtipos intrínsecos, que nos permitan predecir mejor la respuesta a los IA.

2.4. **Objetivo 9:** Evaluar el valor pronóstico en cuanto a riesgo de recaída de los diferentes subtipos intrínsecos moleculares, genes individuales, firmas génicas en CM RE+/HER2+ y nuevos subgrupos moleculares.

Materiales, métodos y resultados principales en CM precoz RE+/HER2-:

Manuscrito 1

<u>Métodos:</u>

Hemos evaluado la expresión génica pre y post tratamiento en muestras de tejido tumoral de: 1) un subgrupo de pacientes tratadas dentro del estudio fase III POETIC en el que las pacientes se aleatorizaron 2:1 a recibir IA durante dos semanas previas a la cirugía (rama tratamiento) versus no tratamiento (rama control) y 2) una segunda cohorte independiente de pacientes tratadas en el hospital Royal Marsden con HT neoadyuvante durante más de 1 mes en el estudio NeoAI (media de tratamiento de 6.24 meses +/- desviación standard 3.9 meses). Para evaluar la expresión génica basal y los cambios moleculares que se produjeron tras diferentes

duraciones de tratamiento con HT, así como evaluar su valor predictivo de respuesta a HT, se extrajo el ARN del tejido tumoral pre y post- IA de estas pacientes del estudio POETIC (137 pacientes que habían recibido 2 semanas de IA y 47 de la rama control) y de 80 pacientes del estudio NeoAI).

Posteriormente comparamos esos cambios moleculares producidos tras 2 semanas de HT con los observados tras un período más largo de HT neoadyuvante (>1 mes). Se determinaron los subtipos intrínsecos y mediante la expresión de genes individuales se calculó la puntuación de más de 600 módulos/firmas génicas relacionados con diferentes vías moleculares del cáncer y con la inmunidad pre y post tratamiento. Un valor p de menos de 0.05 por ambas colas se consideró estadísticamente significativo. Se calcularon t-student para todas las comparaciones no apareadas. Se llevaron a cabo pruebas t-pareadas seguidas de correcciones de Benjami-Hochberg para comparaciones múltiples, correlaciones de Spearman para explorar la correlación de los cambios de expresión de algunos genes individuales o módulos/firmas génicas particulares con la duración del tratamiento con IA. Se utilizó el Análisis de Significación de Microarrays (análisis SAM) para seleccionar firmas génicas y genes individuales cuya expresión basal y cambios en los mismos estuvieran asociados con resistencia precoz a los IA.

Para establecer el valor pronóstico de estas características moleculares asociadas a resistencia a IA, en términos de tiempo hasta la recurrencia (TTR) y supervivencia global, establecimos modelos de regresión de Cox multivariables ajustados por las variables clínico-patológicas estándares: grado de diferenciación histológica, tamaño tumoral, estado ganglionar y edad.

Resultados:

Objetivo 1: La HT neoadyuvante produce porcentajes de cambio en el subtipo intrínseco muy similares, independientemente de la duración del tratamiento con IA. En la cohorte del estudio POETIC, la mayoría de los tumores Luminal B (90%) y HER2-E (50%) cambiaron a Luminal A o normal-like. En la cohorte de NeoAI se obtuvieron resultados similares, con el 76.3% de tumores Luminal B y el 50% de HER2-E siendo re-asignados a Luminal A. No se observó una asociación entre la duración de tratamiento con IA y la frecuencia de cambios de subtipo intrínseco.

Objetivo 2: A diferencia de los subtipos intrínsecos, el tratamiento con IA neoadyuvante durante un período más prolongado mostró un impacto más marcado a nivel transcripcional, con magnitudes de cambio mayores y en un mayor número de genes, algunos involucrados en

vías de señalización clave en cáncer de mama como MAPK, *PI3K/AKT/mTOR* o en la respuesta immune.

Objetivo 3: En las muestras pareadas (biopsia basal diagnóstica y a la cirugía) de la rama control, reportamos y validamos un aumento significativo en la expresión génica de algunos genes relacionados con las vías de *FOS* y *JUN* implicadas en la mitosis y procesos de diferenciación, a pesar de no haber recibido ningún tratamiento. Estas diferencias moleculares se tratan probablemente de artefactos que se producen como consecuencia de la manipulación de muestras, por ejemplo, secundaria a distinto tiempos a la fijación al formol entre la biopsia y la cirugía. Su existencia puede derivar en resultados sesgados e inapropiados, por lo que es necesaria su caracterización más profunda.

Objetivo 4: Las pacientes con CM RE+/HER2- que presentaban al diagnóstico un subtipo intrínseco con alto índice de proliferación que cambiaron a un subtipo con menor índice proliferativo (por ejemplo, de Luminal B o HER2-E a Luminal A), presentaron una mejor respuesta al tratamiento medida por cambios de Ki67. Asímismo, la expresión pre-quirúrgica de diversas firmas génicas relacionadas con la respuesta inmune y con el control tumoral se asociaron a resistencia a IA. De igual modo, los cambios observados entre la biopsia basal y la cirugía en algunas firmas génicas relacionadas con la inmunidad tumoral y con vías de proliferación celular se asociaron también con resistencia a IA.

Objetivo 5: Varios de los cambios moleculares observados entre la biopsia basal y la cirugía tuvieron un impacto en el riesgo a la recaída de estas pacientes, en términos de TTR. Por ejemplo, en cuanto a cambios en los subtipos intrínsecos moleculares, observamos una asociación entre el cambio de subtipo hacia Luminal B y un mayor riesgo de recurrencia, independientemente de la duración de la HT prequirúrgica. Algunas de esos cambios moleculares asociados con distinta supervivencia fueron el aumento de los coeficiente de correlación a Luminal A y normal o la disminución de la proliferación , que se correlacionaron con una mejor supervivencia, así como el aumento del coeficiente de correlación a HER2-E con un aumento en el riesgo de recaída..

Manuscrito 2

Métodos:

Para el segundo estudio de resistencias a HT en CM precoz RE+/HER2+, utilizamos todas las muestras disponibles de pacientes con CM RE+/HER2+ del estudio POETIC (n=342; 237 tratadas y 105 controles). Se extrajo el ARN del tejido tumoral tanto de la biopsia al diagnóstico como de la cirugía, para la posterior evaluación de la expresión génica de 758 genes mediante tecnología Nanostring (panel BC360 para la evaluación de cáncer de mama - que engloba los

subtipos intrínsecos y 46 firmas biológicas clave en CM incluyendo inmunidad tumoral). La tasa de proliferación celular se estimó como porcentaje de tinción de células cancerosas para Ki67. La respuesta se definió en función de: 1) los cambios relativos de Ki67 desde el inicio hasta la cirugía: respuesta pobre (reducción <50%), intermedia (50-75%) y buena (> 75%) y 2) Ki67 residual tras 2 semanas de tratamiento: alto (≥ 10%) frente a bajo (<10%). Asimismo, se identificaron nuevos subgrupos moleculares basados en diferencias en expresión de *ERBB2* (gen de HER2), vía de señalización de los RE, o vías relacionadas con la inmunidad tumoral, mediante agrupación por consenso ("*consensus clustering*"). Para establecer el valor predictivo de respuesta a IA de las distintas características moleculares, se realizaron análisis de regresión logística. Se utilizó el Análisis de Significación de Microarrays (análisis SAM) para seleccionar firmas génicas y genes individuales cuya expresión basal estuviera asociada con la resistencia temprana a los IA. Para establecer el valor pronóstico de las diferentes características moleculares, en términos de TTR, utilizamos modelos de regresión de Cox multivariables ajustados por las variables clínico-patológicas post-quirúrgicas estándares (grado de diferenciación histológica, tamaño tumoral, estado ganglionar y edad).

Resultados:

Objetivo 6: Nuestros resultados validan el subtipo intrínseco conocido como HER2-E como marcador predictivo de resistencia precoz a la HT en tumores RE+/HER2+.

Objetivo 7: La expresión basal elevada de diversas firmas génicas relacionadas con la señalización endocrina tales como *ESR1, FOXA 1, PGR* y la firma de señalización de la vía del RE se asociaron con respuesta a IA. Por el contrario, la expresión basal elevada de *ERBB2* se asoció a resistencia a IA. De igual modo, las firmas génicas relacionadas con la deficiencia de la reparación del ADN tales como la deficiencia de la recombinación homóloga, la firma subrogada de mutaciones en *p53*, la vía de hipoxia y otras firmas génicas involucradas en el componente de punto de control inmunológico *("immune-checkpoint component"*), tales como *IDO1, IFN Gamma, y PD-L1,* se asociaron a resistencia precoz a IA. Resultados similares se observaron a nivel de genes individuales.

Objetivo 8: Mediante el uso de la técnica de "*consensus clustering*", identificamos 5 nuevos subgrupos moleculares según la expresión basal de genes individuales para predecir respuesta a 2 semanas de tratamiento con IA y con valor pronóstico. Se identificaron 3 subgrupos, fundamentalmente conformados por tumores HER2-E y Luminal B,caracterizados por resistencia a los IA: el subgrupo 1 enriquecido con genes inmunes y relacionados con las quemocinas, así como con niveles bajos de *ESR1*; el subgrupo 2 con genes relacionados con la matriz extracelular, niveles también bajos de *ESR1* y con los niveles más altos de *ERBB2*; y el

subgrupo 3 más "inexpresivo", compuesto principalmente por genes relacionados con la deficiencia de reparación del ADN (deficiencia de la recombinación homóloga). Los subgrupos 4 y 5 se caracterizan por una buena respuesta a IA y enriquecimiento con tumores luminales. Ambos subgrupos sobre-expresan genes relacionados con la vía de señalización del RE, pero el subgrupo 5 también genes implicados en la vía de *MAPK / PI3K* y *RAS*. Estos nuevos subgrupos moleculares con distinto valor predictivo de respuesta precoz a los IA presentaron también distinto valor pronóstico; el subgrupo 2 mostró un aumento significativo del riesgo de recaída, permitiendo distinguir aquellos tumores resistentes a IA y con un riesgo de recidiva aumentado más allá del subtipo intrínseco.

Objetivo 9: Observamos un aumento significativo en el riesgo de recaída para el subtipo HER2-E comparado con los tumores luminales (HR 2.14; 95% CI1.11-4.17; p=0.0224). HER2-E se mantuvo como factor predictivo independiente para peor TTR en el análisis multivariado ajustado por variables clínico-patológicas post-quirúrgicas clásicas. Por el contrario, varias firmas génicas relacionadas con la inmunidad tumoral y otras involucradas en el componente de punto de control inmunológico mostraron un valor pronóstico independiente para bajo riesgo de recidiva. Finalmente, los nuevos subgrupos moleculares caracterizados por niveles altos de *ERBB2* y baja expresión de características inmunes, también presentaron un mayor riesgo de recidiva independiente de otras variables.

Discusión

Aunque el tratamiento con IA ha mejorado el pronóstico de mujeres post-menopáusicas con cáncer de mama precoz con RE+, un porcentaje considerable de las mismas (entre un 15-20%) presentan aún un alto riesgo de recaída. Hoy en día, aún falta mejorar el desarrollo de biomarcadores que nos permitan seleccionar y optimizar los tratamientos más adecuados para cada paciente; por lo que es prioritario ampliar nuestro conocimiento sobre la biología molecular en CM.

Este proyecto de doctorado consta de dos estudios principales que se diseñaron con el objetivo de investigar el valor predictivo de respuesta a la HT y el valor pronóstico de las distintas características moleculares del CM precoz con RE+, tanto en población HER2- como HER2+. En este trabajo se ha realizado una caracterización integral de la expresión basada en el ARN de los cambios que ocurren en este tipo de tumores bajo tratamiento con IA, tanto tras una exposición corta (2 semanas) como tras una duración más larga del mismo (>1 mes). En esta caracterización molecular, se ha incluido también la determinación de los subtipos intrínsecos por PAM50, así como otras vías de señalización clave en CM. También se ha evaluado la

asociación de estas características tumorales iniciales, así como las que acontecen tras el inicio del tratamiento con IA, con la resistencia al tratamiento y la supervivencia de las pacientes.

Por un lado, los resultados de este estudio demuestran que las vías de proliferación e inmunidad tumoral están involucradas en la resistencia precoz a IA en pacientes con CM RE+/HER2-. Además, en este subgrupo de CM, los cambios moleculares que se producen sobre los subtipos intrínsecos son similares independientemente de la duración del tratamiento, asociándose además con distinta supervivencia. Estos hallazgos sugieren la utilidad clínica de la evaluación de los cambios en los subtipos intrínsecos tras tan solo 2 semanas de tratamiento. Por otro lado, el subtipo intrínseco HER2-E, niveles altos de *ERBB2*, alteraciones en la recombinación homóloga y en *p53*, así como también elevada inmunidad tumoral, están asociados a resistencia precoz a IA en RE+/HER2+ así como a distinta supervivencia.

Hasta donde sabemos, los resultados incluidos en esta tesis son únicos. Por un lado, comparamos directamente y por primera vez el efecto molecular que se produce tras distintas duraciones de IA. Por otro, incluimos un estudio con la cohorte más grande reportada hasta la fecha de pacientes con CM precoz RE+/HER2+ en la que se ha evaluado el efecto puro del tratamiento con IA (sin tratamiento anti-HER2). Los resultados de esta tesis doctoral pueden ser de una gran utilidad clínica, ya que responden a la necesidad de búsqueda de nuevos biomarcadores de respuesta a HT mediante la correcta evaluación de su valor pronóstico y predictivo de respuesta, mediante la correlación con marcadores subrogados de respuesta como es el Ki67. En esta tesis también hemos incluido un manuscrito que revisa el diseño de ensayos clínicos para la evaluación de fármacos en immuno-oncología, reforzando la necesidad de estudios translacionales como los reportados en esta tesis, con el objetivo de encontrar biomarcadores de respuesta a los distintos tratamientos en cáncer de mama así como fomentar el uso de variables de evaluación de respuesta subrogados adecuados.

1. INTRODUCTION

1.1 Breast Cancer

Breast cancer (BC) is the most common malignancy worldwide and the first cause of cancer-related death among women, with 2.3 million new cases diagnosed in 2020 and accounting for 685,000 deaths in the same year. As of the end of 2020, there were almost 8 million women alive who had been diagnosed with BC in the past five years. As incident cases are expected to increase by 50% in the next two decades, it will become the most prevalent cancer (1). In addition, there are more lost disability-adjusted life years in women due to BC globally than any other type of cancer. BC occurs in every country of the world in women at any age after puberty but with increasing rates in later years (2).

BC is a heterogenous disease that has been classically classified into 3-4 clinically and biologically different subtypes, according to hormone receptor (oestrogen [ER] and/or progesterone [PR]), and human epidermal growth factor receptor 2 (HER2) status, hormone receptorpositive (ER-positive and/or PR-positive) being the most common type of BC. These classic BC subtypes are based on immunohistochemistry (IHC) markers such as the expression of ER, PR, and HER2, with a prognostic and predictive value for different treatments in BC (3).

Overall, treatment of early-stage BC may include surgical removal, radiation therapy, and systemic therapy (including chemotherapy, endocrine therapy (ET), and targeted therapy depending on tumour and patient characteristics) to treat the microscopic disease that has potentially spread from the breast tumour through the blood and or lymph vessels. Such treatments, which can prevent cancer growth and spread, have improved BC prognosis over the years (4). However, the cumulative risk of hormone receptor positive BC distant recurrences, especially in node-positive cases, has remained steady for decades, being a well-recognised unmet clinical need.

1.2 Breast cancer molecular classification

New biomarkers based on omic technologies have been increasingly used for cancer diagnosis, prognosis, and therapy guidance, heralding the era of precision oncology. RNA biomarkers or gene expression signatures have emerged as a major class thanks to the widespread development of high throughput technologies. The introduction of these high throughput

technologies has improved our knowledge of tumour biology and has opened new windows for improving diagnosis and targeted treatments based on individual alterations and tumour biomarkers (5,6).

During the last 20 years, gene-expression profiling has improved our understanding of the complex BC portrait by identifying at least four intrinsic molecular subtypes: Luminal A, Luminal B, HER2-enriched (HER2-E), Basal-like, and a normal breast-like group, which differ in terms of incidence, prognosis, and response to therapies (7,8). The training set for the PAM50 subtype predictor, co-developed by Professor Perou and Dr.Cheang amongst others, was composed of tumour samples from 220 patients with ER+ orER-HER2+ orHER2- tumours. One thousand nine hundred and six genes were used and included 12 true normal breast samples constituting the normal breast-like centroid (9). A final set of 50 genes from these samples was used to calculate the four intrinsic subtypes and the normal-like. In addition to the intrinsic subtypes, the PAM50 assay provides a risk of relapse (ROR) score, predicting relapse-free survival for patients with node-negative tumours not receiving adjuvant systemic therapy.

The HER2-E subtype is characterised by high expression of HER2-regulated genes and proliferation/cell cycle-related genes with lower expression of luminal-related genes than the luminal A and B subtypes (9,10). This molecular entity is likely to be driven by the EGFR/HER2 pathway, showing distinct behaviour and epidemiological risk factors. All the intrinsic subtypes can be found in each of the classic pathological-IHC-based subtypes but with a different distribution. In ER+/HER2- BC, around 80-85% are Luminal A or B, 5-10% are HER2-E, and only 5% are basal-like. By contrast, in HER2+ BC, this distribution differs according to ER status, with up to 75-80% of ER-/HER2+ being HER2-E, 15% Luminal A or B, and 5% basal-like while only 35-40% of ER+/HER2+ are HER2-E, 55-60% Luminal A or B and up to 5% being basal-like (11,12).

Each intrinsic subtype presents a different response to each of the available treatments and different behaviours. For example, data have shown that luminal A and luminal B baseline tumours might be more likely to respond to ET than other subtypes (13,14) and that ET treatment could lead to profound changes in them. However, changes in intrinsic subtypes after exposure to ET alone and their predictive and prognostic value have been understudied. The use of the PAM50 assay as a predictive and prognostic biomarker tool has been generalised for adjuvant treatment decision-making in patients with ER+/HER2- early BC. However, in

HER2+ disease, its use in clinical practice has not been generalised yet, although it has been extensively used in clinical research. Studies in HER2+ BC have revealed that the HER2-E subtype is a predictor of higher sensitivity to anti-HER2 targeted therapy (15–18) but is associated with worse outcomes compared with luminal tumours. The role of intrinsic subtypes in patients with BC treated with ET has also been explored in HER2+ BC. In a single-arm, multicentric study (PerELISA), 65 postmenopausal patients with hormone receptor positive/HER2+ operable BC received two weeks of letrozole and underwent a tumour re-biopsy for Ki67 evaluation (17). Patients classified as molecular responders (those presenting a Ki67 relative reduction >20% from baseline to re-biopsy) continued letrozole and started trastuzumab-pertuzumab for five cycles. Patients classified as molecular non-responders started weekly paclitaxel for 13 weeks combined with trastuzumab and pertuzumab. This study reported the association of PAM50 intrinsic subtyping with molecular responders, with 92% of luminal A and B vs. 44% of HER2-E and basal-like being molecular responders ($p < 0.001$). This study also reported a significantly higher pCR rate in HER2-E compared with the other intrinsic subtypes (45.5% vs. 13.8%, $p=0.042$). Although these results were promising, this was a small, single-arm, non-randomised study and no survival outcomes were reported. Nevertheless, these results support a potential strategy for de-escalating treatment in the management of those patients with ER+/HER2+ disease.

1.3 Treatment of oestrogen receptor-positive early breast cancer

Hormone receptor positive BC is the most common BC type accounting for about 70% of all newly diagnosed cases (19). Approximately 15% of them also show an overexpression and/or amplification of HER2. A sample is considered ER-positive if at least ≥1% of tumour cell nuclei are immunoreactive via IHC. However, there are limited data on ET benefit for cancers with 1% to 10% of cells staining ER+ (ER low positive). Reproductive factors such as early menarche, late menopause, the absence of pregnancies, or lack of breastfeeding increase the risk of developing ER+ BC. Other known causes that impact on the risk of having BC are long exposures to exogenous hormone receptor agonists via hormonal contraception or hormonal replacement (20).

Oestrogen deprivation therapy is a major treatment strategy for ER+ BC (21), leading to a reduced risk of relapse in early ER+ BC (22–24). Oestrogen biosynthesis is regulated by the hypothalamus and pituitary gland via the actions of GnRH and follicle-stimulating hormone (FSH). It is synthesised in peripheral tissues such as adipose tissue, breast, and skin via the

action of the aromatase enzyme, which converts androstenedione and testosterone released from ovaries and adrenal glands to oestrone and oestradiol, respectively. The ovaries are the major source of oestrogens in premenopausal women (23). In postmenopausal women, the synthesis of oestrogens from androgens occurs through the aromatase enzyme in peripheral tissues.

The landscape of ET has changed since oestrogen deprivation was first achieved by oophorectomy or surgical removal of the adrenal glands. Current ET strategies are based mainly on targeting ER signalling pathways by reducing circulating levels of oestrogens or by blocking the action of oestrogen at its receptor. There are three main types of ET according to their mechanism of action: selective ER modulators (SERM) such as tamoxifen, selective oestrogen down regulators such as fulvestrant (SERDs), and aromatase inhibitors (AI)(25,26). However, in early-stage BC, only tamoxifen and Ais are currently approved. Two major classes of Ais have been developed: 1) type I steroidal drugs that include exemestane, an androgen substrate analogue that competitively but irreversibly binds the enzyme inactivating it, and 2) type II nonsteroidal inhibitors such as anastrozole and letrozole, which are examples of triazoles, which reversibly bind to enzymes (26).

Other treatments including chemotherapy, targeted treatments, or CDK 4/6 inhibitors are also given to patients with hormone receptor positive early breast cancer according to other clinical and molecular factors such as HER 2 status or genomic risk.

1.3.1 Treatment of oestrogen receptor-positive/HER2-negative breast cancer

According to their risk of recurrence, pre-menopausal women **with ER+/HER2-** early BC can be treated with tamoxifen (low-risk patients) or with the combination of ovarian suppression with gonadotropin-releasing hormone agonists (GnRHa) (or alternatively oophorectomy) plus AI (high-risk patients). Postmenopausal patients are treated with an AI for 5-10 years, depending on their risk of recurrence and tolerance(27).

Chemotherapy is also recommended in high-risk ER+/ HER2-negative tumours [I, A] such as histological grade III, Ki67>25%, pN+, ≤35 years old, lymphovascular invasion, progesterone receptors < 20%, ≥ T3. In cases of uncertainty regarding indication for adjuvant chemotherapy (after consideration of all clinical and pathological factors), gene expression assays, such as MammaPrint [I, A], Oncotype DX [I, A], Prosigna, Endopredict, or the Breast Cancer Index,

can be used. These panels provide a score grading the risk of recurrence to assist clinicians with adjuvant treatment decisions (28). These molecular signatures are used for decision-making, including not only the use of adjuvant chemotherapy but also the duration of adjuvant ET(29).

Cyclin-Dependent Kinase 4/6 inhibitors (CDK 4/6i) have dramatically improved progression-free and overall survival (OS) of patients with advanced hormone receptor-positive/HER2-negative BC and, in combination with ET, are now considered the standard of care for first or second lines of treatment (30). CDK4/6i have also been investigated in the early setting for ER+/HER2-negative disease, with contradictory results. In the monarchE study, 5637 patients with high-risk, early-stage BC were randomly assigned to receive standard adjuvant ET, with or without abemaciclib 150 mg twice daily for two years. At a preplanned second efficacy interim analysis, two-year invasive disease-free survival was 92.2% with abemaciclib vs. 88.7% without CDK4/6inhibitorhazard ratio [HR0.75 [95% CI 0.60–0.93]), a difference that was significant and clinically meaningful(31). However, the PALLAS trial, which was performed in parallel to the monarchE trial and in patients with stage II–III hormone-receptor-positive, HER2-negative BC, did not show a benefit of palbociclib in addition to standard ET in the adjuvant setting(32). Based on these results, the use of two years of abemaciclib has been approved in the adjuvant setting in combination with AI +/- GnRH analogues in patients with high-risk ER+/HER2- early BC considered as more than 4 positive axillar nodes or 1-3 positive nodes with ≥ 5 cm and/or G2, Ki67 ≥ 20%.

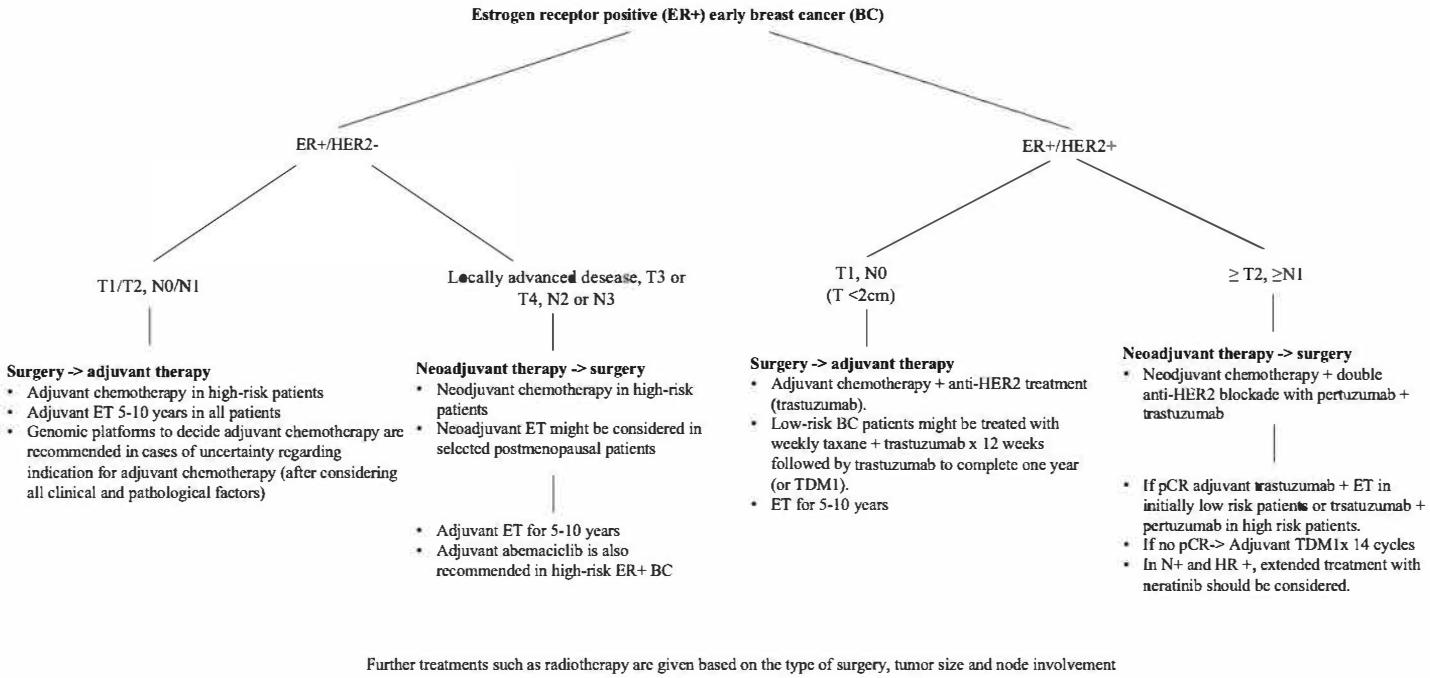
1.3.2 Treatment of oestrogen receptor-positive/HER2-positive breast cancer

Treatment for **ER+/HER2+** early BC patients may include chemotherapy, ET, and anti-HER2 therapy. The addition of trastuzumab to chemotherapy dramatically improved the prognosis of early-stage HER2-positive breast cancer. However, not all patients benefit to the same extent and 15%-31% of patients still recur based on long-term follow-up of adjuvant pivotal trials. A better understanding of tumour biology has led to the development of optimised anti-HER2 drugs and add-on strategies to further improve survival outcomes but also to de-escalating approaches to avoid unnecessary toxicities(33,34). In general, patients with HER2+ early BC should receive neoadjuvant systemic treatment (if tumour size ≥2cm and/or Node +) with dual HER2 blockade with trastuzumab and pertuzumab plus chemotherapy followed by surgery. Adjuvant systemic therapy includes ET for ER+ and anti-HER2 treatment, which differs depending on the achievement or not of a pathological complete response, stressing the importance of using neoadjuvant systemic treatment for patients with stage II or III disease and

administering adjuvant T-DM1 to those patients with residual disease (35). Extended adjuvant therapy with neratinib may be considered in some patients at high risk of recurrence, however, it is unknown what the benefits may be in patients with prior pertuzumab and T-DM1 treatment(36).

Alternatively, surgery can be followed by adjuvant treatment, especially in small, node-negative disease (stage I), and these cases may include a de-escalating regimen with paclitaxel plus trastuzumab for 12 weeks followed by trastuzumab to complete one year of treatment (37). Due to the synergy between trastuzumab and chemotherapy, adjuvant trastuzumab alone is not standard practice, unless the chemotherapy treatment had to be stopped due to toxicity (38). (39). ER+/HER2+ BC patients are also susceptible to receiving hormonotherapy for 5-10 years, depending on their risk of recurrence (28).
The most common strategies for ER+ early BC are summarised in figure 1.

Figure 1. Current treatment strategies for oestrogen receptor-positive early-stage breast cancer. Abbreviations: ER: Oestrogen receptor, T: Tumour, N: Node, ET: Endocrine therapy, HER2: Human epidermal growth factor receptor 2.



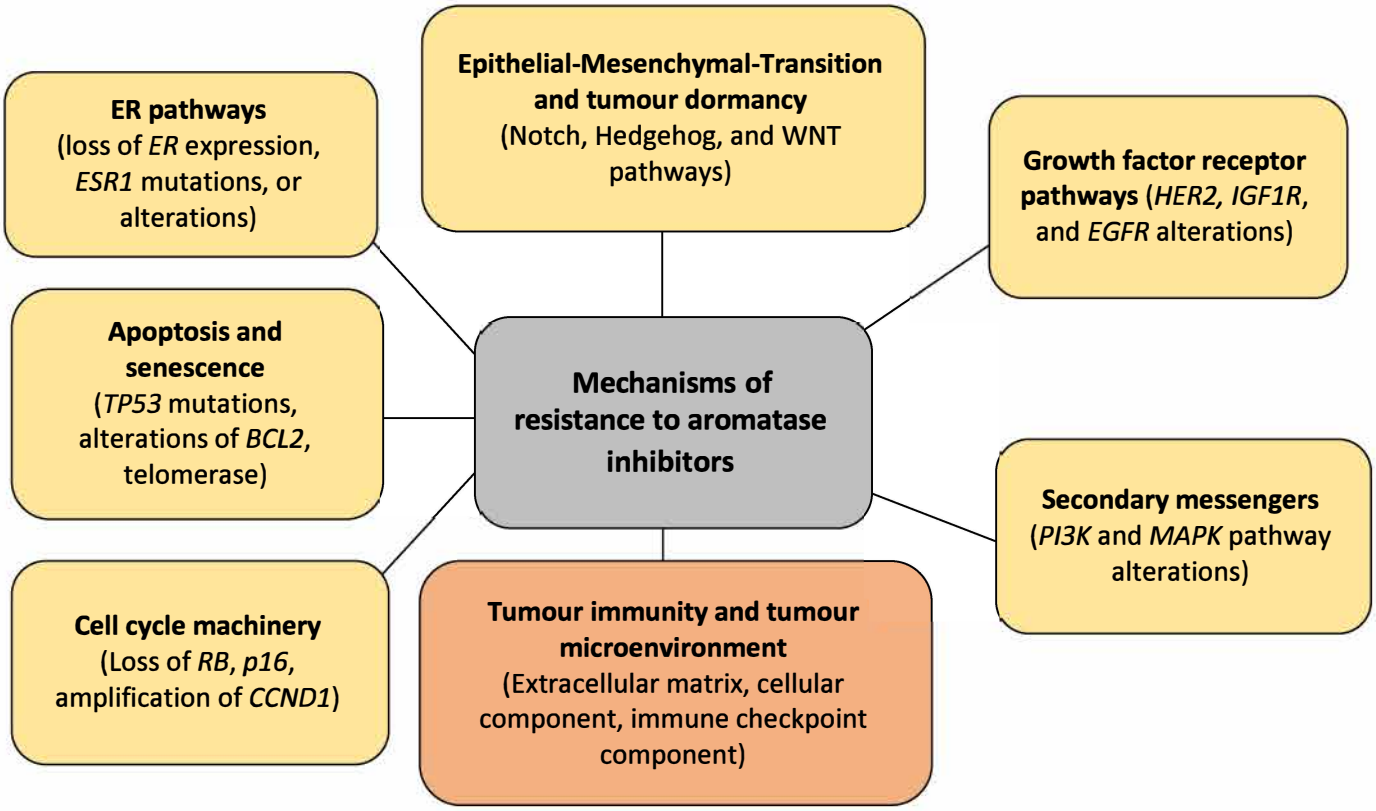
1.4 Mechanisms of breast cancer resistance to endocrine therapy

The majority of patients with advanced disease and up to 30% of patients with hormone receptor-positive BC in the adjuvant setting will become resistant to the inhibitory effects of their ET. A large proportion of patients acquire resistance to ET following initial responsiveness (acquired resistance), and some do not respond from the start (intrinsic resistance). This is associated with tumour growth and disease recurrence or progression and remains an area of unmet clinical need (40).

Over the last decade, several efforts have been made to elucidate the different mechanisms leading to resistance to AI (22). However, the intrinsic nature of ER+ BC, in which the risk of recurrence persists for several decades after diagnosis, has made it challenging to understand the whole picture of ET resistance and whether this resistance is inherent to the primary tumour or results from gained alterations (22,41). A better understanding of these mechanisms would improve the development of new biomarkers to select patients with hormone-dependant BC for better strategies. In addition, the detection of AI resistance in primary tumours, before relapse, is essential because early-stage breast cancer is still within the "curability opportunity".

Different mechanisms associated with resistance to ET have been described, including loss or modification of ERα expression, regulation of signalling pathways such as the PI3K/AKT/mTOR or CDK 4/6 pathways, altered expression of specific microRNAs, growth factor receptors, and cell cycle-related genes, balance of co-regulatory proteins, alterations in ER-related pathways, and co-regulatory proteins amongst others such as the tumour microenvironment (22). Although ER+ BC is less immunogenic than other subtypes, tumour immunity may also be involved in resistance to ET (41). Figure 2 shows the main mechanisms of AI resistance known to date (22).

Figure 2. Summary of the main known mechanisms of resistance to aromatase inhibitors (22).



1.4.1 Somatic mutations

One of the main known mechanisms of resistance to AI is the presence of somatic mutations in, for example, *PI3K, TP53, MAPK1, GATA3*, E-cadherin, or *RB1* (42). Data suggest that AI resistance is encoded by the mutation patterns present in individual tumour genomes; however, detailed and validated studies are still needed. Overall, the mutational maps developed from neoadjuvant AI studies emphasize the genomic heterogeneity that underlies the clinical heterogeneity of the disease. One of the main known mechanisms of AI resistance includes mutations in the PI3K-AKT-mTOR pathway, frequently altered in breast cancer. Activation of *PI3K* has been shown to regulate *ER* expression. Therefore, inhibition of *PI3K* induces *ER* expression, thus double blockade might be the cause of synthetic lethality (22).

1.4.2 *ESR1* alterations

ESR1 mutations are known acquired AI-resistance mechanisms. These mutations were first described in ER+ advanced BC in patients whose disease had progressed after long-term ET. *ESR1* mutations are rare in treatment-naïve primary tumours but frequently appear after ET in metastatic patients. Other molecular alterations of *ESR1* that lead to AI resistance include

amplifications and translocations. All these alterations are more commonly associated with acquired rather than intrinsic resistance (43,44).

1.4.3 Crosstalk between the activation of growth factor receptors (GFRs) and ER signalling

Aberrant activation of HER2 (45), fibroblast growth factor receptor 1 (FGFR1) (46), and insulin-like growth factor 1 receptor (IGF1R) have been identified as other mechanisms of AI resistance (47,48). Their downstream signalling components, such as MAPKs and the PI3K–AKT pathway (49) have also been associated with acquired ET resistance. Activation of the PI3K pathway has been shown to regulate ER expression (50) and mutations in the α-catalytic subunit have been associated with the development of acquired ET resistance (51,52). Prior data have demonstrated that the overexpression of different growth factor receptors (GFRs) signalling through the epidermal growth factor receptor (EGFR) or HER2 activates MAPK in ER+ BC, leading to the loss of ERα expression (50,53), and that inhibition of *MAPK* can reactivate ER expression and tumour responsiveness to ET (54). This crosstalk is particularly relevant in ER+/HER2+ BC (45). Therefore, targeting both ER and HER2 pathways is crucial for patients with ER+/HER2+ early BC (17). Another strategy is to target *HER2* mutations that occur mainly in ER+ breast cancer. However, the adequate selection of patients with potentially resistant tumours and the different strategies needed are still required (36).

1.4.4 Alterations in other signalling pathways

Cell-cycle regulation, including deregulation of cyclin D-CDK4 or CDK6 and enrichment of cyclin D1 (CCND1) have also been associated with resistance to ET (6,42). Data have also shown that epigenetic regulators such as aberrant histone and DNA modifications play a role in ET resistance(6). The host microenvironment, including the extracellular matrix (ECM) and various stromal immune components, are associated with ET resistance (55). Moreover, the chronic inflammation observed in BC is known to promote disease progression and metastasis development (56–58).

1.4.5 Role of tumour immunity in resistance to aromatase inhibitors in oestrogen receptor-positive early breast cancer

Although ER+\HER2- BC tumours are known to be less immunogenic than other types of BC, prior studies have shown that tumour immunity could be involved in resistance to ET (59). Tumour immune-enrichment and immune-checkpoint component-related genes are

upregulated in most luminal B tumours showing poor response to ET as measured by higher Ki67 and worse prognosis (41,60). Some of the immune-related genes involved in AI resistance include *IDO1*, *LAG3*, *CTLA4*, *STAT 1*, and *IFNδ*. A high expression of these genes is associated with higher tumour immune tolerance, which has been related to early resistance to ET (60–62). These findings suggest a potential benefit for immune-checkpoint inhibition in this setting.

By contrast, there is strong evidence suggesting that immune cell pathways and the tumour microenvironment play a crucial role in HER2+ disease (63). HER2+ BC has higher stromal tumour-infiltrating lymphocytes (TILs) compared with HER2- BC, thus conforming a more immunogenic subgroup when compared with other BC subgroups (64–67). HER2-positive BC is generally considered more immunogenic than hormone receptor-positive/HER2-negative BC, but less immunogenic than triple negative (TN) BC. Differences in immunogenicity also exist among intrinsic molecular subtypes, with HER2-enriched tumours being one of the most immunogenic. Compared with other subtypes, HER2-enriched tumours show the highest levels of TILs and are associated with higher expression of immune activation genes (68,69).

The interplay between the immune system and tumour is complex and dynamic, involving the interaction with different HER2-targeted treatments and additional treatments such as chemotherapy, ET, and the modulating action of hormone receptor status, and tumour biology (64). For example, TILs have shown prognostic and predictive value for anti-HER2-targeted treatments in HER2+ BC (70). In addition to their association with survival outcomes, the presence of immune cells and immune-related biomarkers is linked to the effect of different treatments in BC (71–73). In HER2+ BC, the NeoSphere (74) and NeoALTTO (75) studies have shown that tumours with low baseline TILs have lower pathological complete response (pCR) rates. Additionally, both the NeoALTTO (75) and TRYPHAENA (76) studies showed that TILs were associated with improved event-free survival when systemic therapy was administered in the neoadjuvant setting. It was also reported in the FinHER study that TILs were predictive of benefit to adjuvant trastuzumab (71). A pooled analysis of six prospective neoadjuvant clinical trials showed that increased TILs were associated with higher pCR rates and improved disease-free survival in HER2+ BC (77). However, the analysis did not show an association between increased TILs and OS. In contrast, data from the adjuvant N9831 study suggested that the increased presence of TILs was associated with an improvement in recurrence-free survival in patients who received chemotherapy alone, but not among patients treated with chemotherapy plus trastuzumab (78).

When looking at the immune cell subtype in the tumour microenvironment, CD8+, CD4+ Th1, and NK cells are generally considered to favour a tumour-suppressive environment, whilst CD4+ T-helper 2 (Th2), FOXP3+ T-regulatory, and dendritic cells play a pro-tumorigenic role (79). Furthermore, the modulation of immune cells that occurs in HER2+ BC has shown a clinical impact on treatment efficacy (80,81).

Altogether, previous research suggests a crucial role of tumour immunity and immune-checkpoint component on prognosis and resistance to different treatments in ER+ both HER2- and HER2+ early BC. However, the impact of these immune features on resistance to AI treatment has been understudied and requires further investigation.

1.5 Limitations in the study of resistance to endocrine therapy. Window-of-opportunity clinical trials and on-treatment tumour biopsy utility

Short pre-surgical trials of an investigational therapy and on-treatment biopsies are a unique setting to correlate treatment with changes in proliferation of, for example, Ki67 as a surrogate marker of drug activity (82,83) Ki67 has been developed as a biomarker of ET efficacy and may predict long-term outcomes (84).

Intrinsic resistance, i.e., lack of response to initial ET, can be diagnosed at 2–4 weeks following neoadjuvant AI therapy, if tumour Ki67 levels are over 10%. This strategy has been explored in the phase III POETIC trial and other clinical trials and will be developed in the following chapters (82,85). Other neoadjuvant studies investigating different ETs have demonstrated that approximately one-third of cases fail to suppress the Ki67 index to below 10%, indicating early resistance to treatment.

The study of endocrine treatment resistance has encountered several limitations:

a) **Patients are disease-free for long periods**. Pre-surgical and neoadjuvant trials facilitate the study of resistance with the collection of viable paired biopsies at baseline and at surgery and the possibility of correlating different molecular characteristics with response and outcomes, even years later (2,41,86–88).

b) **Immunohistochemistry (IHC)-based biomarkers to guide treatment decisions**. IHC biomarkers are not always good predictors of response to the different treatment options in

early BC. For example, HER2+ overexpression or ER+ are not compelling predictors of response to anti-HER2 targeted therapy or ET respectively (19). Biomarker profiles that distinguish between responders and non-responders are usually based on pre-treatment tumour characteristics, without considering the effect of the drug on the tumour itself (89–91). In the last decade, gene-expression profiling has improved our knowledge of the heterogeneity of BC biology (92). Supervised analysis has led to the development of predictive gene-expression signatures and new clinical assays. Unsupervised analysis has identified multiple signatures covering cell type origin, signalling pathway proliferation and included the "intrinsic subtypes" of BC (6,8,12,92,93).

Knowledge about the biology underlying BC has provided a way to sub-classify the disease with the ultimate goal of treatment personalisation. Since gene expression profiling technologies were introduced, grouping genes with similar expression profiles has been essential for a better interpretation and improvement of functional genome annotations. A critical step in the gene expression datasets has been how to group genes into co-expression categories. The main signatures identified in BC group genes are based on similar biology, driven for example by ER and HER2 status and proliferation (5,94). There is a wide range of different approaches to group gene expression in pathways covering certain characteristics of cancer biology. However, clustering genes into signatures or modules has been the method most used as they include cancer pathway biology, identifying more homogeneous classes, and are also associated with a more uniform clinical outcome (5,50,95–97). Gene expression profiles have been previously categorised into molecular signatures covering the main pathways in BC, including tumour biology, immune and tumour microenvironment to facilitate the understanding of BC biology and differential behaviour (95).

c) **The impact of different lengths of AI treatment on molecular features has not been directly compared, thus making it difficult to distinguish intrinsic resistance from *de novo* or acquired resistance** (22). Reduced ER-dependence and E2F-signalling activation after short- and long-term neoadjuvant AI have been associated with poor response (43), while the enrichment of *ESR1* mutations has also been associated with resistance to AI in patients treated with long-term neoadjuvant AI in primary BC (98). Based on these differential mechanisms, for the comparison of the effect of different lengths of neoadjuvant AI therapy on molecular features, it is necessary to elucidate the full impact on molecular alterations that might limit response and lead to clinical resistance.

d) **HER2 status within ER+ BC tumours causes different patterns of recurrence and response to AI, but it has been barely characterised**. (18,94). In general, ER+/HER2+ BC is associated with a reduced antiproliferative effect of ET and lower response rates, including lower pCR rates to anti-HER2 targeted therapy than ER-/HER2+ tumours. However, sensitivity to AI treatment has barely been explored in this setting (51,70,99–103). It is known that there is an impeded anti-proliferative response of HER2+ BC to ET (90,104), but the underlying causes are still unknown. Identifying potential biomarkers of response to ET beyond HER2 and ER may help clinicians to distinguish patient populations and thus could avoid unnecessary treatments and their associated costs and toxicities.

e) **Tumour immunity and resistance to AI.** Previous research has identified key drivers in a subtype-specific manner that are a direct result of copy number alterations rather than somatic point mutations (6). The high heterogeneity in tumour architecture, cell composition, abundance, and distribution also suggest that the tumour microenvironment and immune cells shape tumour evasion, evolution, and response to different treatments, promoting tumour durability and resistance to therapy (65). However, more studies are needed to understand the differential role of tumour immunity in AI resistance and survival in different subgroups of patients with ER+ BC, such as HER2 – and HER2+.

1.6 Ki67 changes and residual levels at surgery as markers of resistance to endocrine therapy and outcome

Antigen Ki-67 is a protein encoded by the *MKi67* gene, which is associated with cellular proliferation. The expression of Ki67 has been widely used as a surrogate marker of cell proliferation and growth in many cancer types. According to the International Ki67 in Breast Cancer Working Group, Ki-67 levels between 5% and 30% are subject to high interobserver and interlaboratory variability, thus recommending that only levels beyond this threshold should be considered clinically actionable (84,85). An accurate test-tool predicting prognosis in hormone receptor-positiveBC was developed based on response to neoadjuvant ET (105). The PEPI score is the sum of the risk points derived from the pT stage, pN stage, Ki67 level, and oestrogen receptor status of the surgical specimen following neoadjuvant endocrine treatment. This prognostic tool was developed based on data from the Z1031 (21) and ATAC trials (91). In these trials, it was observed that the antiproliferative effect measured by Ki67 after 2 and 12 weeks was significantly greater with anastrozole than with tamoxifen ($P = 0.004$ and $P < 0.001$). Noteworthy, the suppression of Ki67 under AI treatment was associated with

a lower risk of recurrence ($p<0.001$). In addition, for patients with PEPI = 0 disease, the relapse risk over five years was only 3.6% without chemotherapy, supporting the use of adjuvant endocrine monotherapy in this group (82,106).

More recently, results from the phase III POETIC trial (Peri-Operative Endocrine Therapy-Individualising Care), which randomised patients with ER+ BC 2:1 to receive either peri-operative AI vs. no-treatment followed by standard of care, indicated that two weeks of preoperative therapy with an AI may significantly reduce tumour proliferation measured by Ki67 (82). The observed reduction in Ki67 was translated into an improvement in outcome in ER+/HER2- BC but not ER+/HER2+ BC (84,107) Patients with Ki67 high at baseline (≥ 10%) remaining high at baseline (≥ 10%) – i.e., baseline Ki67 higher than 10% and remaining so after 2 weeks of AI treatment— have significantly worse prognosis than tumours with baseline Ki67 high (≥10%) turning to low (<10%) and baseline Ki67 low (<10%) turning to low (<10%) at surgery. The POETIC trial clearly provided the validation of baseline Ki67 as a prognostic marker showing that patients whose $Ki67_{2w}$ remains "high" (≥ 10%) after two weeks of AI treatment have a substantially poorer prognosis than those with a "high" baseline Ki67, which is markedly reduced to "low" (<10%) (83).

1.7 Importance of having a reference group of patients (control group) in biomarker research

Beyond the identification of residual Ki67 at the two-week time point as a biomarker of resistance to AI (83), data from the POETIC trial has also shown that there are some unexpected but significant molecular changes between the baseline biopsy and the surgery specimen in the control arm, i.e., in the tumour of patients who did not receive AI treatment. For example, an increase in the expression of some genes, such as *FOS* and *JUN* in both arms (patients treated with IA and in controls) was reported for the first time in the POETIC trial (108,109). These genes are involved in mitosis and differentiation processes and the differences observed between baseline and surgery samples are not clearly understood as yet. Although the causes of these molecular differences that occur without any treatment are still uncertain, it has been suggested that they might be due to the stress occurring during sample manipulation. Some hypotheses of the causes that might explain this effect are different times of formalin fixation (longer in surgical samples than in biopsies) or exposure to pre-surgical procedures and drugs (i.e., mammography, anaesthesia…) that occur in both treated and control cohorts.

The upregulation of the expression of several genes related to *FOS* and *JUN* would likely be considered an exclusive effect of AI in the absence of a control group, which might be relevant to all archival collections of ER+ BC. Thus, the artefactual effect resulting from preanalytical sample processing needs a deeper characterisation for adequate correction; otherwise, those changes would be wrongly attributed to treatment and lead to biased results.

1.8 Development of novel treatments to improve resistance to aromatase inhibitors

Based on the molecular biology of ER+ BC in both HER2- and HER2+ populations, new treatments, and combinations, including drugs such as immunotherapy or CDK4/6 inhibitors, should be explored with the ultimate goal of improving response to current treatments and survival outcomes.

There are two main critical aspects in the development of new onco-specific drugs: 1) the lack of adequate endpoints in the design of the clinical trials testing them and 2) the limited use of biomarker-based selection and stratification strategies in their dosages. Emerging biological endpoints such as changes in Ki67 levels are mainly driven by the antiproliferative effect of certain drugs; however, the use of Ki67 in clinical trials evaluating the combination of, for example, immune-oncology agents with cytotoxic and targeted drugs should be explored.

The POETIC trial (83) aimed to find an adequate and simple endpoint that served to identify patients with ER+ early BC with sufficiently good prognosis such that standard of care medical treatment (often comprising adjuvant ET alone), was sufficient and another group to be considered for additional therapies. Traditional approaches to this problem have used standard prognostic parameters including tumour size, histological grade, nodal involvement, and age, often integrated into a prognostic tool (e.g., Nottingham Prognostic Index, Adjuvant Online, NHS PREDICT) (97,110), but these merely provide the predicted probability of benefit for a patient population with given tumour and demographic characteristics. Thus, Ki67 was developed as a reliable biomarker of ET efficacy, which also helps to predict long-term outcomes (84). A small neoadjuvant trial (IMPACT; N=330) had already suggested that this might be feasible; results showed that tumour Ki67 after two weeks ($Ki67_{2w}$) of ET predicted outcome better than at baseline, remaining significant in the multivariable analysis, whereas Ki67 at baseline did not (111). Similar results have subsequently been reported in other trials

assessing the effect of different ET strategies (91,105). The POETIC trial, with a much larger patient population (N=4480), aimed to build on these findings to provide definitive clinical evidence to inform future practice (83).

Several genomic platforms have been developed to provide more accurate prognostic and predictive information for the individual patient with ER+/HER2-negative BC (91,93,112,113). In HER2-positive early-stage BC, a multivariable prognostic assay to guide systemic therapy has recently been developed. The HER2DX assay was generated from genomic and clinic-pathological data (including tumour size, nodal status, stromal TILs, PAM50 subtypes, and expression of 13 genes) from patients included in the Short-HER trial (the phase III study evaluating nine weeks vs. one year of adjuvant trastuzumab). The HER2DX risk score was validated as a continuous variable in an independent dataset from adjuvant and neoadjuvant trials, showing a significant association with DFS and distant metastasis-free survival (DMFS), suggesting that in some HER2+ early BC, therapy could be de-escalated (114). Moreover, the new HER2DX test also estimates the likelihood to achieve a pCR (115).

However, these genomic tests are expensive, not widely available, and differ in terms of the information they provide. A simple test after a short duration preoperative ET could therefore be helpful in accurately selecting appropriate treatment in patients if it also incorporated an *in vivo* response to AI.

To recapitulate the applicability of the results obtained in this thesis, a manuscript has been included in the introduction.

1.8.1 Manuscript 1. Lights and shadows in immuno-oncology drug development

Given the importance of immune features in BC but with an apparently different role in TNBC, HER2+, and ER+ tumours, there is a need for a better clinical trial design and choice of study endpoints. This was one of the training objectives of this Ph.D. project and my stay at the Institute of Cancer Research (ICR), in London, one of the world's most influential cancer research organisations. An opinion article was written to discuss the pitfalls of current clinical trial designs and to suggest several new biomarkers for biomarker-driven clinical studies, as well as the use of appropriate surrogate endpoints for efficacy.

cancers

MDPI

Perspective

Lights and Shadows in Immuno-Oncology Drug Development

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Simple Summary: The introduction of immunotherapy has had a significant impact on the cancer treatment landscape, with unprecedented survival outcomes in some tumor types. However, clinical development of immune-oncology (IO) agents presents both opportunities and challenges, and not all patients benefit to the same extent. Many factors influence trial designs and could potentially threaten the success of promising IO drugs: 1. Most IO trials still rely on response evaluation criteria based on image assessment only, while new approaches including biomarkers tracking response should be incorporated. 2. Surrogate endpoints for efficacy are still inferred from classical anticancer drugs that have not been specifically validated for IO trials. 3. There is a need for biomarker-driven clinical studies in order to select appropriated patients. 4. Long-term toxicity monitoring is needed, and dosage calculation should not rely on dose-dependent toxicities. 5. Optimizing the design of new IO agents with collaborative approaches assessing multiple drugs on a biomarker-based basis is needed.

Abstract: The rapidly evolving landscape of immuno-oncology (IO) is redefining the treatment of a number of cancer types. IO treatments are becoming increasingly complex, with different types of drugs emerging beyond checkpoint inhibitors. However, many of the new drugs either do not progress from phase I-II clinical trials or even fail in late-phase trials. We have identified at least five areas in the development of promising IO treatments that should be redefined for more efficient designs and accelerated approvals. Here we review those critical aspects of IO drug development that could be optimized for more successful outcome rates in all cancer types. It is important to focus our efforts on the mechanisms of action, types of response and adverse events of these novel agents. The use of appropriate clinical trial designs with robust biomarkers of response and surrogate endpoints will undoubtedly facilitate the development and subsequent approval of these drugs. Further research is also needed to establish biomarker-driven strategies to select which patients may benefit from immunotherapy and identify potential mechanisms of resistance.

Keywords: immuno-oncology; cancer; trial design; endpoints; biomarkers

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1. Introduction

Immuno-oncology (IO) is redefining the cancer treatment landscape and the way that some solid tumors are treated. Almost 5000 new agents from six different main classes of immunotherapies have been in the drug development pipeline within 2020, including adoptive cell therapy, cancer vaccines, T cell-targeted immunomodulators, other immunomodulators, oncolytic viruses and antibody-based targeted therapies [1]. Overall, IO consists of a wide range of drugs with different mechanisms of action that ultimately lead to the enhancement of immunity against tumor cells. The immune checkpoint inhibitors, which reactivate T-lymphocyte mediated immune response against tumor cells, have been

the most successful type of IO developed since the beginning of the "IO era". Their use is now a standard of care across several solid tumors, including melanoma, non-small cell lung cancer (NSCLC), gastric cancer, head and neck squamous cell carcinoma, renal cancer, bladder cancer, cervical cancer and triple-negative breast cancer (BC) among others [2–6].

1.1. Which Are the Current Challenges with IO?

A substantial number of IO therapies do not progress from phase I-II trials, and some fail in late-trial stages [7]. First, clinical trials testing novel IO drugs still rely on biomarkers and endpoints that are validated for conventional treatments such as cytotoxic agents, although their mechanisms of action are different [8]. Second, the adaptative immune response induced by immunotherapeutic agents is often sustained in time, as well as the inflammatory and autoimmune-related adverse events. Those long-term effects make the evaluation of clinical benefits and toxicities extremely difficult [9]. A wide range of exclusive toxicities to IO agents, mainly characterized by significant latency, has been underestimated by many clinical trials as they are only evident in long-term follow-up or pharmacovigilance studies [10–12]. In addition, the calculation of the IO drug doses still relies on dose-limiting toxicities seen in the first cycles of classical anticancer treatments, which seems inadequate for IO treatment. Furthermore, the differences in the intrinsic biology between IO agents and conventional therapies further complicate their comparisons. Finally, some particular pathways in IO are overcrowded with similar drugs within the same therapeutical setting but led by different pharmaceutical companies. However, these resources could be better relocated to the development of new biomarkers to select the best in class and to test mechanistically different drugs.

BC, for example, is much in need of a paradigm shift of better IO drug development [13]. This cancer type belongs to a group of widely considered "cold tumors", characterized by low mutation and neoantigen burden and low counts of tumor-infiltrating cells (TILs). Although the number of clinical trials assessing the use of immunotherapy in BC is increasing, to date, the approval of its use is only for a selected subset of advanced triple-negative (TN) BC patients with >1% of programmed death ligand 1 (PD-L1) expression by immunohistochemistry (IHC) [6,14]. Overall, the main problem in the development of IO agents in this type of cancer has been the lack of biomarker-guided patient selection for trials and the reliance on a reduced number of "classic" biomarkers such as PD-L1. In particular, PD-L1 remains at least insufficient to fully explain the therapeutic success and durable clinical benefit seen in some patients with PD-L1 non-expressing tumors, especially when treated with other IO agents beyond checkpoint inhibitors [15–19]. However, new genomic alterations such as those in DNA damage response or specific mutated gene pathways have shown promising results as immunomarkers in some translational studies, and their validation in clinical trials should be encouraged [20]. In addition, the main focus of IO development in BC has been put in TNBC due to its general enrichment of TILs and the lack of effective therapies other than chemotherapy. Although the use of IO agents in other subsets of BC, such as luminal B tumors or pretreated HER2-positive BC, has also been explored, there is still much controversy on its use in those settings [21–23]. It is still unclear whether a better design based on a more accurate selection and less pretreated patients would have led to positive results. The other main issue is the lack of assessment of IO agents' combinations, which is now believed to be an alternative strategy to achieve immune response enhancement in "colder tumors". Especially in BC, in which many different pathways, such as estrogen receptor signaling, seem to have major implications for the tumor immune scape, and further combinations of different IO treatments with classical anticancer therapies could potentially help to overcome them [24,25].

1.2. How Can We Do Better?

Despite the great improvement in the field of IO in the past years, most new agents still offer a modest rate of objective responses and poor long-term outcomes compared to conventional treatments. In addition, immune-mediated serious adverse events remain a

potential issue [26]. In order to optimize new IO trials' design to further improve survival outcomes and minimize toxicity rates, new strategies are needed. We have identified five main domains in the development of promising IO treatments that should be redefined for more efficient results and accelerated approvals.

First, there is a crucial need for a biomarker-driven selection of a patient population for each of the new IO agents. Second, the evaluation of efficacy, toxicity and comparisons with current treatments should be based on surrogate endpoints and response criteria for those specific IO agents and not just inferred from conventional drugs. Monitoring long term-outcome seems to be mandatory as IO agent efficacy and toxicity may have long-term effects [27]. Finally, changing the current scenario of IO clinical trial-design with more collaborative approaches that facilitate the assessment of multiple diseases and drugs on a biomarker basis seems crucial [28].

In this manuscript, we will analyze each of those points and suggest some potential improvements in the field.

2. Challenges and Opportunities in IO Drugs Development

A summary of the main critical aspects in IO drug development and strategies for their optimization is illustrated in Figures 1 and 2, respectively.

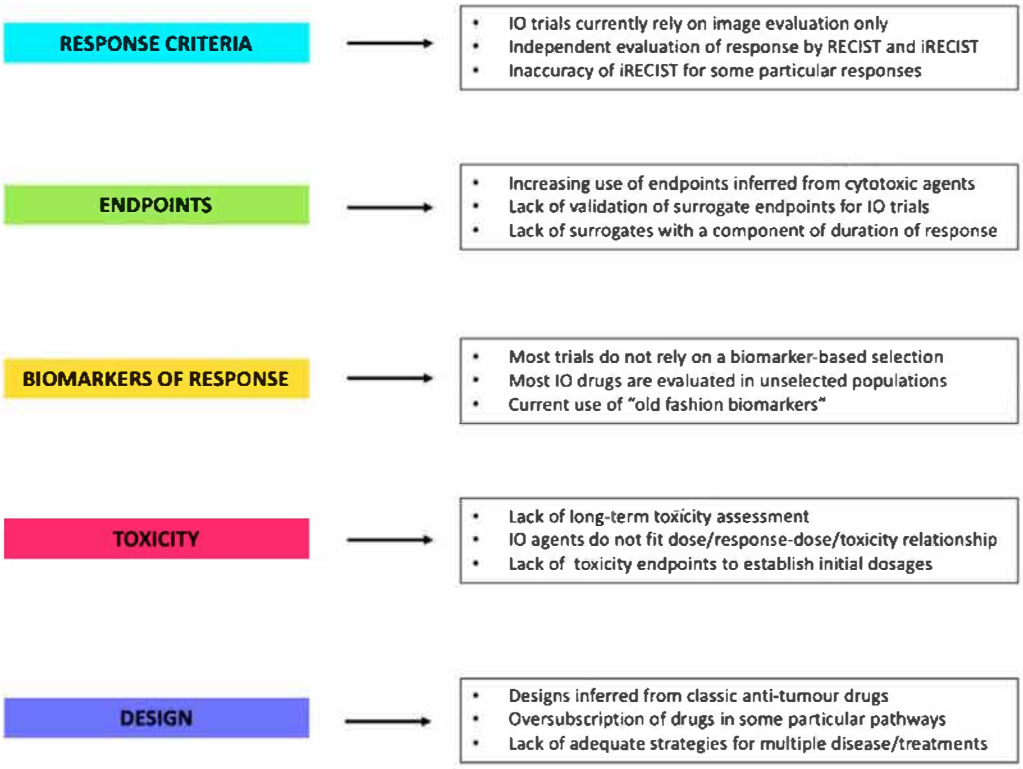

Figure 1. The major drawbacks found in immune-oncology trials designs to date. Abbreviations: IO: immuno-oncology, RECIST: response evaluation criteria in solid tumors, iRECIST: immune response evaluation criteria in solid tumors.

Figure 2. Proposed strategies to optimize the clinical trial design for new immuno-oncology agents. Abbreviations: RECIST: response evaluation criteria in solid tumors, iRECIST: immune response evaluation criteria in solid tumors.

2.1. Response Criteria

From the beginning of the "IO era," researchers realized that the standard response evaluation criteria in solid tumors, namely RECIST alone, would not be suitable for immunotherapy due to the indirect effect caused by the participation of inflammatory cells and their interactions [29]. RECIST relies on the early suppression of tumor growth by chemotherapy and may consequently underestimate the benefit of immunotherapy. The immune RECIST (iRECIST) and other immune-specific related response criteria were developed to evaluate the heterogeneity of responsiveness in patients receiving immunotherapy [30]. Although iRECIST takes into account pseudoprogressions or hyperprogressions, which are observed exclusively with IO agents, demonstration of actual response to treatment may not be distinguishable from such patterns for another several months. This particular timeline may have caused many clinicians to have considered patients as no treatment response and/or stable when the patient may have conversely obtained benefit from treatment since iRECIST still recommends that assessment of response durability may occur between 4 and 8 weeks [31].

The use of iRECIST for evaluation of response has mainly been used for studies evaluating the efficacy of immunotherapeutic agents only. It becomes rather complex when the primary objectives of trials are to perform head-to-head comparisons with non-immunotherapeutic agents or combinations with other cytotoxic or molecular targeted treatments. On the other hand, particular responses observed in patients undergoing immunotherapy treatments are not well captured by iRECIST, such as dissociated responses with some lesions growing, some shrinking or the slow progressions, features linked with clinical benefit. In those cases, classic RECIST could still remain as a meaningful method of evaluation [32]. In addition, most IO clinical trials that compare immunotherapy with other cytotoxic antitumor agents are designed around the evaluation of response on

superiority, inferiority or equivalence [33]. These comparisons may not be appropriate when assessing two mechanistically different treatments, in which case integrative clinical benefit and long-term outcome should be taken into consideration. Although the American Society of Clinical Oncology-Society for Immunotherapy of Cancer (ASCO-SITC) has recently recommended reporting responses according to both the conventional RECIST and iRECIST criteria in parallel [34], this approach is still suboptimal as their evaluation is done independently. Harmonizing and integrating both measurement methods into an adequate tool rather than just separately considering both criteria is urgently warranted for more precise measurements of actual responses.

It is worth noting that some methods beyond image evaluation have also shown promising results for the assessment of response in IO. Some biomarkers assessed in peripheral blood such as interleukin 8 (IL-8), tumor circulating DNA (cDNA) or CD8+ memory effector cytotoxic T cells have recently shown to assist in tracking immune response in different tumors like NSCLC cancer and melanoma [35–37]. Given the strong correlation between detected changes on those biomarkers with subsequent clinical responses, biological assays exploring changes in their expression levels could lead to promising results. Based on that, decisions could be made on the basis of patient-specific immunological tracking biomarkers. Moreover, the pathological assessment of lymphocytes and immune infiltrating cells in tumor biopsies during treatment could also be considered as a useful monitoring tool of clinical response in clinical trials and help to gain a deeper understanding of the changes in tumor characteristics under immunotherapy. However, the application of on-treatment biopsies in clinical practice remains unclear and should be further investigated and refined as it could be considered invasive [38].

In summary, combining an integrative image tool with response markers such as liquid biopsies could become better tailoring of response evaluation in the future of IO.

2.2. Long-Term Efficacy Endpoints and Surrogates

Drug approval is generally based on safety and efficacy assessed by clinically relevant endpoints in phase 3 randomized trials. Overall, survival (OS) is considered to be the gold standard as it reflects the ultimate survival benefit from cytotoxic and other targeted therapy regimens, and there are minimal measurement errors in OS. Meanwhile, progression-free survival (PFS) and objective response rate (ORR) are used as surrogate endpoints in cancer; these measurements provide inferred conclusions from clinical trials and facilitate accelerated approval of new drugs that fill an unmet clinical need [39,40]. Other emerging biological endpoint, such as changes in Ki67 level after short-term treatment with endocrine therapy in BC, has been validated as a surrogate of long-term benefit [41] and are increasingly becoming used as primary endpoints in clinical trials. Surrogate endpoints for the assessment of IO agents' efficacy have primarily been adopted for cytotoxic and molecular targeted drugs; questions remain whether they are suitable to determine benefit from immunotherapy. In particular, there are increased doubts concerning the use of short-term benefit endpoints such as PFS or ORR as primary endpoints in most clinical trials of new IO agents [7,42,43].

A recent meta-analysis of 60 published immunotherapy randomized clinical trials suggested that ORR could be a meager surrogate of response to evaluate the efficacy of IO, and the use of PFS as a surrogate of OS is still indeterminate [44]. Another meta-analysis of 12 randomized clinical trials did not find a significant positive correlation between the OS and PFS hazard estimates, suggesting that PFS assessment is not sufficient to capture the benefit of PD-1-inhibitors in patients with solid tumors [42]. This is not surprising as the unique mechanism of immunotherapy's impact on tumors shows different patterns of response and progression from other conventional agents [45]. Emerging biological endpoints such as changes in Ki67 level are mainly driven by the antiproliferative effect of certain drugs; however, its use in trials evaluating the combination of IO agents with cytotoxic and targeted drugs should be further explored. Recent studies have also shown that endpoints taking into account a component of the duration of response such as milestone

survival or durable response rate may better capture the delayed and persistent responses derived from IO agents and should be further studied [46]. For instance, milestone survival is the survival probability at a given time point, defined a priori as two years, and durable response rate can be measured as a continuous response, such as complete or partial objective responses, beginning within 12 months of treatment and lasting ≥6 months [47]. The advantages of such types of integrative endpoints are that they take into account particular behaviors seen exclusively in IO and allow the use of predefined cutoff time intervals that lead to the rapid characterization of survival probability and inference to long-term survival data.

To date, overall survival still remains the gold standard for the evaluation of clinical efficacy of IO agents in late-phase clinical trials, new biomarker-driven surrogate endpoints that capture the mechanisms of action in early phases of immunotherapy drugs development should be explored.

2.3. Biomarkers of Response

Despite the rapid advance and reduced cost of high-throughput sequencing technologies, most current trials in oncology have limited use of biomarker-based selection and stratification strategies in their designs. However, studies in IO treatments tested in unselected populations are generally negative [48,49]. The lack of new and robust predictive markers is particularly concerning for the selection of the most appropriate subpopulations in relative "colder tumors" such as BC, in which most patients will not benefit from those new IO agents.

In recent years more attention has been paid to the identification of predictive biomarkers of the efficacy of IO drugs to identify patients who benefit from those agents. Most approved immunotherapeutic treatments show efficacy only in selected populations, mainly based on the immunohistochemical (IHC) levels of PD-L1 checkpoint target leading to PD-L1 being the compulsory companion diagnostic assay for the administration of many checkpoint inhibitors in oncology. Many efforts are currently focusing on the reproducibility and standardization of laboratory protocols for the IHC assessment. However, the evaluation of single IHC biomarkers does not completely explain the heterogeneity of tumors, and their use seems to be suboptimal in the "genetics and omics era". In particular, multiplex diagnostic assays would be better, especially when testing IO agents beyond immune checkpoint inhibitors [14–19].

Emerging predictive biomarkers as defined by both the host and tumor factors are promising measurement for a clinical response; the use of these measurements are still in infancy stage due to a lack of standardization and harmonization of reporting methods [50]. Tumor mutational burden, microsatellite instability, and tumor neoantigen loads are some examples. Mutational burden and high microsatellite instability assessment based on mutations in mismatch repair genes have been associated with better response to immunotherapy, especially to anti-programmed cell death protein 1 (anti-PD1) agents [51,52]. Next-generation sequencing has led to a more accurate method of quantification, but it is still difficult to achieve a homogenization on their quantification. Thus, the implementation and standardization of robust bioinformatics methodologies and analytical techniques across laboratories are necessary [53]. Additional exploration to identify the type of mutations is much needed for generating the most relevant neoantigen for recognition by the T cells. Other challenges are that these emerging predictive biomarkers assays for IO agents are usually expensive, technically demanding and not widely available.

Multi-plex and multi-omics based biomarkers indicating higher tumor immune tolerance such as immune-related genes and signatures have thrown some light on the field of predictive biomarkers in IO [20,54]. Some studies have shown that high expression of some particular gene expression signatures is associated with response to PD-L1 inhibitors regardless of their PD-L1 status in NSCLC and melanoma [55]. Other signatures, including some targetable immune checkpoint components such as indoleamine 2,3-dioxygenase (IDO1), lymphocyte-activation gene 3 (LAG-3), or interferon-gamma (IFNγ) genes can pre-

dict benefit from immunotherapy in "colder tumors" such as luminal B BC patients [56,57]. Recent studies have also demonstrated that exhausted CD8+. T cell signatures can predict immunotherapy response in ER-positive BC [58]. These signatures are yet to be validated in clinical trials.

Identification of robust pharmacodynamic biomarkers of IO response remains a challenge [59], and it is likely that combinations of two or more biomarkers to capture immune status more accurately will be needed [20].

2.4. Definition of Toxicity and Treatment Dosage

Immunotherapies have the potential to induce toxicity profiles distinct from those from other cancer treatments. Immune-related adverse events are often underdiagnosed, as patients can remain asymptomatic for long periods of time [10,12]. In addition, toxicity is mostly inflammatory-related, and assumptions from cytotoxic or molecular targeted treatments are not appropriate for IO agents. In particular, precise considerations must be taken during their development [60] as they do not fit the dose–response/dose-toxicity relationships seen with cytotoxic therapies.

In contrast to what usually happens with cytotoxic drugs, an increase in the dosage above the biologically optimal does not always correlate with an increase in efficacy or toxicity in IO [7]. Due to the lack of reliable toxicity endpoints to establish optimal dosages in immunotherapeutic trials, some FDA approvals on immune-checkpoints inhibitors have been based on varying dosages and schedules across different tumor types. This has resulted in some confusion on the clinical implication and implementation for future trial designs [32]. The best approach to define the optimal administration dosages and schedules with the highest efficacy is still open-ended, and deficient toxicity profiles may have unfortunately an impinged on the results from several clinical trials assessing efficacy with IO agents [61].

The optimal design of early phase clinical trials should aim to evaluate doses and schedules at the minimal doses that are biologically active. Based on the distinct behavior of IO agents, flat dosing administration instead of weight-based dosing may be a better approach facilitating smoother administration and avoiding drug waste [62,63]. Long-term follow-up of IO related adverse events is encouraged in trial designs evaluating new-IO agents. Consensus guidelines for recognizing each of the adverse events under immunotherapy and specific management of these reported events should also be incorporated.

Aforementioned, defining doses and schemes of IO agents seem relatively more complicated than with cytotoxic drugs. New approaches in trial designs, including the homogenization of the optimal dosages that will be carried over later phases of drug development, are urgently needed. Finally, the incorporation of additional endpoints especially validated for IO agents in early phases of trials for dose selection to improve efficacy and reduced toxicity, are also warranted.

2.5. The Trial Design Itself

Due to the particular impact on tumor biology by IO treatments, conventional phase 3 clinical trial designs to demonstrate the effects of an experimental therapy compared to standard of care are unlikely to provide definitive answers on the efficacy of IO within reasonable time and cost. The anti IDO1 epacadostat, which was evaluated in a late-phase trial in melanoma, is an example of such a conundrum [6]. This phase III trial ECHO-301/KEYNOTE 252 trial was designed to assess the efficacy of IDO1 inhibition in combination with pembrolizumab, but there were several problems associated with the trial, including the use of endpoints such as PFS and ORR and no pre-planned translational studies to study the tumor biology leading no collection of biological samples that could be studied further to explain the unexpected clinical results [62,63]. This study has posed that translational studies are important elements to be incorporated in the trial design whenever possible.

Furthermore, the field of IO is currently overcrowded with several drugs competing for the same therapeutic space. Advancements in "precision oncology" urge therapy selection based on tumor molecular characteristics. The conventional trial designs, lack of pairing tumor characteristics with therapeutic targets, are not adequate to investigate the broad-spectrum of genetic makeup in tumors that may benefit from different IO agents and targeted therapies. The incorporation of "master protocols" in collaborative clinical study designs can allow multiple disease assessments and multiple strategies at a time. Some "modern" strategies also include several trial designs that enable more personalized and adaptative assessment of new drugs, such as platform trials, which can be multi-arm, multi-stage adaptive studies, pairing targeted therapy with molecular characterization of tumors [64]. Other simpler approaches include umbrella trials, which evaluate multiple targeted therapies for a single disease as defined by specific molecular characteristic subgroups, and basket trials, which use biomarkers for molecular screening and allocation of patients into different trials according to their molecular biology [65,66].

These new approaches in trial designs alleviate the field of IO by optimizing resources and improving the efficiency of the broad amount of emergent clinical trials. However, those new designs require considerable effort, cost and multidisciplinary collaboration. Regulatory approvals and standardization of their use warrant further exploration. The question remains whether a systematic implementation of renewed IO drug designs and translational studies should be encouraged to avoid superfluous numbers of clinical trials.

3. Conclusions

The current clinical development of IO agents has both strengths and weaknesses that provide us with challenges and opportunities for improvement. Given the rapid growth in the IO field, only a greater level of understanding of the underlying mechanisms of resistance, tumor heterogeneity and host and tumor microenvironment can shed light on IO for patient selection through robust biomarkers testing. The silhouette in clinical trial design and response evaluation criteria of IO should be redefined with new approaches like amalgamating integrative tools with response biomarkers such as the use of liquid biopsies. Applying innovative but appropriate clinical trial designs, incorporating multiple robust biomarkers assays of response and surrogate endpoints, will lead to the best possible development and accelerated approvals of new IO agents that are here to stay. Finally, the precise definition of adverse events following long-term evaluation and dosage definition is also needed for successful results. With advances in molecular sequencing technologies and development in machine-learning methods, biomarker-driven strategies to assist the selection of patients for future trials with immunotherapy will soon be a reality.

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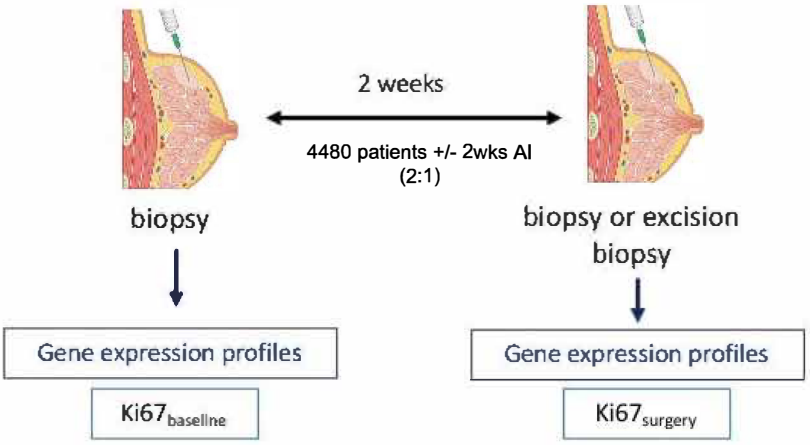
2. HYPOTHESIS

The main objective was to better characterise the mechanisms of AI resistance in early BC in both ER+/HER2- and ER+/HER2+ subtypes and determine whether they are driven by baseline and/or on-treatment genomic alterations. We aimed to find prognostic and predictive biomarkers of early response/resistance to AI which may help select the optimal treatment for each patient.

In this thesis, we have used biological samples from different subsets of BC receiving neoadjuvant AI-based treatment. We obtained gene expression data from both ER+/HER2- and ER+/HER2+ BC populations including the POETIC study. The POETIC clinical trial is the world's largest "window of opportunity" study that randomised nearly 4,500 patients to receive either preoperative AI (treatment arm) or no treatment (control arm) (85). We also analysed the molecular changes that occurred in an independent cohort of patients with RE+/HER2- early BC who received neoadjuvant ET for more than one month (and up to two years) to compare them with the results of the POETIC study.

The collection of viable paired tumour biopsies (at diagnosis and surgery) has provided a huge resource of information on determinants of early response to short-term ET (**Figure 1).**

Figure 3. Design of the POETIC trial



- Our main hypothesis was that mechanisms of AI resistance in patients with ER+ early BC may be driven not only by baseline genomic signatures but also by genomic alterations that occur in response to therapy.

- Our second hypothesis was that some of these genomic characteristics driving AI resistance involve immune-related mechanisms and immune checkpoint-related characteristics and, therefore, they could serve as predictive and/or prognostic biomarkers.
- In ER+/HER2- early BC, we hypothesised that the impact of AI treatment on genomic characteristics differs according to the length of treatment (two weeks vs. >one month). We hypothesised that there would be more and deeper molecular changes after longer-term AI treatment compared with a shorter treatment duration, and that those changes, together with some baseline characteristics, would play a role in predicting response to treatment and survival.
- In ER+/HER2+ early BC, our main hypothesis was that the HER2-E subtype determined in the pre-treatment sample (at baseline) predicts poor response to AI in postmenopausal women. In addition, we expected that additional baseline gene expression profiles and/or alterations beyond the HER2-E subtype would predict response to treatment and are associated with different survival outcomes. These findings may improve the identification of patients with ER+/HER2+ disease that would benefit the most from anti-HER2 targeting therapy and ET.

3. OBJECTIVES

3.1 Primary objective

Our primary objective was to look for prognostic and predictive molecular biomarkers of early response to AI in postmenopausal women with early-stage ER+/HER2- and ER+/HER2+ BC. We also aimed to integrate the immune-related landscape of these tumours for a comprehensive molecular characterisation.

3.2 Objectives in ER+/HER2- early breast cancer

We aimed to better characterise gene expression changes, including the molecular intrinsic subtypes, in ER+/HER2- BC patients treated with short-term AI within the POETIC trial and compare them to those occurring after longer neoadjuvant AI treatment (>1 month). We also wanted to assess the predictive and prognostic role of these observed changes.

3.2.1 **Objective 1:** To characterise changes within the intrinsic molecular subtypes induced by short-term AI treatment (two weeks) and by longer-AI treatment (>one month). We would also evaluate any correlation between the length AI treatment exposure and the magnitude of changes in the intrinsic subtype.

3.2.2 **Objective 2:** To characterise and compare molecular changes beyond the intrinsic subtypes (both at a single gene and pathway level) induced by both short-term (two weeks) and longer-term AI treatment (>one month) in paired tumour biopsies (at baseline and surgery).

3.2.3 **Objective 3**: To characterise tumour molecular differences observed in paired tumour samples from control patients (i.e., who did not receive any pre-surgical treatment in the POETIC trial) and compare them to those observed in the treatment arm: the artefactual effect.

3.2.4 **Objective 4:** To evaluate the predictive value of AI response of both baseline gene-expression profiles and molecular changes induced by short-term and longer-term neoadjuvant AI therapy. We would correlate those molecular characteristics with Ki67 changes between the baseline biopsy and at the two-week time point ($Ki67_{2w}$) at surgery as a surrogate biomarker of response.

3.2.5 **Objective 5:** To assess the impact on time to recurrence (TTR) of the significant molecular changes occurring under short-term and longer AI treatment.

3.3 Objectives in ER+/HER2+ early breast cancer

We aimed to characterise baseline and on-treatment molecular expression profiles of the entire cohort of ER+/HER2+ BC within the POETIC trial. Our main objective was to identify early resistance mechanisms to ET in this understudied BC subgroup and determine whether they are driven by baseline genomic signatures or by genomic alterations that lead to Ki67 changes in response to two weeks of peri-operative AI therapy at surgery. Our specific objectives within the ER+/HER2+ BC cohort were:

3.3.1 **Objective 6:** To evaluate the ability of the HER2-enriched (HER2-E) intrinsic subtype to predict poor response to AI measured by 1) percentage of reduction of Ki67 levels from baseline to surgery and 2) tumour Ki67 at two weeks of treatment ($Ki67_{2w}$) ≥10% or <10%.

3.3.2 **Objective 7**: To identify both baseline single genes and gene signatures predicting early response to AI measured by 1) percentage of reduction of Ki67 levels and 2) tumour $Ki67_{2wk}$ at surgery ≥ 10% or <10%.

3.3.3 **Objective 8:** To identify new molecular subgroups beyond the intrinsic subtypes based on gene expression to predict response to AI.

3.3.4 **Objective 9:** To evaluate the prognostic value of molecular features including intrinsic subtypes, gene expression, and the new molecular subgroups in terms of TTR.

4. MATERIALS, METHODS, AND RESULTS

4.1 Manuscript 2. Impact of duration of neoadjuvant aromatase inhibitors on molecular expression profiles in oestrogen receptor-positive BCs.

Impact of Duration of Neoadjuvant Aromatase Inhibitors on Molecular Expression Profiles in Estrogen Receptor–positive Breast Cancers

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ABSTRACT

Purpose: Aromatase inhibitor (AI) treatment is the standard of care for postmenopausal women with primary estrogen receptor–positive breast cancer. 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 AIs.

Experimental Design: Global gene expression profiles from the PeriOperative Endocrine Therapy for Individualised Care (POETIC) trial (137 received 2 weeks of AIs and 47 received no treatment) and targeted gene expression from 80 patients with breast cancer treated with NET for more than 1 month (NeoAI) were assessed. Intrinsic subtyping, module scores covering different cancer pathways and immune-related genes were calculated for pretreated and posttreated tumors.

Results: The differences in intrinsic subtypes after NET were comparable between the two cohorts, with most Luminal B (90.0% in the POETIC trial and 76.3% in NeoAI) and 50.0% of HER2 enriched at baseline reclassified as Luminal A or normal-like after NET. Downregulation of proliferative-related pathways was observed after 2 weeks of AIs. 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 tumors and differential outcome were observed in the NeoAI study.

Conclusions: Tumor 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 tumors 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 is molecularly and clinically heterogeneous, and approximately 60% to 80% of cases are estrogen receptor–positive (ER^+). The standard of care for postmenopausal women with ER^+ breast cancer includes aromatase inhibitors (AIs) over a 5- to 10-year period. However, 20% to 25% of patients with ER^+ breast cancer will eventually relapse, and additional biomarkers to identify resistance mechanisms to AIs are warranted (1–4).

Global gene expression analyses in breast cancer have shown molecular heterogeneity with a far more complex portrait beyond clinicopathologic classification (5–7). The elucidation of the molecular intrinsic subtypes has led to the categorization of breast cancer tumors into clinically relevant but molecular distinct subgroups that can be optimally defined by the 50 gene–based PAM50 classifier (8–10). These molecular subtypes are associated with different incidence and racial disparity, response to treatment and prognosis (8). However, there are still insufficient data about changes of those molecular characteristics under different lengths of AI treatment and whether pretreatment or posttreatment 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-signaling activation after short- and long-term neoadjuvant AIs are associated with poor response (15, 16). However, we also reported the enrichment of *ESR1* mutation with long-term neoadjuvant AI in primary breast cancer using a real-world cohort of patients treated in the Royal Marsden Hospital (RMH; London, United Kingdom; ref. 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 signaling pathways was comprehensively evaluated. Gene expression

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Our study shows that neoadjuvant treatment with short- and longer-term aromatase inhibitors (AIs) in primary estrogen receptor-positive (ER^+) breast cancer 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 patients with high-risk ER^+ breast cancer tumors. The role of immune-checkpoint component inhibition for endocrine therapy–resistant ER^+ tumors in this setting warrants careful investigation.

profiles from two cohorts of patients with early primary ER^+ breast cancer were analyzed: (i) the PeriOperative Endocrine Therapy for Individualised Care (POETIC) trial, in which patients were treated for 2 weeks (15) and (ii) patients treated for more than 1 month, named in the current study as NeoAI (16).

Materials and Methods

Patients' populations

Data from two different cohorts of postmenopausal women with primary ER^+ breast cancer treated with different lengths of neoadjuvant AI were analyzed (Supplementary Fig. S1).

POETIC subset

The POETIC trial was a phase III, randomized study of 4,486 postmenopausal patients with ER^+ breast cancer. Patients were randomized 2:1 to receive 2 weeks of preoperative AIs (letrozole 2.5 mg, anastrozole 1 mg per day orally) versus no treatment to determine whether perioperative AIs followed by standard adjuvant therapy would improve survival (15). The subset used in this study comprised 184 patients with paired samples: 137 tumors treated with AIs [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 perioperative AIs as a control group.

NeoAI study

This was a retrospective cohort of patients treated with neoadjuvant AIs (letrozole 2.5 mg, anastrozole 1 mg or exemestane 25 mg per day orally) for at least 1 month (mean, 6.24 months ± SD, 3.9) at the RMH between 2003 and 2016 (16). Data from 80 patients from this study were analyzed: 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. To provide a view of real-world AI-resistance mechanisms in ER^+ breast cancer, both $HER2^+$ and $HER2^-$ were included in this study, with subsequent subgroup analyses focused on ER^+ $HER2^-$ tumors.

Gene expression profiles

In the POETIC subset, gene expression data from microarray were obtained as described previously (15). Probes targeting 16,528 expressed genes (detection $P < 0.01$ in at least 20% of samples) were included in this analysis. Expression data were then $\log_2$ transformed and quantile normalized for downstream analysis, and probes were collapsed to gene-level expression based on the highest SD 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 $\log_2$ expression of 744 different genes covering the most important aspects of breast cancer—such as proliferation, invasion, *PI3K-AKT-mTOR* pathways, *MAPK* signaling, inflammation and the PAM50 gene set—previously analyzed 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 tumor sample was classified into one of the five intrinsic subtypes, namely Luminal A, Luminal B, Her2 enriched (Her2-E), basal-l, ike and normal-like using the 50-gene PAM50 classifier after subgroup-specific centering as reported previously (7, 15).

In the NeoAI study, the 46 genes raw expression values used in Prosigna were first normalized to eight housekeeping genes (*ACTB*, *GUS*, *MRPL19*, *PSMC4*, *PUM1*, *RPLP0*, *SF3A1*, and *TFRC*) and then normalized to a cohort of 229 sample $ER^+/HER2^-$ tumors 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 previously (21).

Biomarker analysis

ER status was measured locally and centrally reviewed by IHC. HER2 status was measured locally using IHC and/or ISH. Ki67 proliferation rate was obtained by IHC from staining on formalin-fixed samples using anti–MIB-1 (M7240, DAKO UK). Ki67 rate was categorized into High (≥10%) and Low (<10%) at baseline and surgery. Tumors were also classified into four classes according to Ki67 changes between the two time points: $High_{baseline}$-$High_{surgery}$ (H-H), $High_{baseline}$-$Low_{surgery}$ (H-L), $Low_{baseline}$-$Low_{surgery}$ (L-L), and $Low_{baseline}$-$High_{surgery}$ (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 Benjamini–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 $P_{adjusted} < 0.05$ and $\log_2$ 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. Because of 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* 0.03). Multivariable Cox regression models adjusted for standard clinicopathologic variables including PR, HER2 status, tumor grade, pathologic tumor size, histologic type, nodal status, and vascular invasion were performed to assess the independent prognostic value of changes in gene expression and intrinsic subtypes.

Ethics statement

The POETIC trial was approved by the London–South East Research Ethics Committee (reference 08/H1102/37). For the NeoAI study, ethical approval was received from an NHS research ethics committee (reference 17/EM/0145). Both studies were adopted by the Declaration of Helsinki and patients from both studies provided written informed consent to molecular analysis of their samples for research purposes.

Data availability

Gene expression data from POETIC study can be found at Gene Expression Omnibus with the accession number: GSE126870. Additional data are available upon request by contacting the corresponding author or poetic-icrctsu@icr.ac.uk.

Results

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 S1. Baseline molecular characteristics were different between POETIC and NeoAI cohorts with the majority of samples being Luminal A (88/137; 64.2%) in the POETIC-treated samples subset and Luminal B (38/121; 47.5%) in NeoAI. The rest of the baseline clinicopathologic characteristics were similar among the two subsets.

The differences of intrinsic subtype between baseline and surgery were more frequent in the POETIC treatment group than in the controls (38% vs. 23.4%). In the treated group, most Luminal B tumors at baseline (90.0%, 27/30) and 50.0% (6/12) of Her2-E were redesignated as Luminal A or normal-like subtypes (**Fig. 1A** and **B**), whereas 41.7% of Her2-E and most Luminal A, 85.2% (75/88) and basal-like 66.7% (2/3) tumors remained unchanged after 2 weeks of AI. **Figure 1B** illustrates the changes within Luminal B tumors at baseline after 2 weeks of AI, and that the tumors were increasingly more similar to prototypical Luminal A and normal-like tumors at the 2-week time point. In the control group, although 33.3% (4/12) of Luminal B tumors were reclassified into Luminal A, the majority did not change (**Fig. 1C**). The difference in intrinsic subtypes after 2 weeks (untreated) was likely due to cases that had close similarity with more than one subtype. In particular, baseline Luminal B tumors that were reclassified into Luminal A had close proximity to prototypical Luminal A tumors as illustrated in **Fig. 1D**, in contrast to the clear shift from Luminal B to Luminal A seen in the treatment arm. In addition, in the treated samples 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 Fig. S2A).

Similar to the POETIC trial, in the NeoAI study, most Luminal B tumors (76.3%, 29/38) were redesignated as Luminal A or normal-like; only 13.2% remained unchanged, while 10.5% were classified as basal-like or Her2-E. Fifty percent (5/10) of Her2-E tumors remained as Her2-E while 20.0% (2/10) were redesignated to Luminal A and 30.0% (3/10) to normal-like (**Fig. 1E** and **F**). 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 Fig. S2B).

To further investigate the impact of AI duration on intrinsic subtypes, we tested the correlation of duration of AI with changes of the intrinsic subtype, and there was no statistically significant observed relationship ($P = 0.19$; Supplementary Fig. S3). 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 with the treated samples in POETIC (67.5% vs. 38.0%), likely due to a higher proportion of Luminal B tumors at baseline in the NeoAI study.

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

Gene expression data in the POETIC subset were computed in module scores according to annotated pathways, immune-response, and selected drug-target response signatures. Two module scores (FOS-JUN modules) were significantly upregulated and 11 significantly downregulated after short-term AI therapy, and these modules included protumorigenic signaling modules associated with proliferation, RB loss, and chromosome instability (**Fig. 2A**). Within Luminal A samples, 12 also showed a significant change including the upregulation FOS and JUN. Within Luminal B tumors, eight modules' scores increased, and 19 decreased significantly posttreatment (**Fig. 2B**). As expected for a highly proliferative ER-dependent intrinsic subtype, Luminal B tumors 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 (**Fig. 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 preanalytic sample processing due to handling methodology (25, 26). In this study, the expression of the 17 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, $\log_2$ FC = 0.5–1.8) and controls (*P* value all genes < 0.0001, $\log_2$ FC = 0.5–2.2; **Fig. 3**).

Looking at the changes of expression profiles at a single-gene level from baseline to surgery in the two studies (Supplementary Table S2), a higher number of genes involving proliferation, keratin expression, and endocrine-related pathways like *PGR*, were downregulated in NeoAI when compared with POETIC. More genes from key pathways in breast cancer such as *MAPK* and *PI3K-AKT* (i.e., *IGF1*, *NR4A1* and *NGFR*) or mTOR (*BTG2*; ref. 15) were upregulated in the NeoAI study (Supplementary Table S3).

The differential expression of genes in common between both datasets are shown in **Fig. 4A** and **B**. Genes from FOS-JUN modules *(FOS*, *JUN*, and *ERG1*), *MAPK/ERK*, *PI3K-AKT* and *JAK/STAT* pathways, and *IGF1* involved in tumor growth and resistance to AI, were upregulated 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

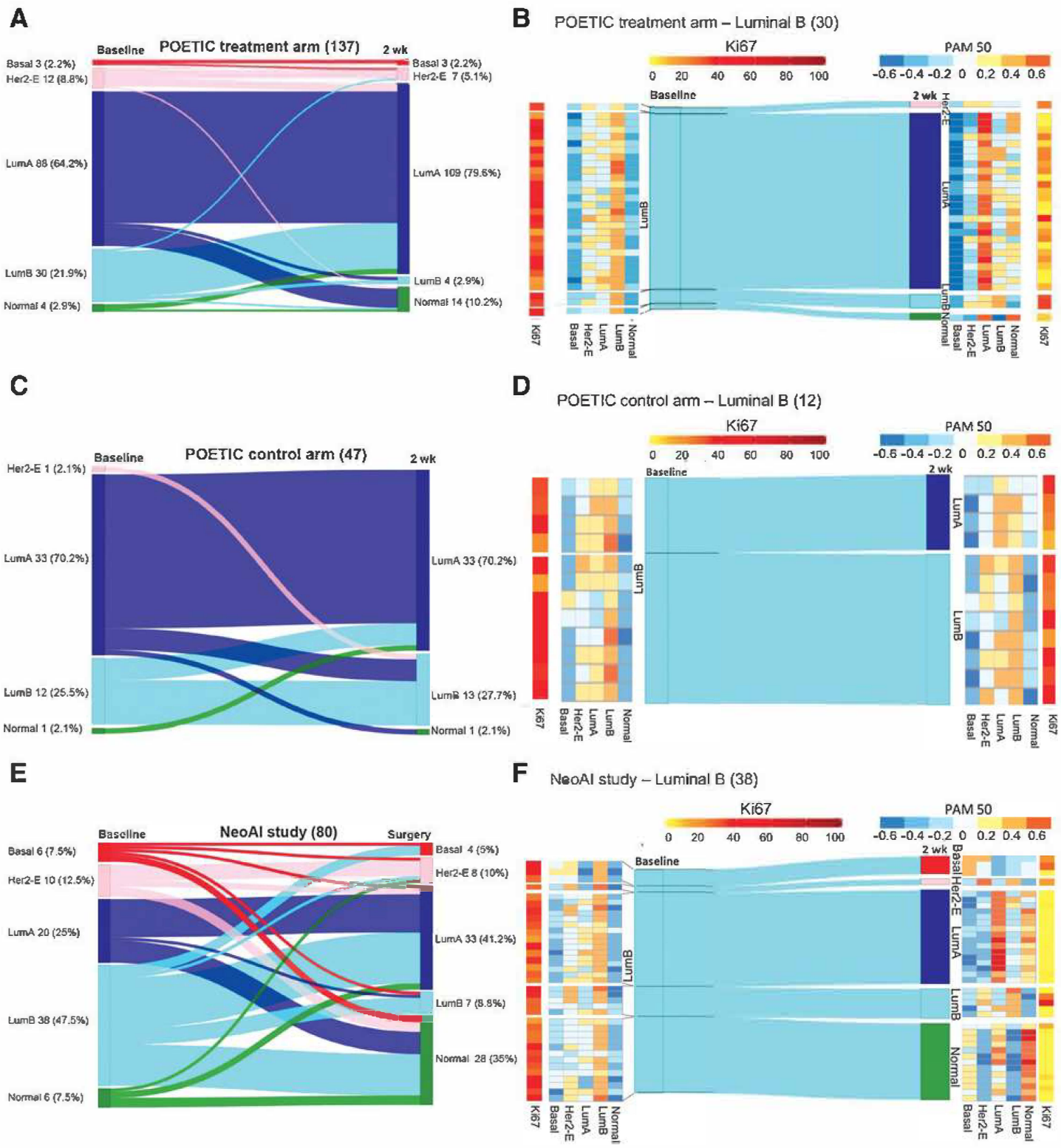

Figure 1.

Differences in intrinsicsubtype 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**). Her2-E, Her2 enriched; LumB, Luminal B; LumA, Luminal A; 2 wk, 2-week time point.

changed significantly from baseline to surgery after longer-term treatment compared with shorter AI therapy in the overall populations (**Fig. 4A**) and in Luminal B tumors only (**Fig. 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 with the NeoAI Nanostring data matrix.

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: (i) All POETIC-treated, (ii) POETIC Luminal A treated, (iii) POETIC Luminal B treated, (iv) POETIC controls, (v) all NeoAI, (vi) NeoAI Luminal A, and (vii) NeoAI Luminal B. **Figure 4C** shows the exclusive genes that

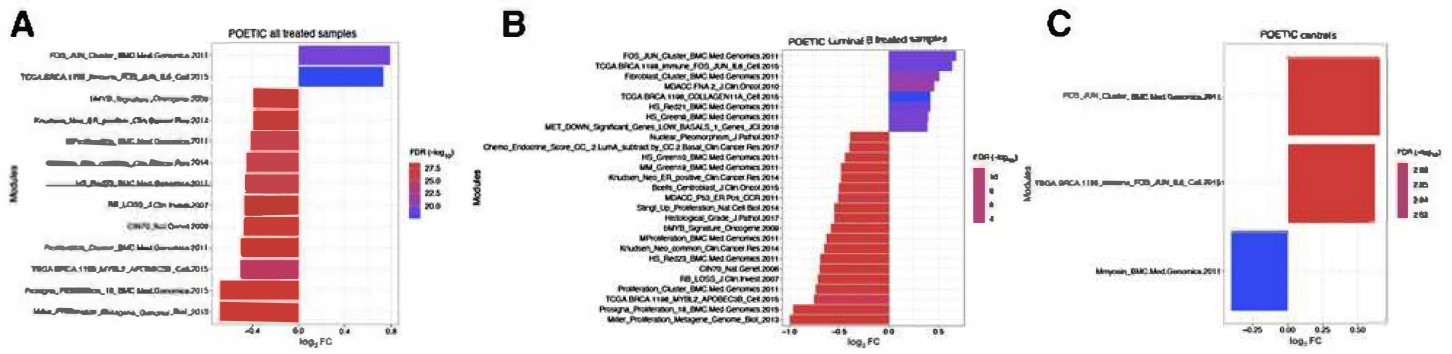

Figure 2.
Module scores expression changes in the POETIC cohort. **A**, Barplots showing the significant module scores expression changes between baseline and after 2 weeks of AI in the POETIC dataset for all samples, for Luminal B samples only (**B**) and for controls (**C**). The *x*-axis shows the $\log_2$FC and the *y*-axis shows the significant module scores that changed. Bars are colored by the degree of significance of the *P* value by paired *t* test. FDR; false discovery rate; $\log_2$ FC, $\log_2$ fold change.

were found significantly different 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 2 weeks 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 tumors of NeoAI study were also significantly affected by AI treatment despite being thought to be associated with nonresponse 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) versus longer (>2 months) AI treatment in the NeoAI dataset and Kruskall–Wallis tests to compare patients grouped in 1–2 months versus >2–6 months versus >6 months, respectively. There were not significant differential changes in gene expression among those categories. We also investigated whether there were positive correlations between the length of AI treatment with the changes in the 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 ($\log_2$ FC) with length of AI treatment was observed (*P* value range = 0.68–0.90; Supplementary Fig. S4).

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 classified 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 subtype. There was a statistically 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 tumors 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 exact test $P < 0.0001$).

Most treated Luminal B tumors in the POETIC subset (17/27; 63%), and all Luminal B tumors in the NeoAI study (33/33; 100%) that were reclassified 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 tumors were AI sensitive (Fisher 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

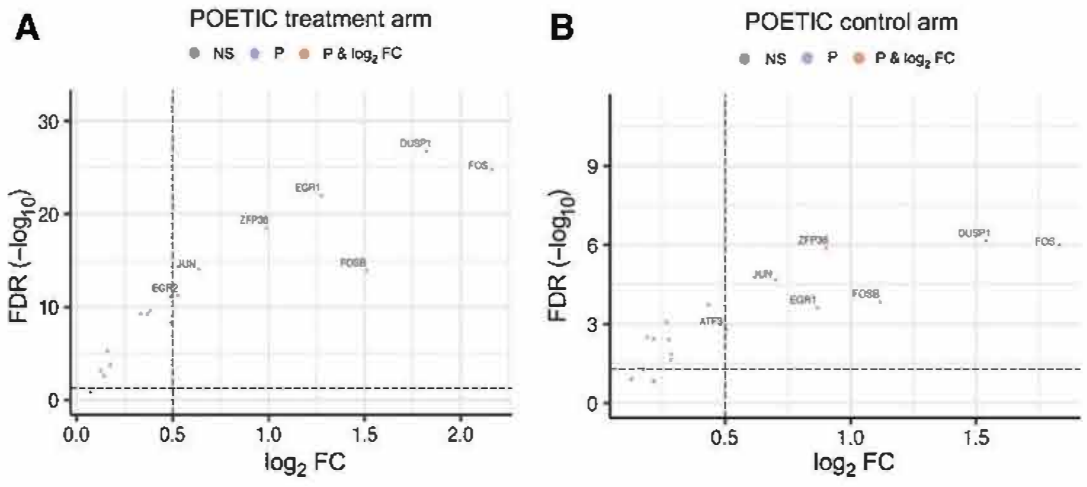

Figure 3.
Differential single-gene expression changes between baseline and surgery of the 17 genes included in the FOS and JUN module scores in the POETIC cohort. Differential expression in the treated samples from POETIC subset (**A**) and in the controls (**B**). In red, there are the significant genes by *P* values by paired *t* tests and $\log_2$FC. FDR, false discovery rate; $\log_2$ FC, $\log_2$ fold change; NS, nonsignificant; P, significant by *P* value paired *t* tests; P&$\log_2$FC, significant by *P* value and $\log_2$ fold change.

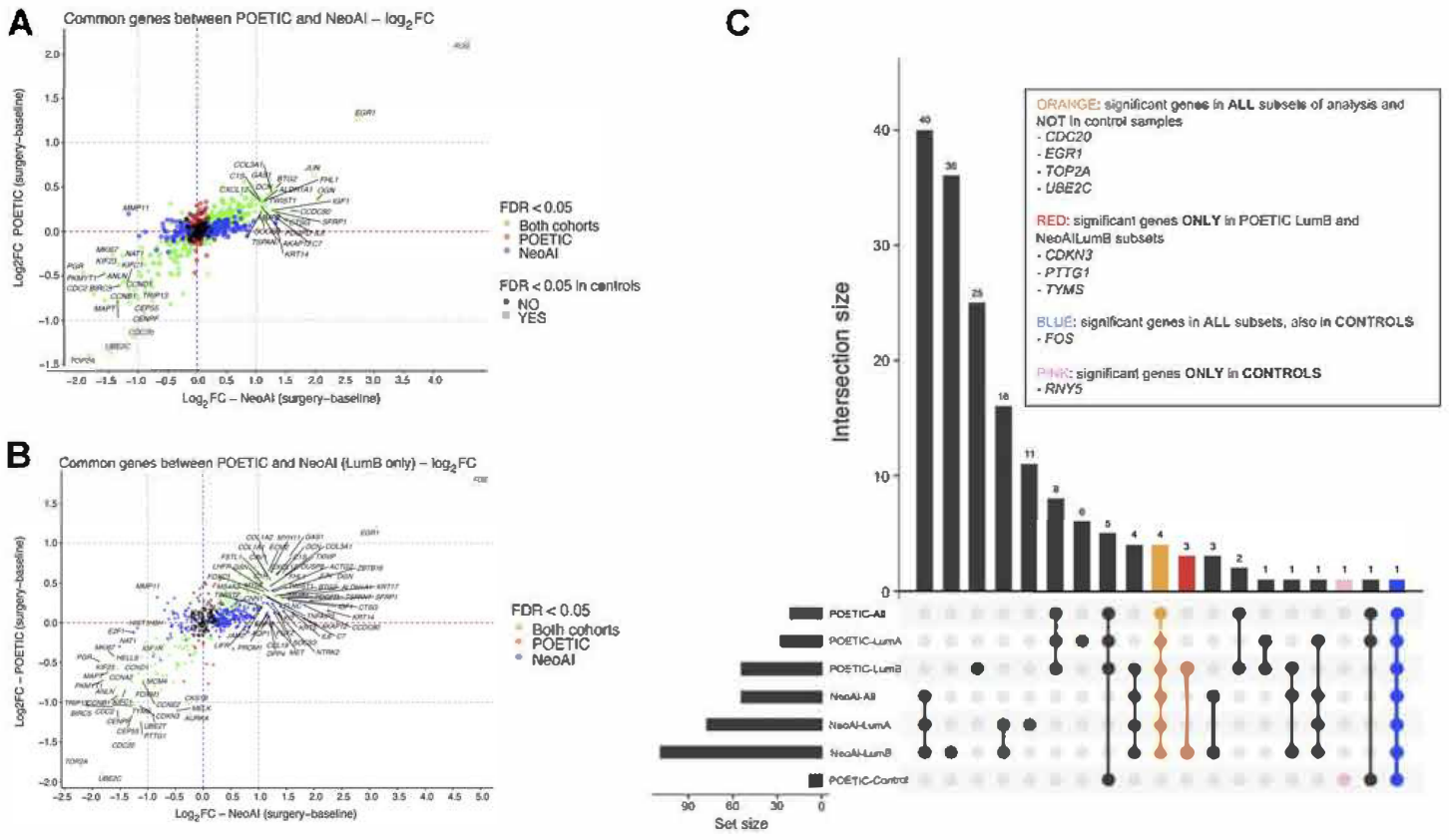

Figure 4.

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 $\log_2$ FC among the entire short-term and long-term AI cohorts. **B,** Scatterplot of differentially expressed genes between baseline and surgery measured by $\log_2$ FC among Luminal B–treated tumors only in the short-term and long-term AI cohorts **C,** Boxplot showing the intersections of common genes differentially expressed between baseline and surgery among different combinations of subgroups of sample patients within the POETIC and NeoAI cohorts: all treated patients in POETIC, only treated with Luminal A in POETIC, only treated with Luminal B in POETIC, all patients in NeoAI, only Luminal A in NeoAI, only Luminal B patients in NeoAI, only controls in POETIC. FDR, false discovery rate; $\log_2$ FC, $\log_2$ fold change.

among 105 ER^+ HER2-negative tumors 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 with Luminal A tumors (Supplementary Fig. S5A). There were 24 immune-related gene modules covering immune-cell pathways, immune-checkpoint component, and IFNγ biology high-expressed at baseline that were associated with early AI resistance (Supplementary Fig. S5A). These gene modules include some genes that have been previously associated with Luminal B–resistant tumors such as *IFNG*, *STAT1*, *IDO1*, *LAG 3*, and *CTLA4* (19). Visualizing 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 (**Fig. 5**). To assess the differential gene expression changes between responder and nonresponder tumors (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 Fig. S5B). Supplementary Figure S6 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 Fig. S6). Gene Ontology enrichment analysis showed that genes related to proliferative and cell-cycle pathways were upregulated in the H-H group compared with H-L.

To investigate further the changes of immune-related features associated with resistance to AI by duration of neoadjuvant endocrine therapy, two immune-related module scores were calculated: (i) The "durvalumab signature" (median of *PD-L1*, *LAG3*, *CXCL9*), previously reported to predict response to immunotherapy in melanoma (20) and (ii) 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 tumors in the neoadjuvant setting (19).

In the POETIC subset, the higher expression of "durvalumab signature" and "immune-tolerance signature" was associated with H-H tumors (Supplementary Fig. S7A). In that setting no significant changes of the signatures' expression from baseline to surgery were seen in either H-H or H-L categories (**Fig. 6A**; Supplementary Fig. S7A). On the other hand, in the NeoAI cohort, there was also a higher expression of the immune-related signatures in H-H tumors compared with H-L at baseline (Supplementary Fig. S7B) 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 tumors compared with H-H tumors (**Fig. 6B**). Although the expression of both immune signatures at surgery remained significantly higher in H-H tumors compared with H-L in the POETIC subset (**Fig. 6C**), it was not significantly different after long-term AI therapy (**Fig. 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.

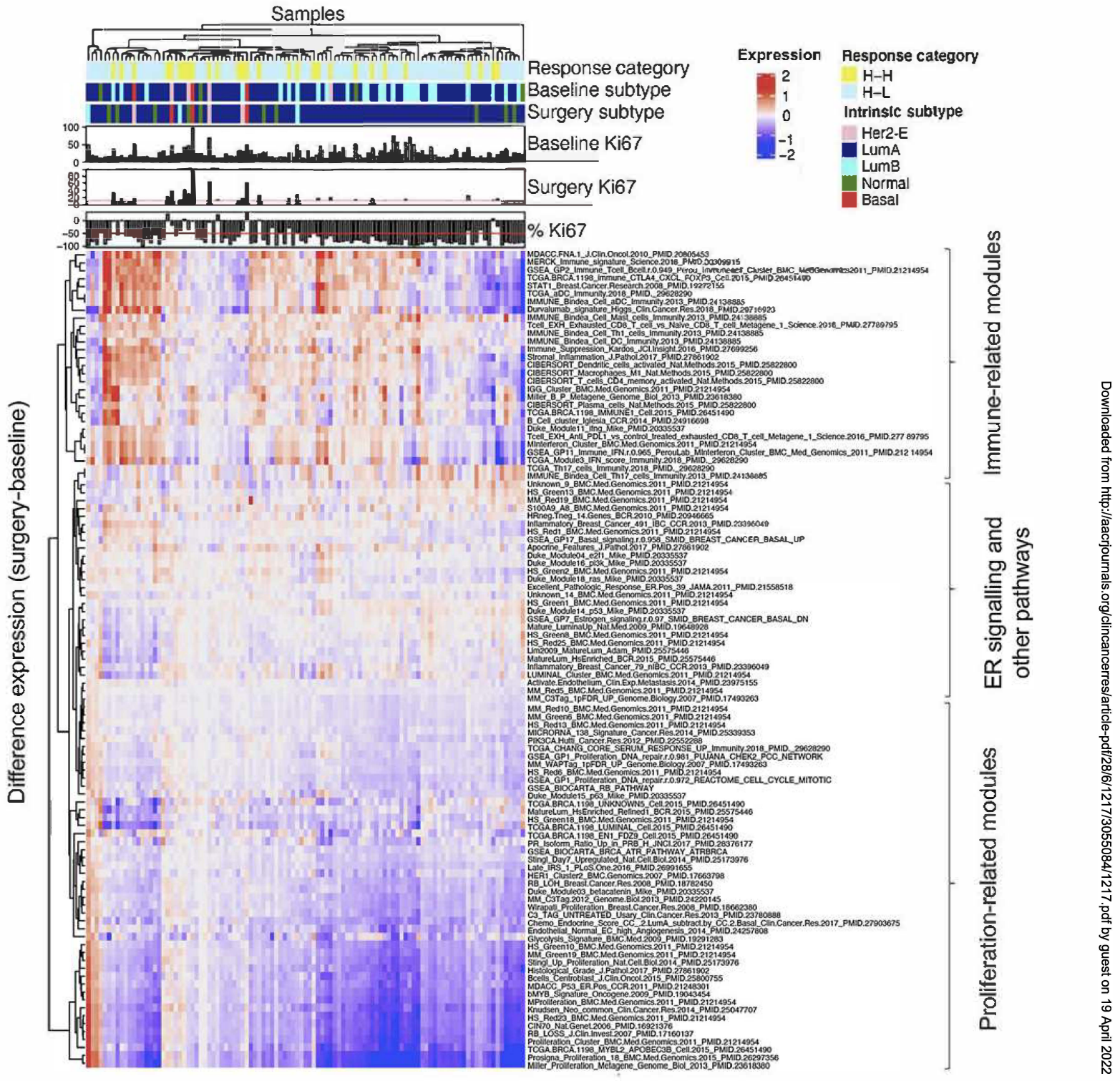

Figure 5.

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 SAM analysis between Ki67 H-H versus H–L, categories in the POETIC subset and annotated by the main categories. ER, estrogen receptor; expression, gene expression; H-H, Ki67 $High_{baseline}$- Ki67 $High_{surgery}$; H-L, Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$; Her2-E, Her2 enriched; LumB, Luminal B; LumA, Luminal A; 2 wk; 2-week time point.

Our results suggest that the enrichment on *PDL1* after longer AI might be the key driving the differences between responders and nonresponders in the NeoAI study (Supplementary Fig. S7C).

Finally, to explore the impact of AI duration on the expression of these two early endocrine-resistance immune-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 (log_2FC) with duration of AI (Supplementary Fig. S8).

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 previously with patient

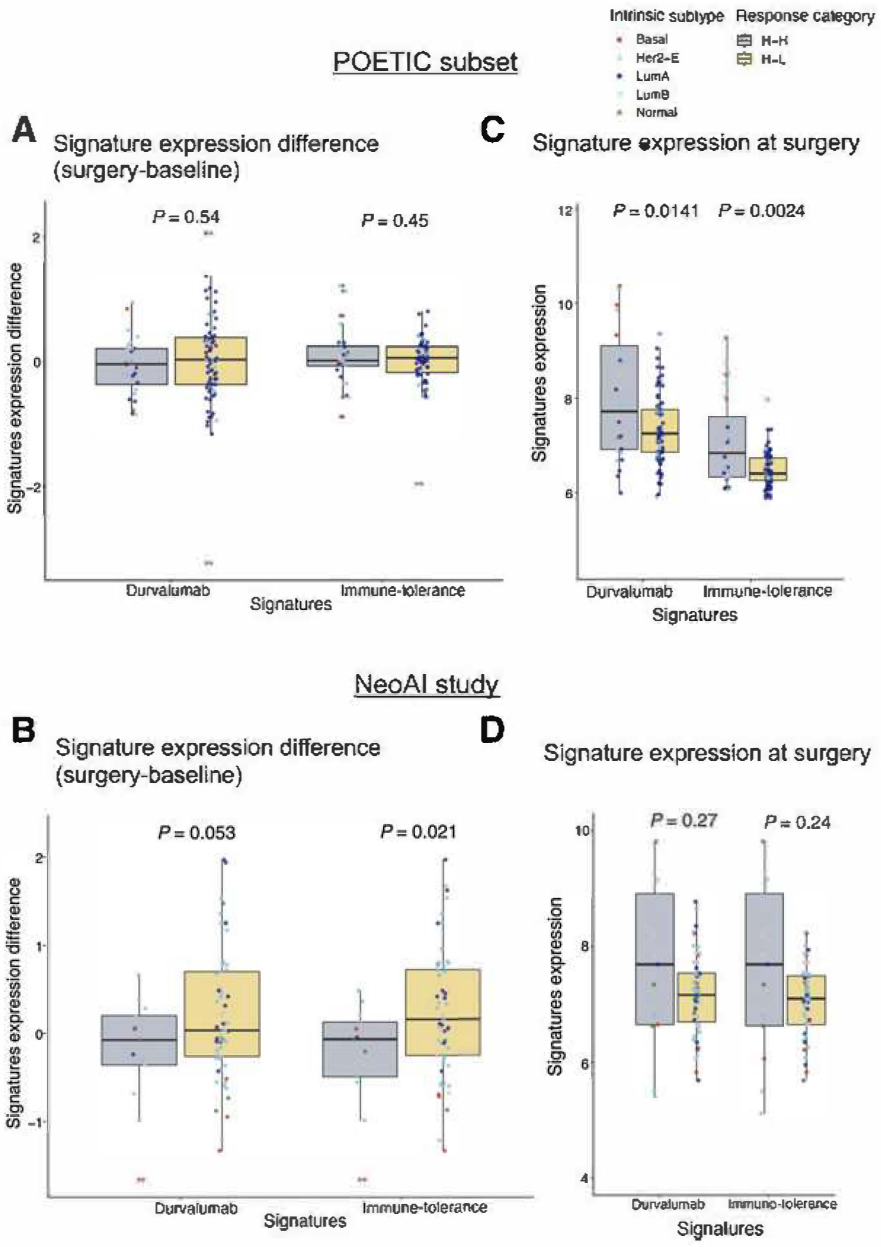

Figure 6.

Boxplots showing changes in gene expression from baseline to surgery of the two immune-related signatures ("durvalumab" and "immune-tolerance") among H-H and H-L Ki67 response categories in the POETIC-treated subset (**A**) and in the NeoAI study (**B**). Boxplots showing gene signature expression of the two immune-related signatures at surgery stratified by H-H and H-L tumors in the POETIC-treated subset (**C**) and in the NeoAI study (**D**). H-H, Ki67 $High_{baseline}$-Ki67 $High_{surgery}$; H-L, Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$; Her2-E, Her2 enriched; LumB, Luminal B; LumA, Luminal A; 2 wk, 2-week time point.

survival data, in each of the two datasets as follows: (i) changes in the correlation coefficient scores to prototypical intrinsic subtype centroids from baseline to surgery, (ii) significant changes that associated with resistance to AI, (iii) significant changes from baseline to surgery in all tumors, (iv) significant changes from baseline to surgery in Lumina B tumors.

Results are shown in Supplementary Table S4 (POETIC) and Supplementary Table S5 (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 that were associated with resistance to AI (H-H tumors) and a subset of the reported significant changes from baseline to surgery found in both datasets, were associated with differential survival. There were no statistically significant associations of the immune features such as the increase of *LAG3*, and the increase of "durvalumab" signature expression with differential outcome.

Our results suggest 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 postmenopausal women with early ER^+ breast cancer, 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-E tumors change their intrinsic subtype within just 2 weeks of treatment, mainly from Luminal B toward Luminal A or normal-like; these changes are associated with differential response to AI and outcome; (ii) in contrast, longer AI treatment may induce additional and greater gene expression changes than 2 weeks only; (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) breast cancer tumors showing early resistance to AI are characterized by a greater expression of immune-checkpoint component, immune-cell enrichment and proliferation, and these signatures were more impacted by longer AI treatment.

$ER^+/HER2^-$ tumors 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 breast cancer (27–29). Previous data have 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" tumors—the majority were Luminal A at baseline. From a biological perspective, our study also shows that most Luminal B or Her2-E tumors 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" breast cancer such as basal-like and some Luminal B and Her2-E will not change despite prolonged AI treatment. On the basis of prior studies, Luminal A and Luminal B baseline tumors are more likely to respond to endocrine therapy than other intrinsic subtypes (30, 31); however, our study also suggests that changes toward a lower-risk subtype correlate with sensitivity to AI treatment and better survival, beyond baseline intrinsic subtypes. Thus, an early reassessment of the intrinsic subtype at the 2-week time point could be essential to distinguish those "sensitive" tumors 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 with longer AI treatment. As expected, most of the downregulated module scores in the POETIC subset involved a decrease of the "high-risk" characteristics toward 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, prevents 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). Second, a PAM50-based CES in $ER^+/HER2^-$ early disease is capable of predicting response to ET in comparison with chemotherapy (38). Higher CES values are associated with endocrine sensitivity and chemoresistance, hence, the estrogen 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, dedifferentiation, 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 signaling 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 tumor-infiltrating cells and immune-related characteristics (43–45) as well as the importance of tumor microenvironment 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 signaling and other chemokines and ILs, as well as the two immune-related gene modules in H-L tumors. In this article, we have also reported the prognostic value of some of those gene expression changes induced by 2 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 preanalytic sample processing (24). In the current 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 nontreated 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^+ breast cancer.

Finally, the 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%; refs. 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 tumors 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 and colleagues have recently demonstrated that immune checkpoint–related genes are upregulated in most Luminal B tumors 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 tumor 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 2 weeks of AI with nonresponder tumors 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 the literature, a small subgroup of $ER^+/HER2^-$ breast cancer could potentially benefit from immunotherapy, currently approved for metastatic triple-negative breast cancer 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 tumors after longer AI treatment has a role in acquired therapy resistance and survival.

Our study has some limitations and strengths. First, we analyzed data from two subsets with very different backgrounds, data collection and analytic methodology. However, our targeted analyses were focused on pathways/modules, facilitating the evaluation of changes under AI treatment and comparison among datasets. Moreover, we included a control group that enabled 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 presurgical setting. Additional whole transcriptome work in a much larger subset of the POETIC treatment arm is ongoing 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 tumors. 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 also with patient outcome, thus providing a supporting rationale to consider the continuation with ET for higher-risk tumors if changes in transcriptional gene expression signatures are desired. Finally, further investigation on the use of immune-checkpoint component inhibition in this setting is warranted.

Authors' Disclosures

J. Parker reports a patent for PAM50 with royalties paid from Veracyte. H. Tovey reports grants from Merck Sharp & Dohme Ltd., Pfizer Inc., and Bayer outside the submitted work. J.M. Bliss reports grants from Cancer Research UK during the conduct of the study; grants and nonfinancial support from AstraZeneca, Merck Sharp & Dohme, Puma Biotechnology, Clovis Oncology, Pfizer, Janssen-Cilag, Novartis, Roche, and Eli Lilly outside the submitted work. J.F.R. Robertson reports grants from AstraZeneca, Novartis, and AstraZeneca and personal fees from AstraZeneca outside the submitted work. M. Dowsett reports personal fees from AstraZeneca and Nanostring outside the submitted work. M.C.U. Cheang reports other support from Nanostring and personal fees from Veracyte during the conduct of the study; in addition, M.C.U. Cheang has a patent for Breast Cancer Classifier: US Patent No. 9,631,239 with royalties paid. No disclosures were reported by the other authors.

Authors' Contributions

M. Bergamino: Conceptualization, formal analysis, investigation, visualization, methodology, writing–original draft. **G. Morani:** Software, formal analysis, investigation, methodology, writing–original draft. **J. Parker:** Conceptualization, data curation, formal analysis, supervision, writing–review and editing. **E.F. Schuster:** Conceptualization, software, visualization, methodology, writing–review and editing. **M.F. Leal:** Conceptualization, data curation, visualization, writing–review and editing. **E. López-Knowles:** Conceptualization, data curation, visualization, writing–review and editing. **H. Tovey:** Formal analysis, visualization, methodology, writing–review and editing. **J.M. Bliss:** Methodology, writing–review and editing. **J.F.R. Robertson:** Writing–review and editing. **I.E. Smith:** Writing–review and editing. **M. Dowsett:** Conceptualization, supervision, funding acquisition, visualization, writing–review and editing. **M.C.U. Cheang:** Conceptualization, data curation, investigation, visualization, methodology, writing–original draft, writing–review and editing.

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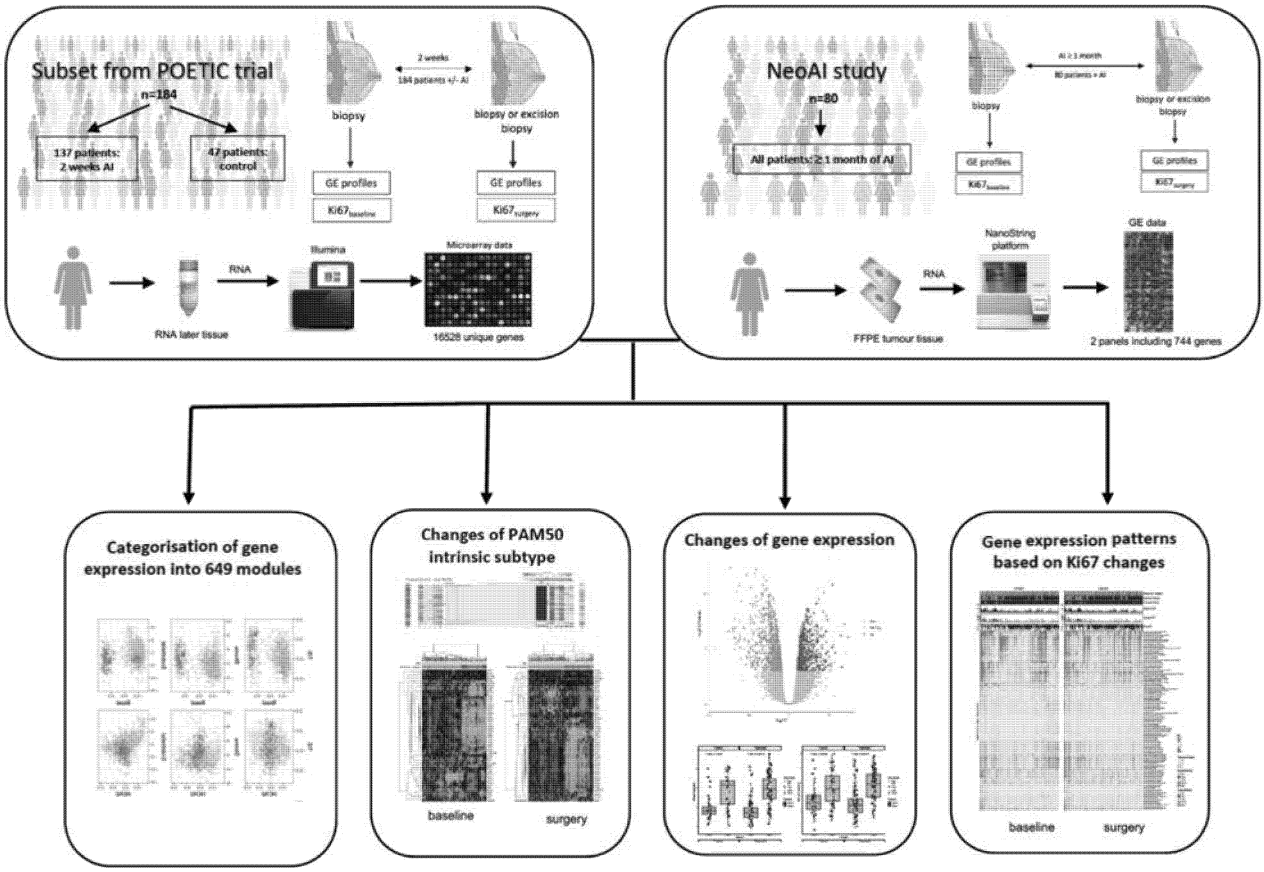
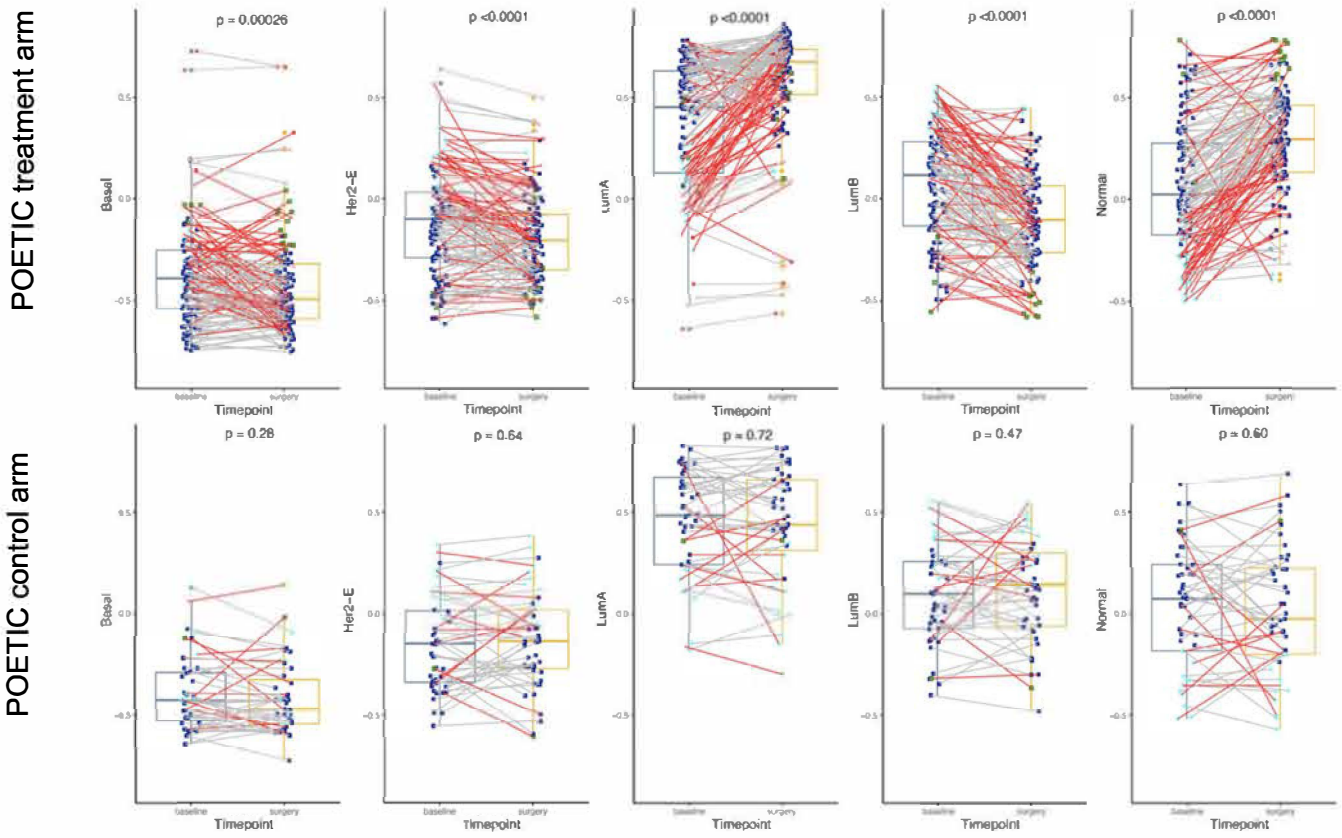
4.1.1 Supplementary materials from manuscript 2

Supplementary figure S1. Overview of the study including POETIC and NeoAI cohorts. **Abbreviations:** n: number, GE: Gene expression, AI: Aromatase inhibitors, FFPE: Formalin-Fixed Paraffin-Embedded samples

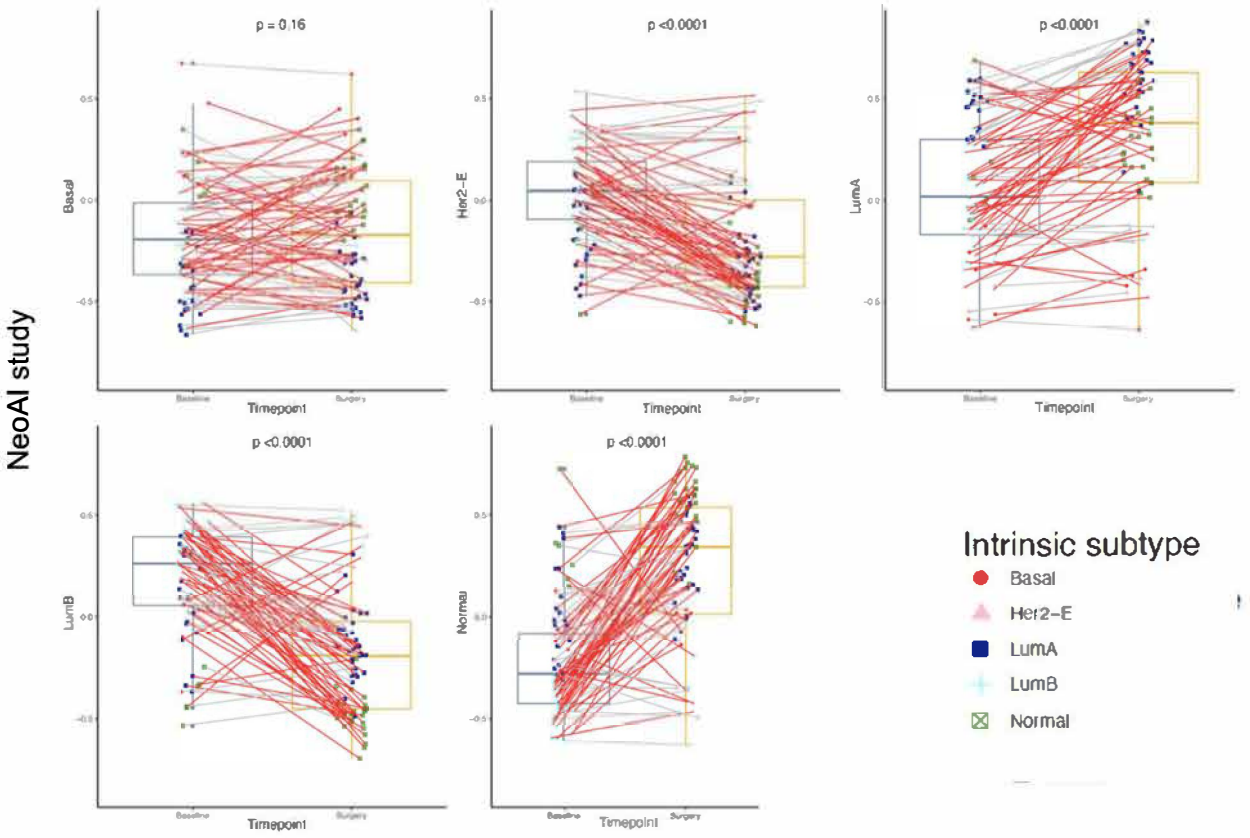


Supplementary figure S2. Changes on subtype correlation coefficients **A.** Changes on subtype correlation coefficients in treated and control samples in the POETIC cohort. Red lines: Tumours showing change in intrinsic subtype after 2-weeks (Treated: 38.0%; Controls: 23.4%). **B.** Changes on subtype correlation coefficients in the NeoAI study. Red line: Tumours showing changes in intrinsic subtype after neoadjuvant treatment (NeoAI study: 67.5%). **Abbreviations:** Her2-E: Her2 enriched, LumB: Luminal B, LumA: Luminal A.

A.

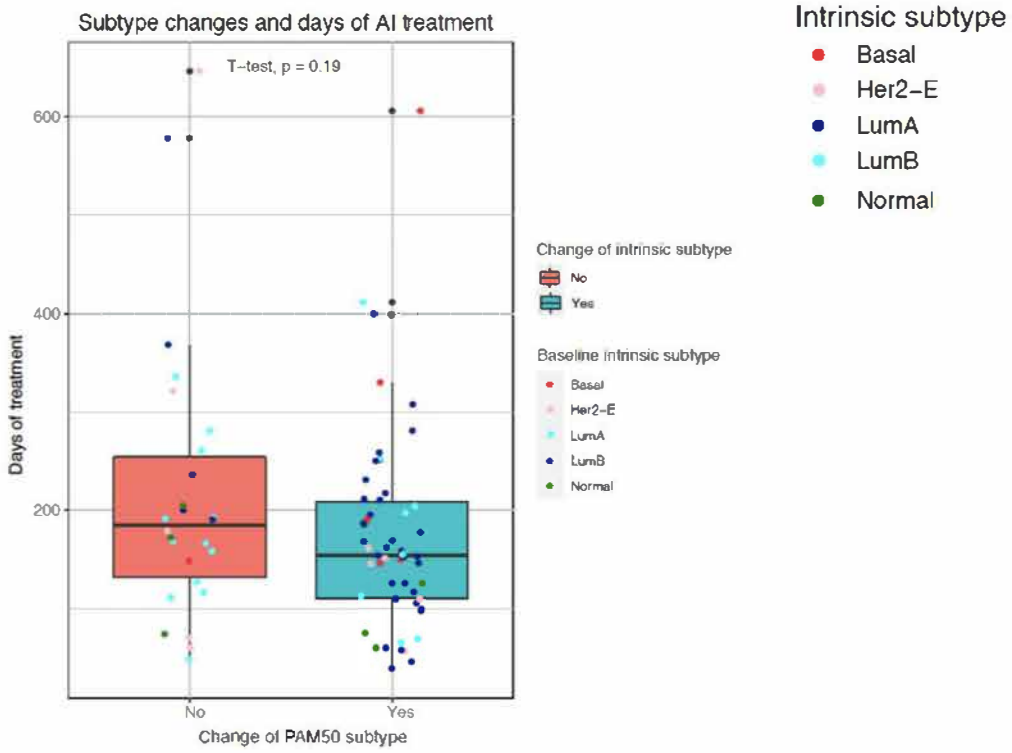

POETIC treatment arm
POETIC control arm
p = 0.00026
p <0.0001
p <0.0001
p <0.0001
p <0.0001
p = 0.28
p = 0.64
p = 0.72
p = 0.47
p = 0.60
Basal
Her2-E
LumA
LumB
Normal
Timepoint

B.


NeoAI study
p = 0.16
p <0.0001
p <0.0001
p <0.0001
p <0.0001
Basal
Her2-E
LumA
LumB
Normal
Timepoint
Intrinsic subtype
Basal
Her2-E
LumA
LumB
Normal

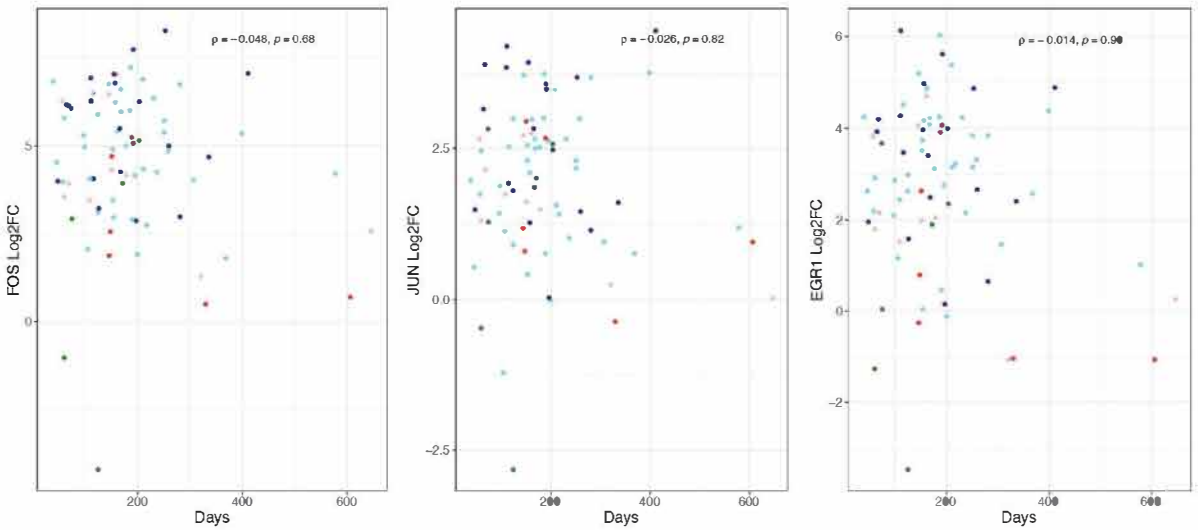
Supplementary figure S3. Association test of the median number of days under AI treatment and changes on PAM50 intrinsic subtype (yes/no).

Abbreviations: Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A



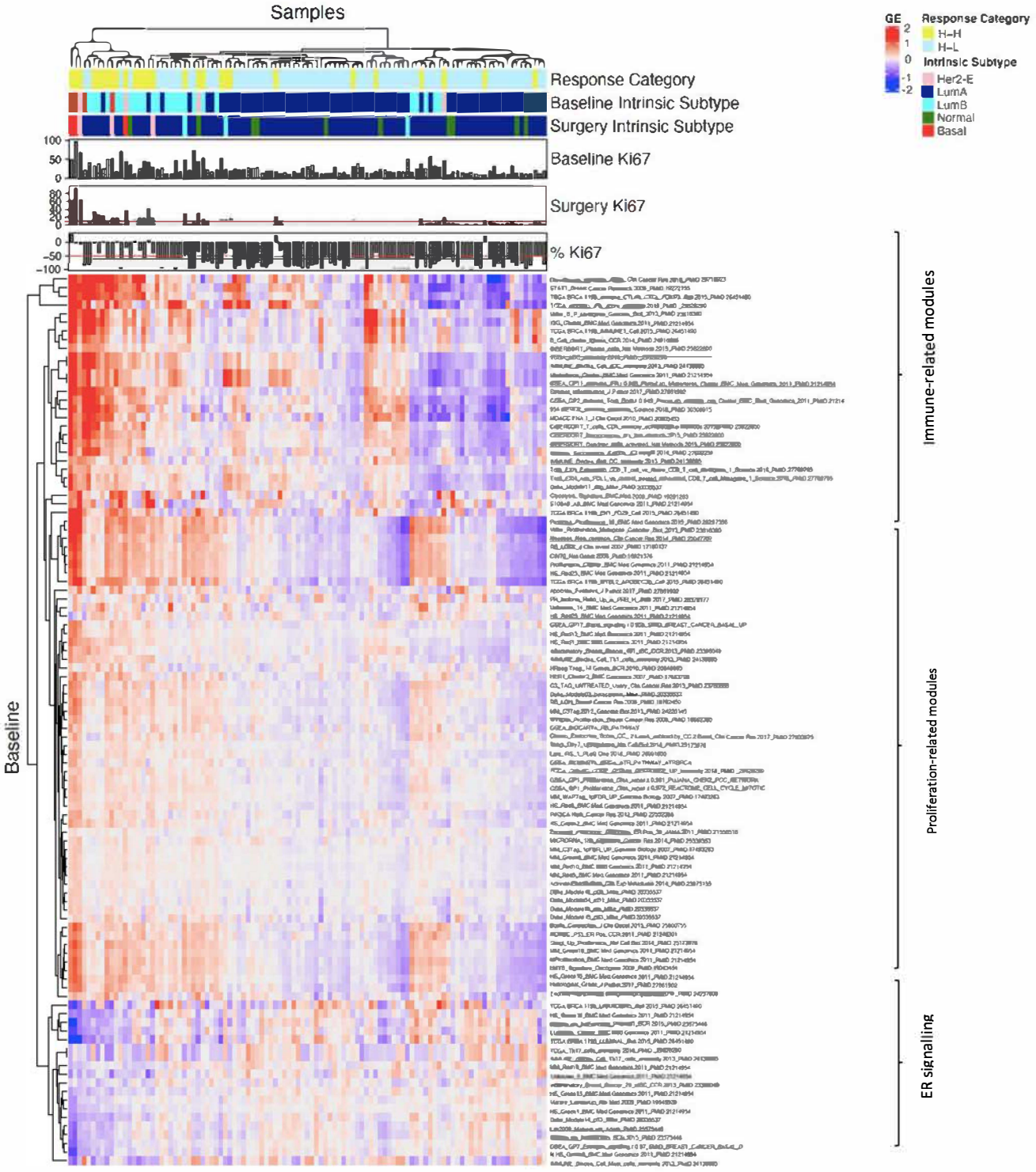
Supplementary figure S4. Spearman Rank-Order Correlation test of the changes of expression from baseline to surgery (Log2FC) under AI treatment of available genes from FOS and JUN modules in the POETIC cohort (FOS, JUN and EGR1) with time in the NeoAI study.

Abbreviations: FC: Fold change, Her2-E: Her2-enriched, lumB: luminal B, lumA: luminal A



Supplementary figure S5. A. Unsupervised hierarchical clustering of module scores at baseline in the POETIC treated subset selected by two unpaired SAM analysis for Ki67 H-H vs H-L categories and median centered. **B.** Significant modules using two unpaired SAM analysis of the differential changes in module scores between H-H vs H-L in POETIC treated samples. **Abbreviations**: H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery}$, H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$, Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A, 2wk: two-week time point, GE: Gene expression**.**

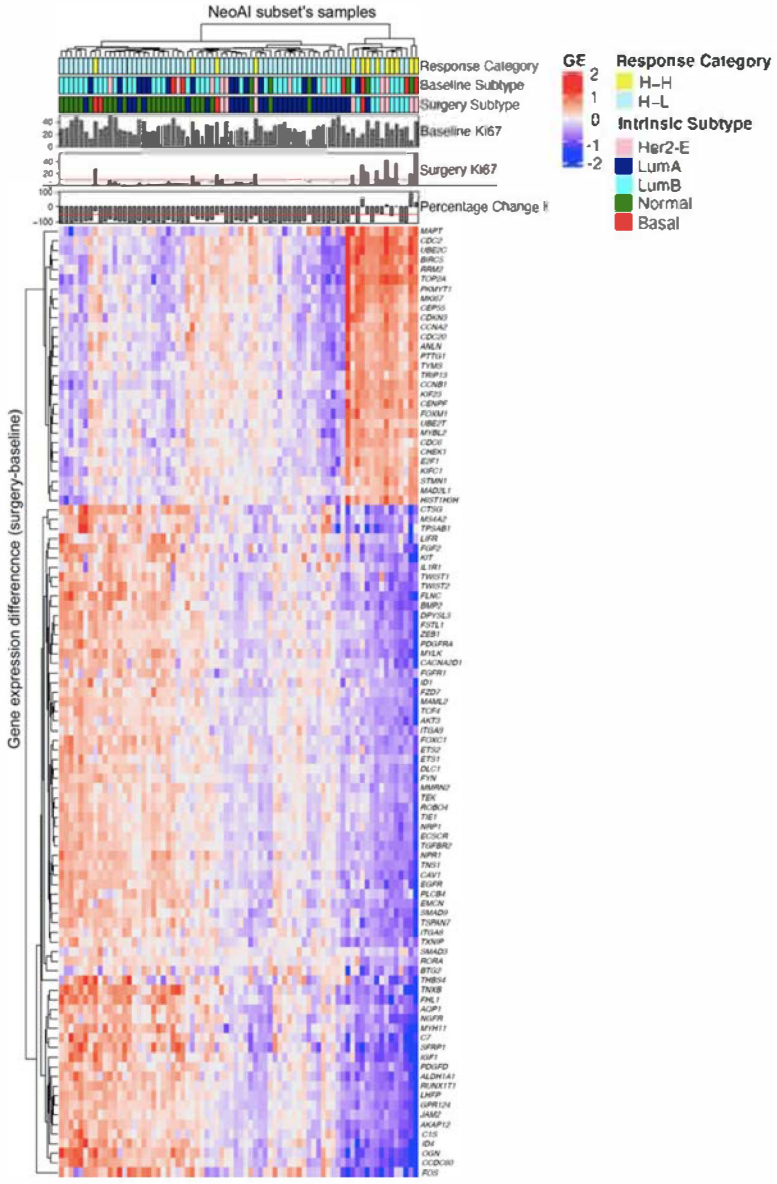
A.



B.

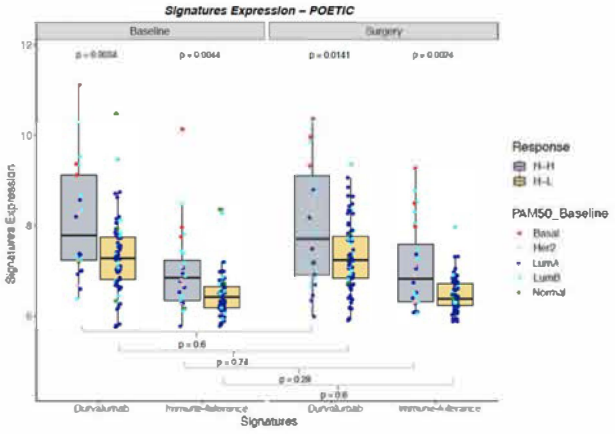
Knudsen_Neo_ER_positive_Clin.Cancer.Res.2014_PMID.25047707
Nuclear_Pleomorphism_J.Pathol.2017_PMID.27861902
Prosigna_Proliferation_18_BMC.Med.Genomics.2015_PMID.26297356
Bcells_Centroblast_J.Clin.Oncol.2015_PMID.25800755
Miller_Proliferation_Metagene_Genome_Biol_2013_PMID.23618380

Supplementary figure S6. Unsupervised hierarchical clustering of single gene expression changes in the NeoAI subset selected by two unpaired SAM analysis for Ki67 H-H vs H-L. **Abbreviations**: H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery}$, H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$, Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A, 2wk: two-week time point, GE: Gene expression.

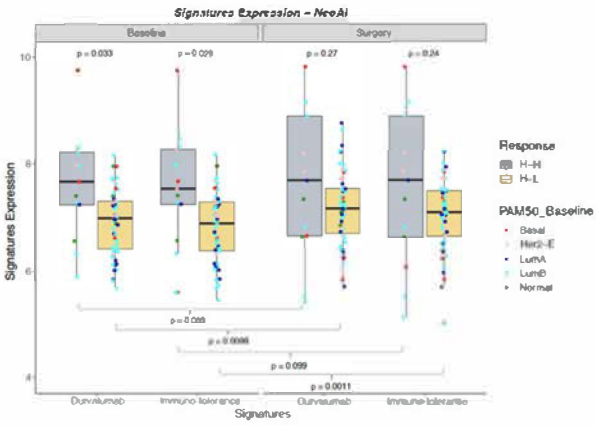


Supplementary figure S7. Boxplots showing gene signature expression of the two immune-related module-score ("Durvalumab" and "Immune-tolerance") at baseline and at surgery amongst H-H and H-L Ki67 response categories in A. POETIC and B. NeoAI; C. Differential changes in gene expression for each individual gene included in the two immune-related modules: "Durvalumab" and "Immune-tolerance" between H-H and H-L tumours in the NeoAI subset. **Abbreviations**: H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery}$, H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$, Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A, 2wk: two-week time point

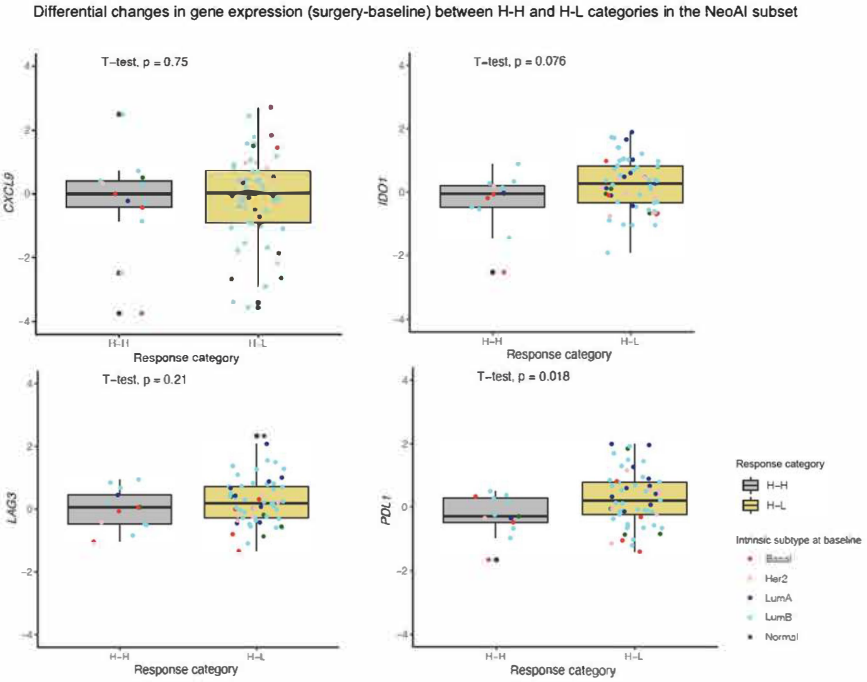
A.



B.

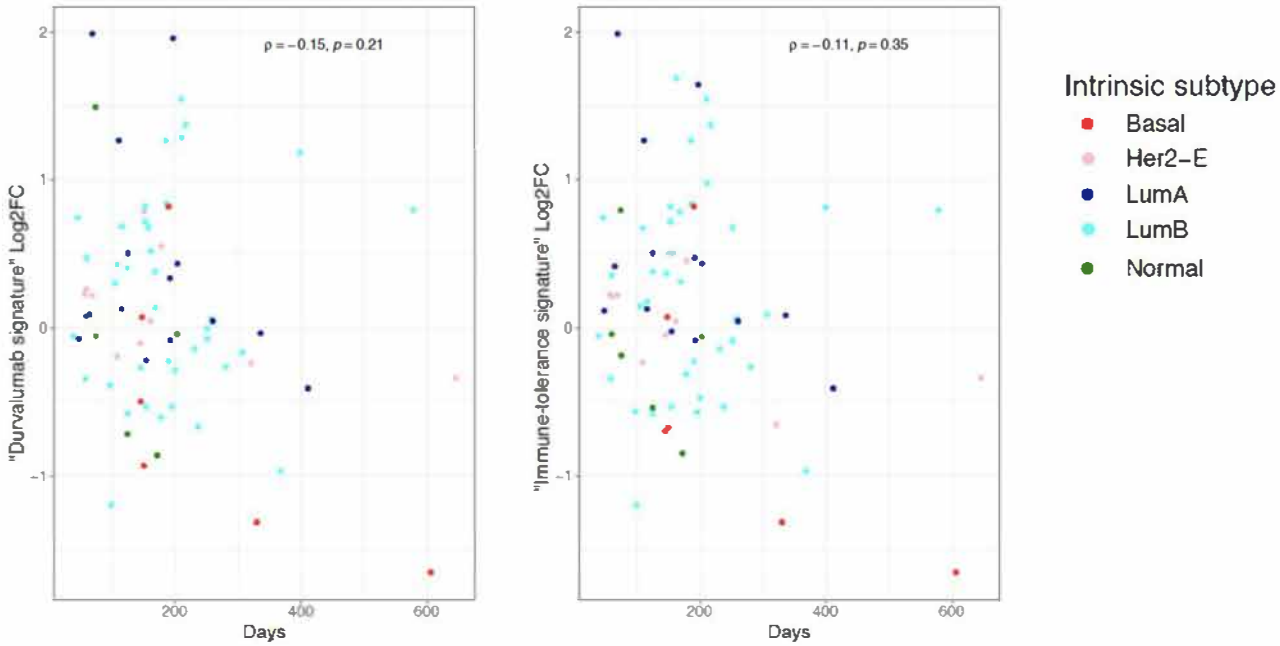


C.



Supplementary figure S8. Spearman rank order correlation of the changes in gene expression from baseline to surgery (Log2FC) under AI treatment of the two immune related signatures "Durvalumab" and "Immune-tolerance" with time in the NeoAI study.

Abbreviations: FC: Fold change, Her2-E: Her2-enriched, lumB: luminal B, lumA: luminal



Supplementary table S1. Demographics and molecular characteristics in POETIC subset (treatment and control arms separately) and in the NeoAI study. **Abbreviations:** H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery}$, H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$, L-L: Ki67 $Low_{baseline}$- Ki67 $Low_{surgery}$, L-H: Ki67 $Low_{baseline}$-$High_{surgery}$, Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A.

	POETIC (treatment arm)	**POETIC (control arm)**	**NeoAI study**
	n=137	**n=47**	**n=80**
	Surgery Tumour size (cm)		
≤2	39 (28.5%)	13 (27.7%)	23 (28.8%)
>2 & ≤5	94 (68.6%)	29 (61.7%)	46 (57.5%)
>5	4 (2.90%)	5 (10.6%)	11 (13.7%)
	Nodal status		
Negative	69 (50.4%)	27 (57.4%)	43 (53.8%)
Positive	68 (49.6%)	20 (42.6%)	22 (27.5%)
NA	0 (0.0%)	0 (0.00%)	15 (18.7%)
	Histological type		
Ductal	109 (79.6%)	38 (80.9%)	60 (75.0%)
Lobular	25 (18.2%)	9 (19.1%)	12 (15.0%)
Mixed ductal and lobular	0 (0.0%)	0 (0.0%)	8 (10.0%)
Mucinous	1 (0.73%)	1 (0.0%)	0 (0.0%)
Papillary	1 (0.73%)	2 (0.0%)	1 (0.0%)
Tubular	1 (0.73%)	3 (0.0%)	2 (0.0%)
	HER2 status		
Negative	118 (86.1%)	39 (82.9%)	75 (93.8%)
Positive	19 (13.9%)	8 (7.10%)	5 (6.2%)
	PAM50 subtype at baseline		
Basal Like	3 (2.2%)	0 (0%)	6 (7.5%)
Her2-E	12 (8.80%)	1 (2.10%)	10 (12.5%)
LumA	88 (64.2%)	33 (70.2%)	20 (25.0%)
LumB	30 (21.9%)	12 (25.5%)	38 (47.5%)
Normal	4 (2.9%)	1 (2.1%)	6 (7.5%)
	Change of subtype		
Yes	52 (38.0%)	11 (23.4%)	54 (67.5%)
No	85 (62.0%)	36 (76.6%)	26 (32.5%)

	Ki67 changes		
H-H	42 (30.7%)	34 (72.3%)	13 (16.3%)
H-L	82 (59.8%)	6 (12.8%)	61 (76.3%)
L-H	0 (0.0%)	6 (12.8%)	1 (1.2%)
L-L	13 (9.5%)	1 (2.1%)	5 (6.2%)

Supplementary table S2. Number of genes significantly differentially expressed between baseline and surgery in each of the two cohorts according to different subgroups within each cohort.

	POETIC subset	**NeoAI study**
All tumours	21 genes	54 genes
Luminal tumours	26 genes	100 genes
Luminal A	27 genes	77 genes
Luminal B	54 genes	109 genes
Controls	8 genes	

Supplementary table S3. Significant differential changes for single gene level between baseline and surgery in the NeoAI study and in POETIC subset according to treatment arm and ranked by Log2FC values. **Abbreviations:** ID: Identification, Log2FC: Log2 Fold Change, FDR: False Discovery Rate.

NeoAI			*POETIC (treated)*			*POETIC (controls)*		
Gene ID	**Log2FC**	**p-adjusted value (FDR)**	***Gene ID***	**Log2FC**	**p-adjusted value (FDR)**	***Gene ID***	**Log2FC**	**p-adjusted value (FDR)**
Inhibited								
PGR	-1.4	<0.0001	***TFF1***	-1.6	<0.0001	***HBB***	-1.6	0.01
TOP2A	-1.3	<0.0001	***TOP2A***	-1.4	<0.0001	***HBA2***	-1.5	0.01
BIRC5	-1.0	<0.0001	***UBE2C***	-1.4	<0.0001	***HBA1***	-1.3	0.017
PKMYT1	-1.6	<0.0001	***HBB***	-1.3	<0.0001	***RNY5***	-1.2	0.022
MAPT	-1.2	<0.0001	***HBA2***	-1.3	<0.0001			
UBE2C	-1.8	<0.0001	***CDC20***	-1.2	<0.0001			
RRM2	-1.4	<0.0001	***NUSAP1***	-1.2	<0.0001			
MKI67	-1.8	<0.0001	***HBA1***	-1.1	<0.0001			
CENPF	-1.2	<0.0001	***NEK2***	-1.1	<0.0001			
KIF23	-1.3	<0.0001	***SUSD3***	-1.1	<0.0001			
CDC2	-1.9	<0.0001	***ASPM***	-1.0	<0.0001			
NAT1	-1.2	<0.0001	***UHRF1***	-1.0	<0.0001			

ANLN	-1.5	<0.0001	***PRC1***	-1.0	<0.0001			
MYBL2	-1.1	<0.0001	***FGFR3***	-1.0	<0.0001			
KIFC1	-1.2	<0.0001	***AGR2***	-1.0	<0.0001			
CCNB1	-1.1	<0.0001						
MMP11	-1.6	<0.0001						
CEP55	-1.5	<0.0001						
CDC20	-1.3	<0.0001						
CCND1	-1.3	<0.0001						
TRIP13	-1.2	<0.0001						
Activated								
FOS	4.6	<0.0001	***FOS***	2.2	<0.0001	***FOS***	1.83	0.00051
EGR1	2.0	<0.0001	***RGS1***	1.8	<0.0001	***RGS1***	1.55	0.0012
NR4A1	2.7	<0.0001	***DUSP1***	1.8	<0.0001	***DUSP1***	1.54	0.00027
OGN	2.7	<0.0001	***FOSB***	1.5	<0.0001	***FOSB***	1.12	0.021
JUN	1.3	<0.0001	***CYR61***	1.3	<0.0001			
NR4A3	2.0	<0.0001	***EGR1***	1.3	<0.0001			
CCDC80	1.2	<0.0001						
IL6	1.2	<0.0001						
CTSG	1.5	<0.0001						
C7	1.1	<0.0001						
BTG2	1.2	<0.0001						
ID4	1.0	<0.0001						
SFRP1	1.2	<0.0001						
KRT14	1.6	<0.0001						
IGF1	1.6	<0.0001						
COL3A1	1.4	<0.0001						
TNXB	1.2	<0.0001						
GAS1	1.2	<0.0001						
DCN	1.3	<0.0001						
PDGFD	1.3	<0.0001						
TWIST1	1.1	<0.0001						
CCL4	1.4	<0.0001						
AKAP12	1.2	<0.0001						
CMA1	1.3	<0.0001						
ALDH1A1	1.1	<0.0001						
FHL1	1.1	<0.0001						
SOCS3	1.0	<0.0001						
TSPAN7	1.2	<0.0001						
MMP2	1.3	<0.0001						
CXCL12	1.1	<0.0001						
C1S	1.3	<0.0001						
SMAD9	1.1	<0.0001						
NGFR	1.3	<0.0001						

Supplementary table S4. Multivariable cox regression models for TTR and OS for each of the significant findings in the POETIC subset: in blue changes in the correlation coefficient scores to prototypical intrinsic subtype centroids; in orange changes of the modules scores that were significantly different from baseline to surgery in all tumours including lumB, in green

those significant changes from baseline to surgery in lumB only, in grey those changes in modules score observed significant differentially expressed between H-H and H-L tumours by SAM analysis (light grey also includes modules significantly different expressed between baseline and surgery in lumB tumours and dark green in lumB and in all tumours). The multivariable cox models were adjusted for the standard clinicopathological variables: PR status, HER2 status, tumour grade, pathological tumour size, histology subtype, nodal status, and vascular invasion. **Abbreviations:** TTR: Time to Recurrence, OS: Overall Survival, CI: Confidence Interval, FDR: False discovery rate, Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A, 2wk: two-week time point, H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery}$, H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$,

	Multivariable cox models for TTR					Multivariable cox models for OS				
	Hazard Ratio	95% CI		p-value	FDR	Hazard Ratio	95% CI		p-value	FDR
Basal_centroid_change	3.81	0.16	91.45			0.07	0.01	0.82	**	*
Her2E_centroid_change	4.12	0.12	138.19			1.42	0.08	24.21		
LumA_centroid_change	0.11	0.01	1.04	*	*	1.21	0.2	7.2		
LumB_centroid_change	10.66	1.03	110.8	**	*	12.18	1.74	85.47	**	*
Normal_centroid_change	0.1	0.01	0.72	**	**	0.22	0.04	1.14	*	
TCGABRCA1198_MYBL2_APOBEC3B_Ce	12.23	3.51	42.54	***	***	2.38	0.89	6.37	*	
Proliferation_Cluster_BMCMedGe	12.19	3.36	44.26	***	***	3.22	1.07	9.66	**	*
CIN70_NatGenet2006_PMID169213	15.78	3.99	62.36	***	***	3.81	1.18	12.26	**	*
RB_LOSS_JClinInvest2007_PMID	13.61	3.47	53.39	***	***	3.21	1.03	10	**	
HS_Red23_BMCMedGenomics2011_P	14	3.55	55.17	***	***	3.13	0.98	9.98	*	
Knudsen_Neo_common_ClinCancerR	15.31	3.65	64.26	***	***	5.54	1.67	18.37	***	*
MProliferation_BMCMedGenomics	18.63	3.93	88.25	***	***	2.9	0.81	10.39		
bMYB_Signature_Oncogene2009_PMI	25.28	4.92	129.83	***	***	3.9	0.97	15.64	*	
TCGABRCA1198_immune_FOS_JUN_IL	0.32	0.15	0.67	***	***	0.53	0.3	0.94	**	*
FOS_JUN_Cluster_BMCMedGenomics	0.31	0.15	0.65	***	***	0.59	0.34	1.02	*	
Histological_Grade_JPathol2017	32.64	4.94	215.45	***	***	6.16	1.24	30.51	**	*
Stingl_Up_Proliferation_NatCell	27.03	4.7	155.49	***	***	5.47	1.25	23.98	**	*
MDACC_P53_ERPos_CCR2011_PMID2	26.36	4.06	171.29	***	***	3.32	0.77	14.34		
MM_Green19_BMCMedGenomics2011	53.55	6.56	437.33	***	***	7.23	1.3	40.22	**	*
HS_Green10_BMCMedGenomics2011	48.32	6.45	362.14	***	***	3.23	0.56	18.71		
Chemo_Endocrine_Score_CC_2LumA	28.65	3.59	228.56	***	***	5.84	0.93	36.73	*	
MET_DOWN_Significant_Genes_LOW_B	0.57	0.2	1.59			0.58	0.27	1.26		
HS_Green9_BMCMedGenomics2011_	0.67	0.23	1.95			0.57	0.25	1.28		
HS_Red21_BMCMedGenomics2011_P	0.67	0.25	1.79			0.58	0.28	1.21		
TCGABRCA1198_COLLAGEN11A_Cell	0.66	0.3	1.47			0.59	0.32	1.09	*	
MDACCFNA2_JClinOncol2010_PM	0.5	0.16	1.59			0.48	0.2	1.2		

Fibroblast_Cluster_BMCMedGenom	0.59	0.23	1.52			0.55*	0.27	1.1	*	
Nuclear_Pleomorphism_JPathol20	32.76	3.24	331.64	***	***	9.37	1.36	64.79	**	*
Bcells_Centroblast_JClinOncol	21.27	3.77	119.83	***	***	2.05	0.5	8.42		
Prosigna_Proliferation_18_BMCMe	7.07	2.62	19.08	***	***	2.38	1.06	5.39	**	*
Knudsen_Neo_ER_positive_ClinCan	23.14	4.76	112.49	***	***	8.05	2.06	31.47	***	*
Miller_Proliferation_Metagene_Ge	6.6	2.53	17.23	***	***	2.28	1.02	5.1	**	

*** p<0.01, ** p<0.05, * p<0.1

Supplementary table S5. Multivariable cox regression models for Overall survival (OS) and multivariable linear regression models for risk of recurrence score (ROR) as surrogate of recurrence in the NeoAI subset: in blue changes in the correlation coefficient scores to prototypical intrinsic subtype centroids; in orange changes of the genes that were significantly different from baseline to surgery in all tumours including lumB; in green those significant changes in lumB only, in yellow, grey and purple the significantly different changes of gene expression between H-H and H-L tumours: in grey there are genes that also changed significantly from baseline to surgery in lumB only and in purple in all patients. The models have been adjusted for the standard post-surgery clinicopathological variables: PR status, HER2 status, diagnostic-tumour grade, surgical tumour size, diagnostic histological type, nodal status, and vascular invasion. **Abbreviations**: OS: Overall Survival, ROR: Risk of Recurrence Score, CI: Confidence Interval, FDR: False discovery rate, Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A, 2wk: two-weeks time point, H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery}$, H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$,

	Adjusted cox models for OS					**Adjusted linear regression models for ROR at surgery**		
		95% CI						
	Hazard Ratio	5%	95%	p-value	FDR	Correlation Coefficient	p-value	FDR
Basal_centroid_change	0.20	0.03	1.44			-0.25		
Her2_centroid_change	3.76	0.88	16.06			0.69	***	***
LumA_centroid_change	0.63	0.14	2.79			-0.49	***	***
LumB_centroid_change	3.60	1.25	10.35	**		0.68	***	***
Normal_centroid_change	0.33	0.14	0.81	**		-0.71	***	***
CCL4	1.01	0.70	1.45			-0.39		
CMA1	0.75	0.53	1.07			-0.52	***	***
COL3A1	0.68	0.49	0.93	**		-0.59	***	***
CXCL12	0.63	0.45	0.89	**	*	-0.57	***	***
DCN	0.61	0.45	0.82	***	**	-0.66	***	***
EGR1	0.94	0.78	1.15			-0.60	***	***
GAS1	0.68	0.49	0.95	*		-0.61	***	***
IL6	0.81	0.60	1.11			-0.47	***	***
JUN	0.87	0.66	1.14			-0.61	***	***

KRT14	0.82	0.71	0.95	**	*	-0.48	***	***
MMP11	1.22	0.91	1.63			0.09		
MMP2	0.75	0.53	1.05			-0.59	***	***
NAT1	1.32	0.98	1.78			0.40	***	**
NR4A1	0.91	0.76	1.10			-0.51	***	***
NR4A3	0.95	0.74	1.22			-0.42	**	**
PGR	1.02	0.80	1.31			0.31		
SOCS3	0.83	0.66	1.04			-0.45	***	***
ACTG2	0.61	0.44	0.84	**	*	-0.58	***	***
C1R	0.70	0.51	0.96	*		-0.63	***	***
CCL19	0.83	0.62	1.11			-0.19		
CCND1	1.00	0.65	1.55			0.43	***	***
CCNE2	1.72	1.12	2.65	**	*	0.47	***	***
CDCA1	1.30	0.79	2.13			0.47	***	***
CHIT1	1.10	0.80	1.50			-0.19		
CNN1	0.53	0.36	0.78	***	**	-0.58	***	***
COL1A1	0.75	0.55	1.04			-0.48	***	***
COL1A2	0.71	0.52	0.96	*		-0.58	***	***
COL6A6	0.64	0.44	0.93	**		-0.40	***	***
DPP4	0.90	0.62	1.31			-0.50	***	***
DUSP6	0.90	0.55	1.46			-0.45	***	***
ECM2	0.56	0.39	0.82	**	*	-0.61	***	***
FGF7	0.53	0.35	0.80	**	*	-0.53	***	***
FIGF	0.75	0.52	1.07			-0.47	***	***
GSN	0.54	0.38	0.78	***	**	-0.64	***	***
HELLS	1.79	0.96	3.33			0.46	***	***
IGF1R	1.37	0.77	2.42			-0.03		
KRT17	0.79	0.64	0.96	**		-0.54	***	***
KRT5	0.83	0.68	1.01			-0.46	***	***
MCM4	1.50	0.85	2.66			0.56	***	***
MET	0.65	0.42	1.00	*		-0.52	***	***
MS4A1	0.84	0.61	1.15			-0.28		
NEFL	0.91	0.61	1.35			-0.38	**	**
NTRK2	0.78	0.58	1.06			-0.32	*	*
PROM1	0.81	0.59	1.12			-0.37	*	*
TNFAIP3	1.05	0.68	1.61			-0.34	**	**
WIF1	0.73	0.52	1.02			-0.45	***	***
WNT11	1.22	0.75	1.99			-0.37	**	**
ZBTB16	0.97	0.75	1.25			-0.38	***	***
AKT3	0.42	0.25	0.69	***	**	-0.71	***	***
CACNA2D1	0.45	0.30	0.68	***	**	-0.55	***	***
CDKN3	1.27	0.89	1.81			0.69	***	***
CHEK1	1.78	0.98	3.21			0.67	***	***
DLC1	0.54	0.34	0.86	**	*	-0.55	***	***
DPYSL3	0.42	0.29	0.62	***	**	-0.61	***	***
ECSCR	0.43	0.27	0.68	***	**	-0.58	***	***
EMCN	0.52	0.34	0.80	**	*	-0.59	***	***
ETS1	0.27	0.15	0.47	***	**	-0.50	***	***
ETS2	0.59	0.37	0.94	*		-0.54	***	***
FGFR1	0.46	0.25	0.83	**	*	-0.65	***	***
FOS	0.93	0.79	1.09			-0.57	***	***
FYN	0.37	0.23	0.61	***	**	-0.56	***	***
FZD7	0.38	0.22	0.68	***	**	-0.64	***	***
GPR124	0.54	0.38	0.78	***	**	-0.71	***	***

ID1	0.45	0.28	0.72	***	**	-0.58	***	***
IL1R1	0.75	0.49	1.16			-0.42	***	***
ITGA8	0.55	0.35	0.86	**	*	-0.58	***	***
ITGA9	0.45	0.26	0.79	**	*	-0.61	***	***
KIFC1	1.22	0.79	1.87			0.58	***	***
LIFR	0.55	0.33	0.92	*		-0.49	***	***
MAD2L1	2.69	1.31	5.50	**	*	0.56	***	***
MAML2	0.42	0.25	0.70	***	**	-0.63	***	***
MMRN2	0.43	0.25	0.74	***	**	-0.64	***	***
NPR1	0.47	0.31	0.72	***	**	-0.53	***	***
NRP1	0.44	0.27	0.72	***	**	-0.60	***	***
PDGFRA	0.47	0.29	0.74	***	**	-0.62	***	***
PLCB4	0.64	0.40	1.02			-0.55	***	***
ROBO4	0.42	0.26	0.69	***	**	-0.57	***	***
RORA	0.83	0.48	1.43			-0.54	***	***
RUNX1T1	0.68	0.47	1.00	*		-0.66	***	***
SMAD3	0.78	0.37	1.64			-0.32	*	*
STMN1	2.02	1.18	3.47	**	*	0.63	***	***
TCF4	0.56	0.33	0.95	*		-0.65	***	***
TEK	0.41	0.23	0.72	***	**	-0.56	***	***
TGFBR2	0.41	0.26	0.67	***	**	-0.63	***	***
THBS4	0.82	0.63	1.07			-0.43	***	***
TIE1	0.44	0.27	0.70	***	**	-0.63	***	***
TNS1	0.39	0.23	0.67	***	**	-0.68	***	***
TPSAB1	0.89	0.68	1.17			-0.47	***	***
UBE2T	1.41	0.85	2.33			0.60	***	***
ZEB1	0.48	0.32	0.73	***	**	-0.59	***	***
AQP1	0.37	0.23	0.58	***	**	-0.59	***	***
BMP2	0.78	0.56	1.10			-0.56	***	***
CAV1	0.52	0.33	0.81	**	*	-0.60	***	***
CCNA2	1.08	0.68	1.73			0.69	***	***
CDC6	1.29	0.78	2.12			0.60	***	***
E2F1	1.56	0.99	2.48			0.65	***	***
EGFR	0.68	0.46	0.99	*		-0.55	***	***
FGF2	0.58	0.39	0.86	**	*	-0.53	***	***
FLNC	0.57	0.40	0.82	**	*	-0.60	***	***
FOXC1	0.67	0.42	1.06			-0.59	***	***
FOXM1	1.61	1.05	2.45	*		0.66	***	***
FSTL1	0.54	0.36	0.82	**	*	-0.57	***	***
HIST1H3H	1.26	0.84	1.89			0.62	***	***
JAM2	0.49	0.30	0.78	**	*	-0.65	***	***
KIT	0.55	0.37	0.83	**	*	-0.68	***	***
LHFP	0.52	0.35	0.78	***	**	-0.68	***	***
MS4A2	0.97	0.65	1.44			-0.56	***	***
MYH11	0.64	0.45	0.90	**	*	-0.60	***	***
MYLK	0.53	0.36	0.76	***	**	-0.64	***	***
PTTG1	1.72	1.06	2.80	*		0.69	***	***
TWIST2	0.72	0.49	1.07			-0.51	***	***
TXNIP	0.89	0.56	1.41			-0.55	***	***
TYMS	1.41	0.91	2.17			0.62	***	***
AKAP12	0.49	0.33	0.72	***	**	-0.63	***	***
ALDH1A1	0.67	0.49	0.92	**	*	-0.66	***	***
ANLN	1.48	1.01	2.17	*		0.66	***	***
BIRC5	1.28	0.96	1.71			0.71	***	***

BTG2	0.94	0.66	1.33			-0.52	***	***
C1S	0.63	0.45	0.90	**	*	-0.64	***	***
C7	0.64	0.47	0.87	**	*	-0.58	***	***
CCDC80	0.61	0.47	0.80	***	**	-0.64	***	***
CCNB1	1.61	1.08	2.39	**		0.66	***	***
CDC2	1.41	1.02	1.94	*		0.74	***	***
CDC20	1.38	0.90	2.13			0.62	***	***
CENPF	1.48	0.97	2.26			0.69	***	***
CEP55	1.71	1.16	2.53	**	*	0.63	***	***
CTSG	0.77	0.57	1.03			-0.53	***	***
FHL1	0.64	0.47	0.87	**	*	-0.60	***	***
ID4	0.73	0.57	0.94	**	*	-0.63	***	***
IGF1	0.61	0.45	0.85	**	*	-0.55	***	***
KIF23	1.59	1.05	2.40	*		0.66	***	***
MAPT	1.29	0.94	1.77			0.58	***	***
MKI67	1.32	0.92	1.88			0.68	***	***
MYBL2	1.79	1.15	2.79	**	*	0.61	***	***
NGFR	0.53	0.37	0.75	***	**	-0.53	***	***
OGN	0.70	0.54	0.90	**	*	-0.62	***	***
PDGFD	0.46	0.31	0.66	***	**	-0.63	***	***
PKMYT1	1.42	1.05	1.93	*		0.68	***	***
RRM2	1.47	1.08	1.99	**	*	0.74	***	***
SFRP1	0.68	0.52	0.89	**	*	-0.56	***	***
SMAD9	0.58	0.39	0.88	**	*	-0.63	***	***
TNXB	0.62	0.45	0.87	**	*	-0.55	***	***
TOP2A	1.24	0.96	1.61			0.72	***	***
TRIP13	1.66	1.01	2.72	*		0.60	***	***
TSPAN7	0.44	0.27	0.70	***	**	-0.59	***	***
TWIST1	0.83	0.58	1.18			-0.56	***	***
UBE2C	1.36	1.00	1.84	*		0.74	***	***
CD274	1.44	0.86	2.40			-0.27		
LAG3	2.23	1.27	3.95	**	*	-0.22		
IDO1	1.06	0.60	1.88			-0.20		
CXCL9	1.08	0.80	1.47			0.09		
Durvalumab	2.24	1.28	3.92	**	*	-0.20		
Immune.tolerance	1.68	0.81	3.48			-0.22		

*** p<0.01, ** p<0.05, * p<0.1

4.1.2 Additional information on baseline gene expression in ER+/HER2- breast cancer and its predictive value (manuscript 2)

Baseline biomarkers of resistance to AI in ER+/HER2-BC

As part of manuscript 2 and beyond gene expression changes, we also explored baseline gene expression patterns amongst classes of Ki67-level changes between baseline and surgery following two weeks of AI treatment in ER+/HER2- early BC. We finally decided not to include this data in the manuscript as we wanted to perform the analysis in a larger subset of patients within the POETIC trial.

Tumours were then classified into four classes according to Ki67 changes between the two time points: $High_{baseline}$-$High_{surgery}$ (H-H), $High_{baseline}$-$Low_{surgery}$ (H-L), $Low_{baseline}$-$Low_{surgery}$ (L-L), and $Low_{baseline}$-$High_{surgery}$ (L-H). Tumours were categorised into 28 H-H and 77 H-L. Significance Analysis of Microarrays (SAM analysis) was performed to test the expression values against the appropriate response variable in an unpaired Two Class setting to compare (H-H/H-L settings) (116). The L-L category was not included due to the small number of patients. In each analysis, the identification of a specific *delta* parameter determined the False discovery rate (FDR) cut-off for significance. To further identify potentially interesting biological patterns associated with the outcomes of interest, hierarchical clustering of expression profiles at different time points based on the results of the SAM analysis was performed (117). Each analysis was performed separately for each arm in the POETIC subset and the NeoAI study.

RESULTS: Differential gene expression patterns amongst classes of Ki67-level changes between baseline and surgery (H-H and H-L)

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 tumour subtypes with the capacity of lowering the risk (all except luminal A and normal) were classified into changes (if they turned into a lower-risk subtype) or not changes (if they remained as the same subtype or turned into a higher risk one). There was a statistically significant association between "No-changes or changes to a higher risk subtype" with the H-H Ki67 response category in both subsets (POETIC treated cohort: 100% no-changes in H-H tumours, 48.5% no-changes in H-H, and 51.5% in H-L; p=0.001326; NeoAI study: 58.8% no-

changes in H-H, 41.2% in H-L, and 100% of changes in H-L; Fisher's exact test p=8.127E-07).

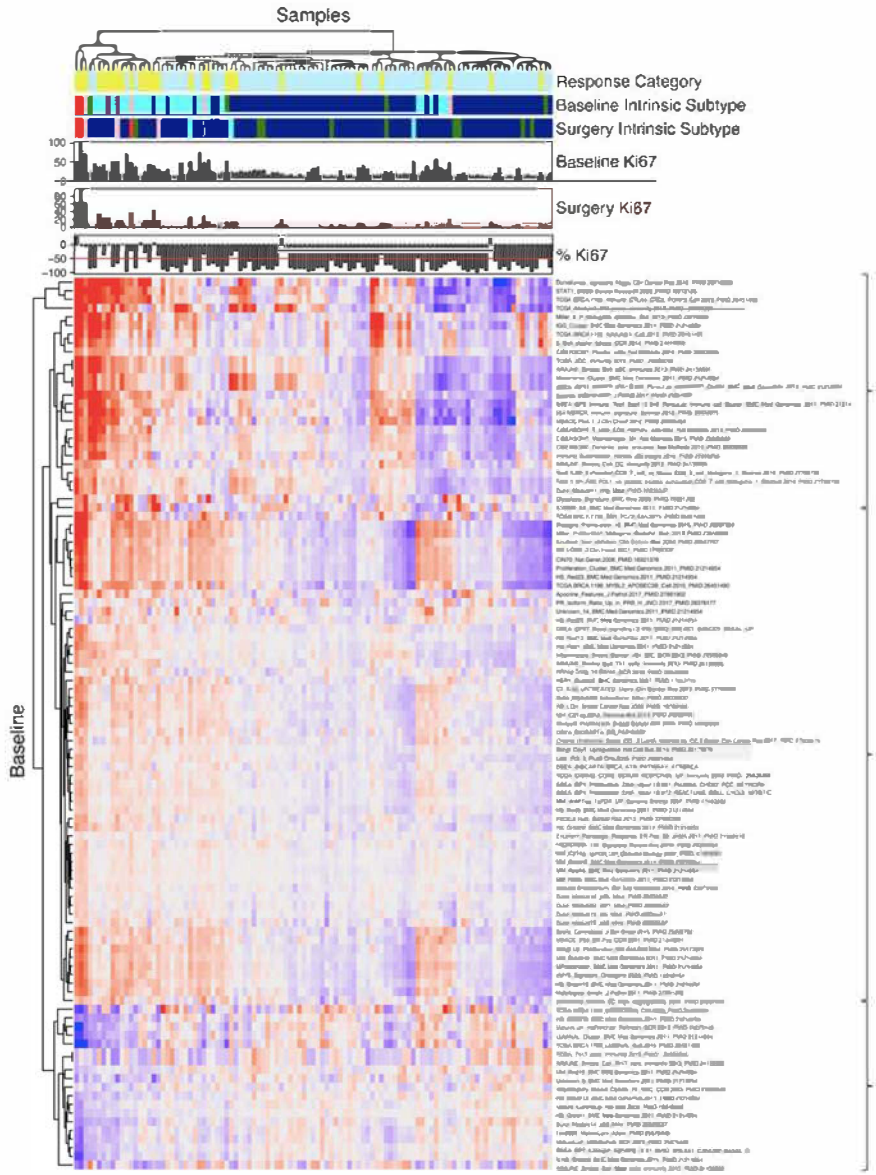
In order to identify other baseline molecular characteristics involved in response to neoadjuvant AI therapy, we performed SAM analysis based on the Ki67 change categories from baseline to surgery (H-H and H-L) in the POETIC trial. Only HER2 negative tumours were included in this analysis. As expected, baseline Ki67 was remarkably higher within luminal B intrinsic subtype samples with a trend to retain high Ki67 after two weeks of AI compared with luminal A tumours. Twenty-four immune-related gene modules covering immune-cell pathways, immune-checkpoint component, and IFNδ biology, were significantly more expressed in H-H tumours compared with H-L both at baseline **(Figure 3**) and surgery **(Figure 4)**. These modules include some genes that have been previously associated with luminal B-resistant tumours such as *IFNG, STAT1, IDO1, LAG 3,* and *CTLA4* (60). A general trend in terms of gene expression stability in H-H tumours was also observed when looking at gene expression in paired (baseline-surgery) tumour samples following short AI treatment, with just a general downregulation of proliferation-related modules (**Figure 4**).

To further investigate the role of immune-related features on resistance to AI, two immune-related modules were calculated: (1) The "Durvalumab signature" shown in the SAM analysis (median of *CD274*, *LAG3*, *CXCL9* - durvalumab signature) and (2) a new module score defined by M. Ellis' group, including significant genes associated with resistance to AI in luminal B tumours in the neoadjuvant setting (median of *CD274*, *LAG3*, *IDO1* - Immune-tolerance signature)(60). In the POETIC subset, the expression of the "Durvalumab signature" ($p_{baseline}$=0.003; $p_{surgery}$=0.014) and the "Immune-tolerance signature" ($p_{baseline}$=0.004; $p_{surgery}$=0.002) was significantly higher in H-H compared with H-L tumours at both baseline and surgery (**Figure 3B**). No significant changes in signature expression from baseline to surgery were seen in either H-H or H-L categories. As observed in short-term treated patients, in the NeoAI cohort, the expression of both immune signatures at baseline were significantly higher in H-H tumours compared with H-L (p=0.033 and 0.029, respectively; **Figure 3C**). However, this association was lost in surgical specimens of patients treated with long-term AI therapy. In addition, a significant increase in the expression of the "Durvalumab signature" (p=0.0098) and the "Immune-tolerance signature" (p=0.0011) at surgery was seen in H-L tumours but not in H-H tumours.

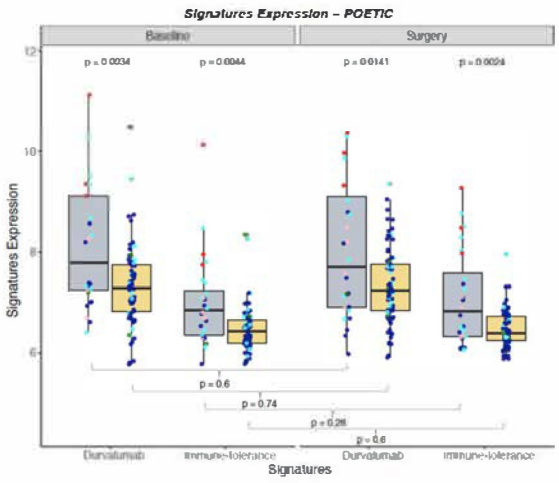
Finally, to explore the impact of AI duration on the expression of those ET-resistance immune-related modules, we looked at the correlation of their expression with AI exposure time in the NeoAI study. No correlation was found between changes in their expression from baseline to surgery (Log2FC) and the duration of AI (**Figure 5)**.

Figure 4. Differential gene expression patterns amongst classes of Ki67-level response changes between baseline and surgery (H-H and H-L) **A.** Unsupervised heatmap of module scores at baseline in the POETIC treated subset selected by two unpaired SAM analyses by Ki67 H-H vs. H-L categories and median centred. **B.** Differences of the two immune-related signature expressions ("Durvalumab" and "Immune-tolerance") between H-H and H-L Ki67 categories according to the two different time points (baseline and surgery) in the POETIC treated subset. **C.** Differences of the two immune-related signature expressions from baseline to surgery in the NeoAI study. **Abbreviations**: H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery,}$ H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery,}$ Her2-E: Her2 enriched, LumB: Luminal B, LumA: Luminal A, 2wk: two-week time point.

A.


Samples
Response Category
Baseline Intrinsic Subtype
Surgery Intrinsic Subtype
Baseline Ki67
Surgery Ki67
% Ki67
Baseline
Immune-related modules
Proliferation-related modules and others
ER-signaling pathways and others

B


Signatures Expression – POETIC
Baseline
Surgery
p = 0.0034
p = 0.0044
p = 0.0141
p = 0.0024
p = 0.6
p = 0.74
p = 0.28
p = 0.6
Signatures Expression
Durvalumab
Immune-tolerance
Signatures

C

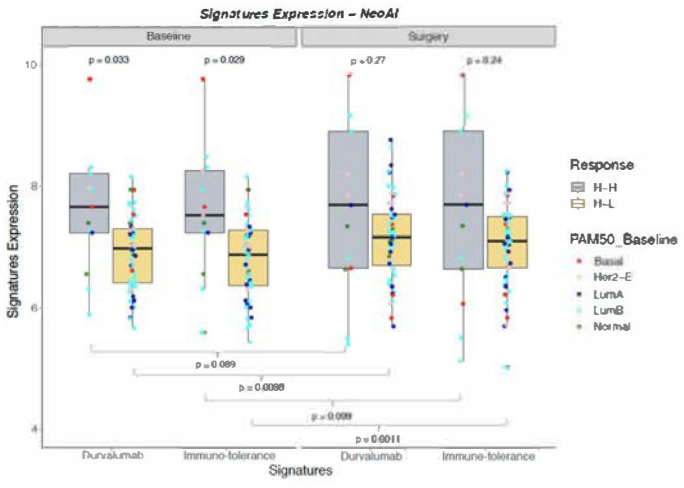

Signatures Expression – NeoAI
Baseline
Surgery
p = 0.033
p = 0.029
p = 0.27
p = 0.24
p = 0.089
p = 0.0098
p = 0.089
p = 0.0011
Signatures Expression
Durvalumab
Immuno-tolerance
Signatures
Response
H-H
H-L
PAM50_Baseline
Basal
Her2-E
LumA
LumB
Normal

Figure 5. Baseline and paired surgery heatmaps of module score expression selected by two unpaired SAM analyses by Ki67 H-H and L-L categories at baseline in the POETIC subset for ER+/HER2- BC patients and median centred. Both heatmaps follow the order of the baseline module expression. **Abbreviations**: H-H: Ki67 $High_{baseline}$- Ki67 $High_{surgery}$, H-L: Ki67 $High_{baseline}$- Ki67 $Low_{surgery}$, Her2-E: Her2 enriched, lumB: luminal B, lumA: luminal A, 2wk: two-week time point.

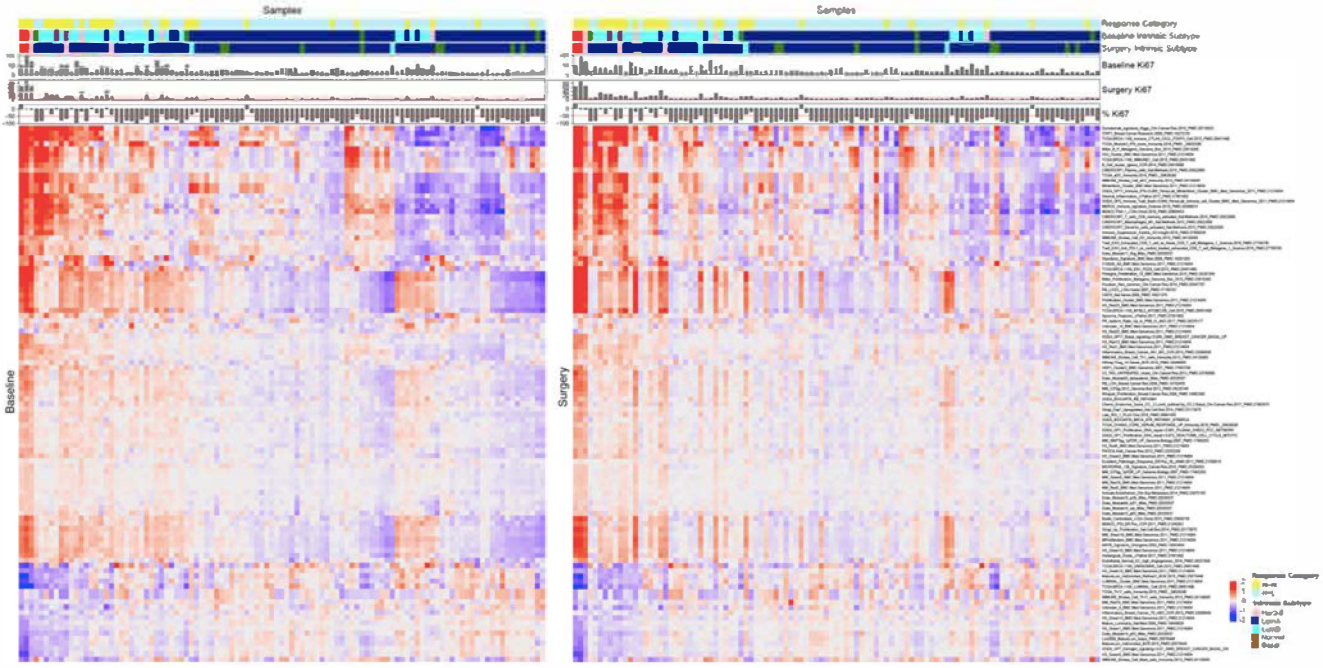
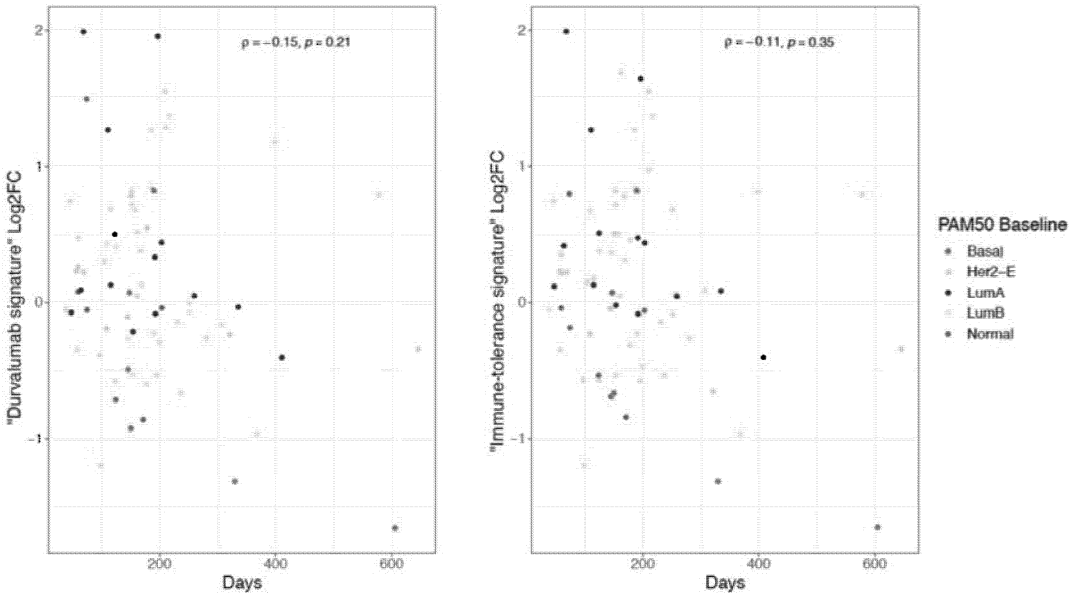


Figure 6. Spearman Rank-Order Correlation of the expression changes from baseline to surgery (Log2FC) under AI treatment of the two immune-related signatures “Durvalumab” and “Immune-tolerance” with time in the NeoAI study



4.2 Manuscript 3: HER2-E subtype and novel molecular subgroups drive aromatase inhibitor resistance and an increased risk of relapse in early ER+/HER2+ BC**.**

HER2-enriched subtype and novel molecular subgroups drive aromatase inhibitor resistance and an increased risk of relapse in early ER+/HER2+ breast cancer

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Summary

Background Oestrogen receptor positive/ human epidermal growth factor receptor positive (ER+/HER2+) breast cancers (BCs) are less responsive to endocrine therapy than ER+/HER2- tumours. Mechanisms underpinning the differential behaviour of ER+HER2+ tumours are poorly characterised. Our aim was to identify biomarkers of response to 2 weeks' presurgical AI treatment in ER+/HER2+ BCs.

Methods All available ER+/HER2+ BC baseline tumours (n=342) in the POETIC trial were gene expression profiled using BC360™ (NanoString) covering intrinsic subtypes and 46 key biological signatures. Early response to AI was assessed by changes in Ki67 expression and residual Ki67 at 2 weeks ($Ki67_{2wk}$). Time-To-Recurrence (TTR) was estimated using Kaplan-Meier methods and Cox models adjusted for standard clinicopathological variables. New molecular subgroups (MS) were identified using consensus clustering.

Findings HER2-enriched (HER2-E) subtype BCs (44.7% of the total) showed poorer Ki67 response and higher $Ki67_{2wk}$ (p<0.0001) than non-HER2-E BCs. High expression of *ERBB2* expression, homologous recombination deficiency (HRD) and *TP53* mutational score were associated with poor response and immune-related signatures with High $Ki67_{2wk}$. Five new MS that were associated with differential response to AI were identified. HER2-E had significantly poorer TTR compared to Luminal BCs (HR 2.55, 95% CI 1.14−5.69; p=0.0222). The new MS were independent predictors of TTR, adding significant value beyond intrinsic subtypes.

Interpretation Our results show HER2-E as a standardised biomarker associated with poor response to AI and worse outcome in ER+/HER2+. HRD, *TP53* mutational score and immune-tumour tolerance are predictive biomarkers for poor response to AI. Lastly, novel MS identify additional non-HER2-E tumours not responding to AI with an increased risk of relapse.

Funding Cancer Research UK (CRUK/07/015).

Keywords: Breast cancer; HER2+; Aromatase inhibitors; HER2-Enriched subtype

Introduction

Human epidermal growth factor receptor 2 positive (HER2+) breast cancer (BC) has been associated with an aggressive phenotype and poor patient outcome.[1] However, the introduction of HER2-targeted therapies dramatically changed the prognosis of these patients

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[1] These authors contributed equally to this work.

[2] Supplementary table: POETIC consortia.

Research in context

Evidence before this study

AI treatment is the standard of care and most effective therapy for post-menopausal women with early oestrogen receptor positive (ER+) breast cancer (BC). ER+ BC tumours that also over-express HER2 are very heterogenous with several treatment options but variable responses to the different available drugs. Prior studies have shown that ER+/HER2+ BC show limited antiproliferative response to endocrine therapy and thus, are at a higher risk of recurrence. This may be PgR dependent, as many of those tumours do express low PgR levels. In addition, they have lower response rates to anti-HER2 targeted therapy compared to ER-/HER2+ tumours. Most studies investigating mechanisms of resistance to endocrine therapy have been performed in ER+/HER2- disease and are not well understood in HER2+ BC. Although endocrine-related gene expression has been previously associated with good response to aromatase inhibitors and high levels of *ERBB2* with poor response, there is an overall lack of optimal biomarkers to pair with the optimal treatment for each patient within ER+/HER2+ BC. As such, identifying robust molecular features and defining novel subgroups based on tumour biology is essential to identify the most adequate treatment strategies for this particular BC subgroup.

Added value of this study

This study establishes HER2-enriched intrinsic subtype as one of the main components driving poor response to AI and higher risk of relapse in ER+/HER2+ BC. Our results indicate the importance of molecular subtyping of BC beyond the standard HR and HER2 assays. Beyond the intrinsic subtypes, *ERBB2*, DNA damage repair signaling, *p53* mutant surrogate signature and immune-tumour tolerance related signatures are also associated with resistance to treatment.

We also identified five new single gene based molecular subgroups that can distinguish HER2-E and Luminal tumours responding or not to AI treatment and at a higher risk of relapse. Molecular subgroups characterised with high expression of immune related features drive an intrinsic lower risk of relapse despite predicting poor response to AI, while higher levels of *ERBB2* and extracellular matrix related genes lead to worse outcome.

Implications of all the available evidence

Firstly, the worse response to treatment and poorer outcome of HER2-enriched BC tumours highlights the potential need of treatment intensification for this intrinsic subtype with additional anti-HER2 targeted therapy, with the limitation, but also "real world" treatment limitation, that not all the patients in the study received the current standard anti-HER2 therapy. The higher sensitivity to aromatase inhibitors and good prognosis associated with luminal tumours, in particular with Luminal A, provides a rational for de-escalation, which has been previously suggested for ER+HER2+ unselected population.

Secondly, the new molecular subgroups show that immune-related features provide ER+/HER2+ BC tumours with an intrinsic good prognosis despite their association with early poor response to AI treatment and might also deserve a de-escalating approach. Furthermore, the assessment of the novel molecular subgroups might be crucial for the identification of some HER2-E BCs patients at a lower risk of relapse and additional non-HER2-E BCs patients with an increased risk of relapse. The combined investigation of the intrinsic subtypes and these new molecular subgroups might be key for the selection of candidate patients for escalated and de-escalated approaches in the future.

and the natural history of the disease.[2,3] Despite the improvement, long-term follow-up data indicate that approximately 15–23% of patients in early stage, still develop recurrent disease.[4]

Fifteen percent of all BC overexpresses *HER2* and approximately 50% of these are also classified as hormone receptor positive (HR+), which confers substantial differences in biology and clinical outcome from HR+/HER2- disease. HR+/HER2+ BCs are molecularly heterogeneous and around 30% of them are HER2-Enriched (HER2-E). This subtype is characterised by a high *HER2/EGFR* pathway activation, increased proliferation and an immune-activated stroma with elevated tumour infiltrating lymphocytes. It has a lower expression of luminal-related genes, than the Luminal A and B subtypes, potentially benefiting greatly from anti-HER2 therapies but poorly from endocrine therapy (ET).[5–7]

Resistance to endocrine therapies has been mainly studied in HR+/HER2- BC and includes down-regulation of oestrogen receptor (ER) expression, altered expression of ER co-regulators, presence of ER mutations, ligand-independent activation of ER and co-activators by growth factor receptor kinases.[8,9] However, those mechanisms might differ between HER2+ and HER2- tumours, in part due to the differential distribution of intrinsic subtypes within each BC subgroup. HER2-targeted therapies might be felt to negate the importance of development of resistance to AI but while anti-HER2 therapy is generally given for no more than 1 year to primary BC patients, ET is given for at least 5 years. Thus, any residual HER2+ disease after the end of the HER2-targeted therapy remains at risk of an incomplete endocrine response.

The PeriOperative Endocrine-Therapy for Individualised Care (POETIC) trial[10] is the framework used to study endocrine resistance mechanisms in a large set of ER+/HER2+ BC patients. In the context of POETIC, we hypothesised that resistance mechanisms to ET are driven by baseline genomic features. Gene expression profiles at baseline were assessed and the key genomic characteristics were tested for association with response

to AI, as measured by residual levels and changes of Ki67 after two weeks of treatment, and with clinical outcome. We sought to develop predictive signatures of AI response and address the clinical challenge of identifying patients who are likely to benefit from each of the specific therapies.

Methods

Patients and samples

All available ER+/HER2+ BC tumours from the POETIC trial in which patients were assigned to 2 weeks of peri-surgical AI or no AI (control) were included in this study.[10] A consort diagram of the study is shown in Supplementary Figure S1. Ki67 staining of 2-week samples from the control group was restricted to a randomly selected subset due to the minimal expected change on Ki67 from baseline to surgery.[11] In summary, of 470 ER+/HER2+ patients included in POETIC, we obtained successful results for 342 patients.

RNA extraction

RNA was extracted from three adjacent macro-dissected 10µm formalin-fixed paraffin-embedded (FFPE) sections from the baseline block of the patients included in the study. The ROCHE High Pure miRNA isolation kit (Roche, Basel, Switzerland) was used following SOP M027 from The Cancer Genome Atlas (TCGA) Program developed by the Biospecimen Core Resource (BCR) at Nationwide Children's Hospital in Columbus, Ohio. Quantification was done using high sensitivity RNA Qubit assays (Thermo Fisher Scientific, Carlsbad, CA).

Gene expression profiling

Gene expression of 758 genes was assessed using the NanoString nCounter Platform (Nanostring Technologies, Seattle, WA) Breast Cancer 360™ codeset (BC360) covering intrinsic subtypes and 46 key biological signatures (Supplementary Table S1). 150ng of RNA was run and processed on a NanoString nCounter™ FLEX Analysis System according to manufacturer's instructions. NanoString raw data was normalised by NanoString according to the BC360 pipeline using 18 house-keeping genes.

Immunohistochemistry

ER status was measured locally and was centrally reviewed by immunohistochemistry (IHC). HER2 status was measured locally using IHC and/or fluorescence *in situ* hybridisation (FISH). Ki67 proliferation rate was obtained by IHC in FFPE tissues using the MIB-1 antibody (M7240, DAKO UK, RRID: AB_2631211).[10,12] Ki67 has been validated in our laboratory previously and we are part of the International Ki67 in Breast Cancer Working Group.

Outcomes

The primary endpoints of this study were based on Ki67 as a measure of tumour's resistance to AI. Two Ki67 endpoints were used: 1) Ki67 change was calculated as the difference between Ki67 expression at surgery and baseline (relative change) and was categorised into Ki67 response categories defined as percentage-changes from baseline to surgery: poor response (PR) (reduction <50%), intermediate response (IR) (50–75%) and good response (GR) (>75%). This reflects the antiproliferative response to AI treatment which relates to the treatment benefit. 2) Residual Ki67 at 2-week timepoint ($Ki67_{2wk}$) High (≥ 10%) and Low (<10%) which correlated to the residual risk after AI treatment. The secondary endpoint was time to recurrence (TTR) (local and metastatic recurrence) to evaluate the prognostic significance of the molecular characteristics analysed.

Statistical analysis

Statistical analysis was performed using the R software (version 3.6.3). P values were considered significant if lower than 0·05. Wilcoxon tests were applied in unpaired comparisons and Kruskall-Wallis tests in all multiple comparisons in both treated and control tumours. Spearman Rank correlation was used to explore the correlation between genes or signatures. Logistic and ordinal regression models were performed to identify signatures significantly associated with 2-week Ki67 and Ki67 response categories respectively. Multiple testing correction was undertaken by the Benjamini & Hochberg (FDR) method.[13] Significance analysis of microarrays (SAM analysis) was performed in multiclass setting to compare the Ki67 response categories and in the unpaired Two Class to compare extreme response classes (GR and PR) and $Ki67_{2wk}$ High versus Low.[14] Hierarchical clustering of the gene expression profiles that were identified by SAM analysis was also performed.[15]

Consensus clustering was used to identify new molecular subgroups and their association with Ki67 response categories and outcome was tested.[16] Controls and treated patients were included to obtain the subgroups, but only the treated patients with Ki67 available, were used to assess their predictive value for AI resistance.

TTR was measured as time from randomisation to local, regional, or distant tumour recurrence or death from breast cancer without previous notification of relapse. Second primary cancers and intercurrent deaths were censored. TTR was estimated using Kaplan-Meier methods and Cox models. Multivariable Cox-regression models were adjusted for standard post-surgery clinicopathological variables: grade, tumour size, nodal status and age. We included age as the main driver/surrogate for the adjuvant treatment choice as most patients ≥70 years old did not receive

chemotherapy or trastuzumab (67·2%, 82/122) compared to patients <70 years (14%, 31/221). The independent prognostic value of those gene-expression based variables with differential survival in the univariate analysis were assessed. Both controls and treated patients were included in the survival analysis. The assumptions evaluated were: 1. that the dependent variable was ordered; 2. that one or more of the independent variables were continuous, categorical or ordinal; 3. that no multi-collinearity i.e. independent variables were independent from each other and the proportional odds. And 4. that the tests were done for the proportional odds assumptions for the ordinal logistic regression analysis for single gene and signature with the Ki67 Change categories GR/IR/PR. All the assumptions were met without any assumptions' violations. Patients with missing data were excluded. Only one patient had a missing variable (which constituted only 0·29% of the entire cohort).

Ethics

The POETIC trial was approved by the London–South East Research Ethics Committee (reference 08/H1102/37) and adopted by the Declaration of Helsinki. Patients provided written informed consent to molecular analysis of their samples for research purposes.

Role of funding source

Funders did not have any role in study design, data collection, data analysis, interpretation or writing of report.

Results

Patient clinicopathological characteristics

In this study, 342 ER+/HER2+ patients with baseline gene expression were included: 237 AI-treated and 105 untreated controls (Supplementary Figure S1). The demographics were well balanced between both groups (Supplementary Table S2). In summary, 93·3% of the tumours were ductal, 48·2% were grade 2 and 38·3% grade 3. At surgery 54·7% had a tumour diameter between 2 and 5 cm and 47·4% had positive nodal status. 60·5 percent of patients received adjuvant chemotherapy and trastuzumab and 98·2% of patients were treated with adjuvant ET.

PAM50 subtypes and Ki67 endpoints

We evaluated whether intrinsic subtypes could predict response to ET. In the entire subset of patients, 44·7% of tumours classified as HER2-E, 36·3% Luminal B, 17·2% Luminal A and 1·8% Basal-Like. In addition, the proportion of intrinsic subtypes was comparable between control and treated groups with the controls slightly enriched with HER2-E (54% vs 41·4%) and reduced Luminal A tumours (8·0% vs 21·1%), not statistically significantly different (p>0·05), Within the treated subgroup, 31% achieved GR, 22·5% were IRs and 46·5% PRs, while 52·0% had $Ki67_{2wk}$ High and 48·0% Low, respectively.

As expected, most control tumours were classified as PRs (96·0%) and $Ki67_{2wk}$ High (96·0%) (Table 1). In the treated group, there was a significant change in Ki67 values in all subtypes (Figure 1A), except for Basal-like, possibly due to the small sample number. Overall, the HER2-E subtype was associated with poorer response to AI compared to non-HER2-E, evaluated as Ki67 response category and residual Ki67. HER2-E tumours were associated with higher grade (68·6% grade 3 in HER2-E vs 30·9% in luminals, $p<0·0001$, Fisher test) and larger tumours (50·3% >3 cm in HER2-E vs 39·1% in luminals, $p<0·0001$, Fisher test). However, the association of the HER2-E subtype and Ki67 response remained significant in each of the categories

	TREATED					CONTROLS				
Arm	**All 226 (100.0%)**	**Basal 3 (1.3%)**	**HER2-E 95 (42.1%)**	**LumA 45 (19.9%)**	**LumB 83 (36.7%)**	**All 50 (100.0%)**	**Basal 0 (0.0%)**	**HER2-E 27 (54.0%)**	**LumA 4 (8.0%)**	**LumB 19 (38.0%)**
				Ki67 Response Categories						
GR	70 (31·0%)	0 (0·0%)	15 (15·8%)	18 (40·0%)	37 (44·6%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)	0 (0·0%)
IR	51 (22·5%)	0 (0·0%)	17 (16·5%)	11 (24·4%)	23 (27·7%)	2 (4·0%)	0 (0·0%)	1 (3·80%)	0 (0·0%)	1 (5·3%)
PR	105 (46·5%)	3 (100%)	63 (66·3%)	16 (35·5%)	23 (27·8%)	48 (96·0%)	0 (0·0%)	26 (96·3%)	4 (100%)	18 (94·7%)
		Chi -squared 27·69, $P<0·00001$					Fisher's exact test $P>0·05$			
				$Ki67_{2wks}$						
HIGH	118 (52·0%)	3 (100·0%)	80 (84·2%)	7 (15·6%)	28 (33·7%)	48 (96·0%)	0 (0·0%)	27 (100%)	3 (75·0%)	18 (94·7%)
LOW	109 (48·0%)	0 (0·0%)	15 (15·8%)	38 (84·4%)	55 (66·3%)	2 (4·0%)	0 (0·0%)	0 (0·0%)	1 (25·0%)	1 (5·30%)
		Chi-squared 67·98, $P<0·00001$					Fisher's exact test $P>0·05$			

***Table 1*: Distribution of the 4 intrinsic subtypes within patients with Ki67 data and by Ki67 categories**
Abbreviations: HER2-E, HER2-Enriched Subtype; LumA, Luminal A subtype; LumB, Luminal B subtype; GR, Good response; IR, Intermediate response, PR, Poor response; $Ki67_{2wk}$, Ki67 at 2 weeks timepoint.

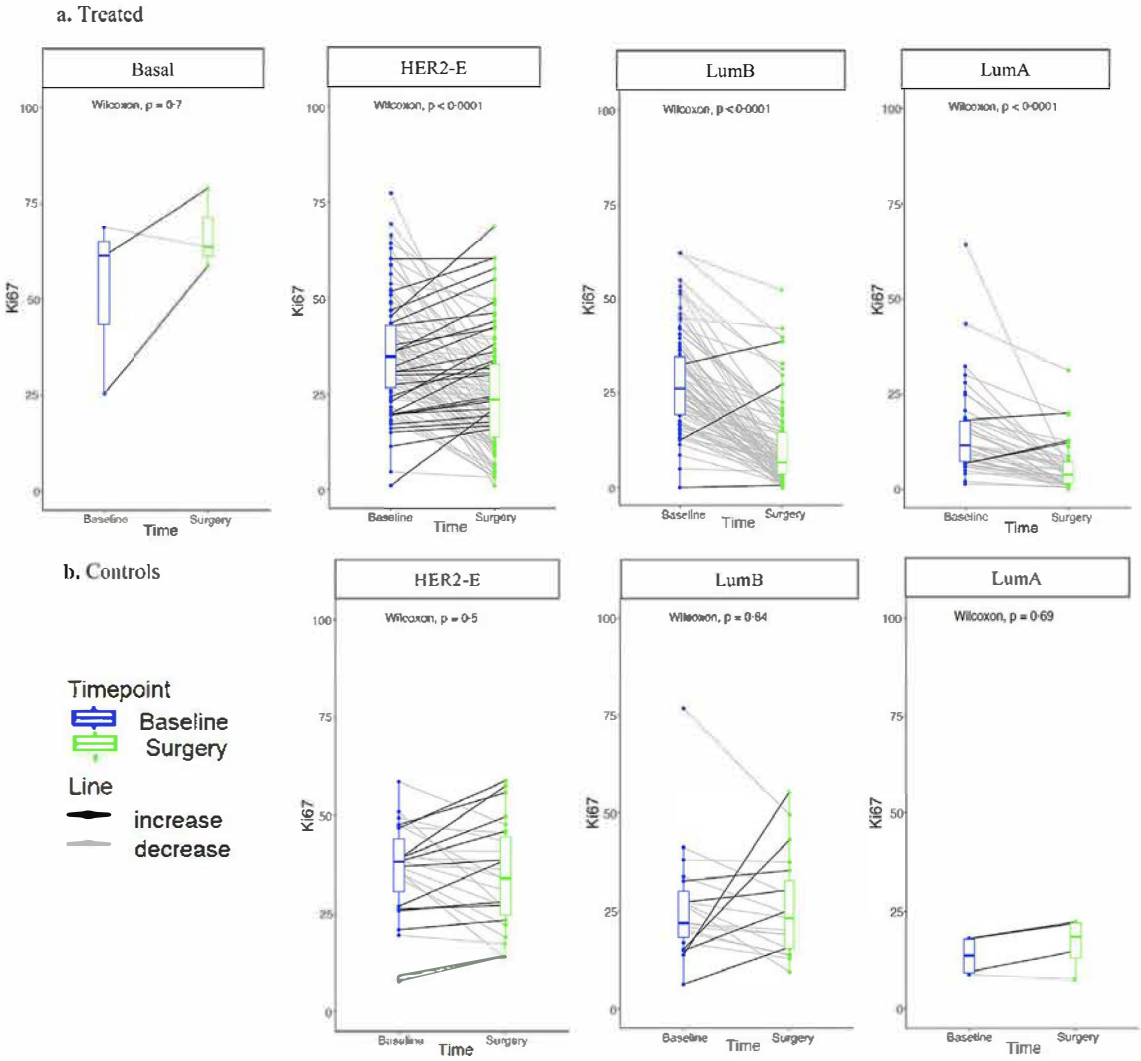


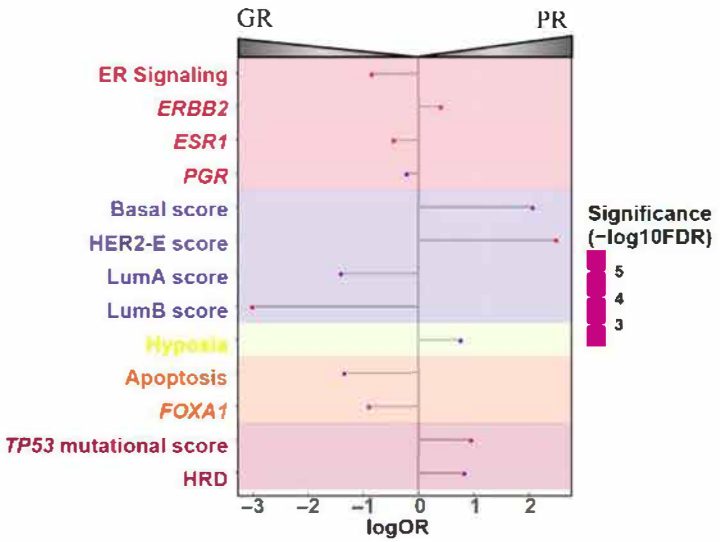
Figure 1. Ki67 changes from baseline to surgery stratified by intrinsic subtype at baseline within a. Treated and b. Controls. Abbreviations: HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype.

within those variables. In the control patients with Ki67 data, no significant changes of Ki67 were observed amongst subtypes (Figure 1B). These findings suggest that HER2-E might be one of the main components driving poor early response to AI in ER+/HER2+ BC tumours.

Signature expression and Ki67 endpoints

We then evaluated the association of other biological molecular features (46 signatures) with the defined Ki67 endpoints (Supplementary Table S3). High expression of endocrine related signatures such as *ESR1*, ER-Signaling, *FOXA1* and *PgR* as well as Luminal A and B correlation coefficient scores were associated with GR and Low $Ki67_{2wk}$ (OR 0·05–0·82; FDR<0·0001–0·0015, ordinal logistic regression model), while high *ERBB2*, Basal-like and HER2-E correlation coefficient scores were associated with PR and High $Ki67_{2wk}$ (OR 1·52–12·31; FDR<0·0001, ordinal logistic regression) (Figure 2). Noteworthy, the high expression of apoptosis signature (pro-apoptotic) was associated with GR (OR 0·26; 95% CI 0·12–0·54; FDR=0·0017, ordinal logistic regression) whilst high DNA-damage repair signatures such as the homologous recombination deficiency (HRD), hypoxia, and the *TP53* mutational status' surrogate signature were associated with PR (OR 2·18–2·65, FDR<0·0001–0·0059, ordinal logistic regression). Additional high expression of signatures involved in immune-checkpoint component and tumour immunity such as *IDO1*, IFN Gamma, *PD-L1* and Tumour Inflammation Signature (TIS) as well as the genomic risk score were associated with High

a. Differential signature expression amongst Ki67 response categories



b. Differential signature expression amongst Ki672wks categories

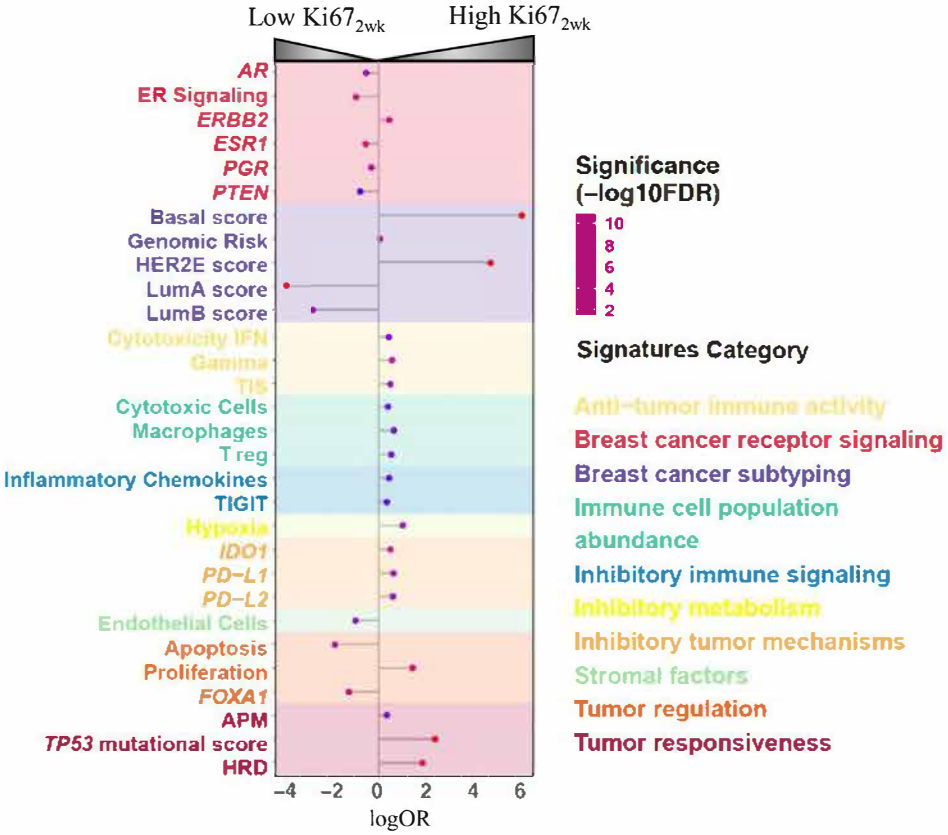


Figure 2. Signatures significantly associated with differential response to AI. a. Ordinal regression models in treated patients with Ki67 data (n=227) coloured by FDR for Ki67 response categories and b. Logistic regression models for Ki67$_{2wk}$. Abbreviations: AI, Aromatase inhibitors; logOR, log Odds Ratio; FDR, False discovery rate; HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype.

$Ki67_{2wk}$ (OR 1·67–1·49, FDR<0·0001–0·0084, logistic regression). Following prior evidence, we also assessed the correlation of the different signatures with Ki67 > or < than 20% to validate our findings.[17] The same signatures were associated with differential response to AI based on this Ki67 threshold (Supplementary Table S4).

To assess the above associations according to the different subtypes, we tested them in the HER2-E and Luminal subtypes separately. The signatures were not significantly differently expressed between response categories amongst HER2-E tumours for any of the two Ki67 endpoints (Supplementary Figure S2A). However, in the Luminal tumours, proliferation, *TP53* mutational score, genomic risk and the Basal-like and HER2-E coefficient scores were associated with High $Ki67_{2wk}$ (OR 1·15–16·84; FDR=0·00056–0·013, logistic regression), while high expression of *AR* signature and the Luminal A coefficient scores were associated with Low $Ki67_{2wk}$ (OR 0·25–0·77; FDR=00019–0·013, logistic regression) (Supplementary Figure S2B). Thus, beyond HER2-E intrinsic subtype: *ERBB2*, *TP53* mutational status, HRD and some immune-related signatures were also associated with early resistance to AI.

Single gene expression and Ki67 endpoints

Multiclass SAM analysis of single gene expression for the three Ki67 response categories (GR/IR/PR) (Δ=0·26, FDR<0·05) identified 8 genes with significantly different expression amongst groups (p<0·05). High expression of *GRB7* and *ERBB2* were associated with PR while high expression of others such as *IGF1R*, *ESR1*, *CHAD* and *BCL2*, were associated with GR (Figure 3A). Two unpaired class SAM analysis for GR versus PR (Δ=1, FDR<0·05) showed the association of 31 genes with GR (p <0·05, Wilcoxon test) (Figure 3B)· Two class unpaired SAM analysis with $Ki67_{2wk}$ categories: High versus Low (Δ=1·63, median FDR=0) identified 128 genes associated to Low $Ki67_{2wk}$ including genes involved in *PI3K/AKT*, *MAPK* and oestrogen signaling and 83 genes associated to High $Ki67_{2wk}$, including genes involved in immune-checkpoint component, proliferation and cell-cycle regulation (Figure 3C).

To further investigate the biological differences between early AI responders and resistant tumours we evaluated the *ESR1* gene expression levels amongst the four intrinsic subtypes. The highest levels of *ESR1* were seen in Luminal B tumours. Noteworthy, within the Luminal A tumours a group of patients showed lower levels of *ESR1* were associated with higher levels of *ERBB2* signaling, especially when compared with other subtypes (Supplementary Figure S3).

Overall, at a single gene expression level, ER signalling-related genes drive responses to AI treatment while *HER2* and immune-related genes associates with early resistance.

Identification of new molecular subgroups predicting Ki67 endpoints

Using consensus clustering, we identified 5 novel molecular subgroups of samples based on single-gene expression that are associated with different Ki67 response and $Ki67_{2wk}$ (Figure 4 and Supplementary Table S5). Interestingly, these new molecular subgroups divided mainly HER2-E samples with lower response to AI, into three groups with differential expression of molecular features such as *ERBB2*, ER signaling or immune-related pathways.

Figure 4 shows the molecular features of the clusters (C): C1, C2 and C3 were characterised by PR to AI (60/111, 54·1%) and high $Ki67_{2wk}$ (82/111, 73·9%) and an enrichment of HER2-E subtype (79/111; 71·2%) and Luminal B tumours (21/111, 18·9%) (Supplementary Figure S4A). C1 (29·2% of the total) showed higher levels of immune and chemokine-related genes and lower levels of *ESR1* (Supplementary Figure S4B). C2 (14·2% of the total) had higher levels of extracellular matrix organization (ECM) related genes, lower levels of *ESR1* and the highest levels of *ERBB2* (Supplementary Figure S4C), also observed when distributed by subtypes. C3 (5.7% of the total) had low expression of most genes but a slight upregulation of genes covering DNA-damage repair deficiency. C4 (22.1%) and C5 (28·8%) were characterised by GR to AI and an enrichment of luminal tumours (Luminal B 81/165; 49·1% and Luminal A 57/165; 34·5%) (Supplementary Figure S4A and Supplementary Table S5). Both luminal subgroups overexpressed ER signaling-related genes. However, while C4 had the highest levels of *ESR1* (Figure 5C), C5 was enriched with genes involved in *MAPK/PI3K* and *RAS* signaling. Figure 4 also shows the signatures' expression within each of the single gene-based molecular subgroups, thus confirming the biologic distinctiveness of each of the new molecular subgroups.

Time to recurrence analysis

Median follow-up was 62·9 months (IQR 58·1–74·1). Univariate analysis was performed to evaluate the prognostic value of each individual genomic feature analysed. There was no overall significant difference of TTR between intrinsic subtypes when all the subgroups were compared together (Figure 5A). However, HER2-E had significantly poorer TTR (HR 2·14; 95% CI 1·11–4·17; p=0·022, univariate regression model) compared to luminal tumours (Figure 5B). C2, which is characterised by low expression of immune-related genes and the highest *ERBB2* expression, showed a significantly higher risk of recurrence compared to the rest of the molecular subgroups (HR 3·05; 95% CI 1·31–7·15; p=0·01, univariate regression model) (Figure 5C).

We performed a series of cox regression models for multivariable survival analysis including standard clinicopathologic factors along with different molecular subgroups for TTR and compared the changes of chi-

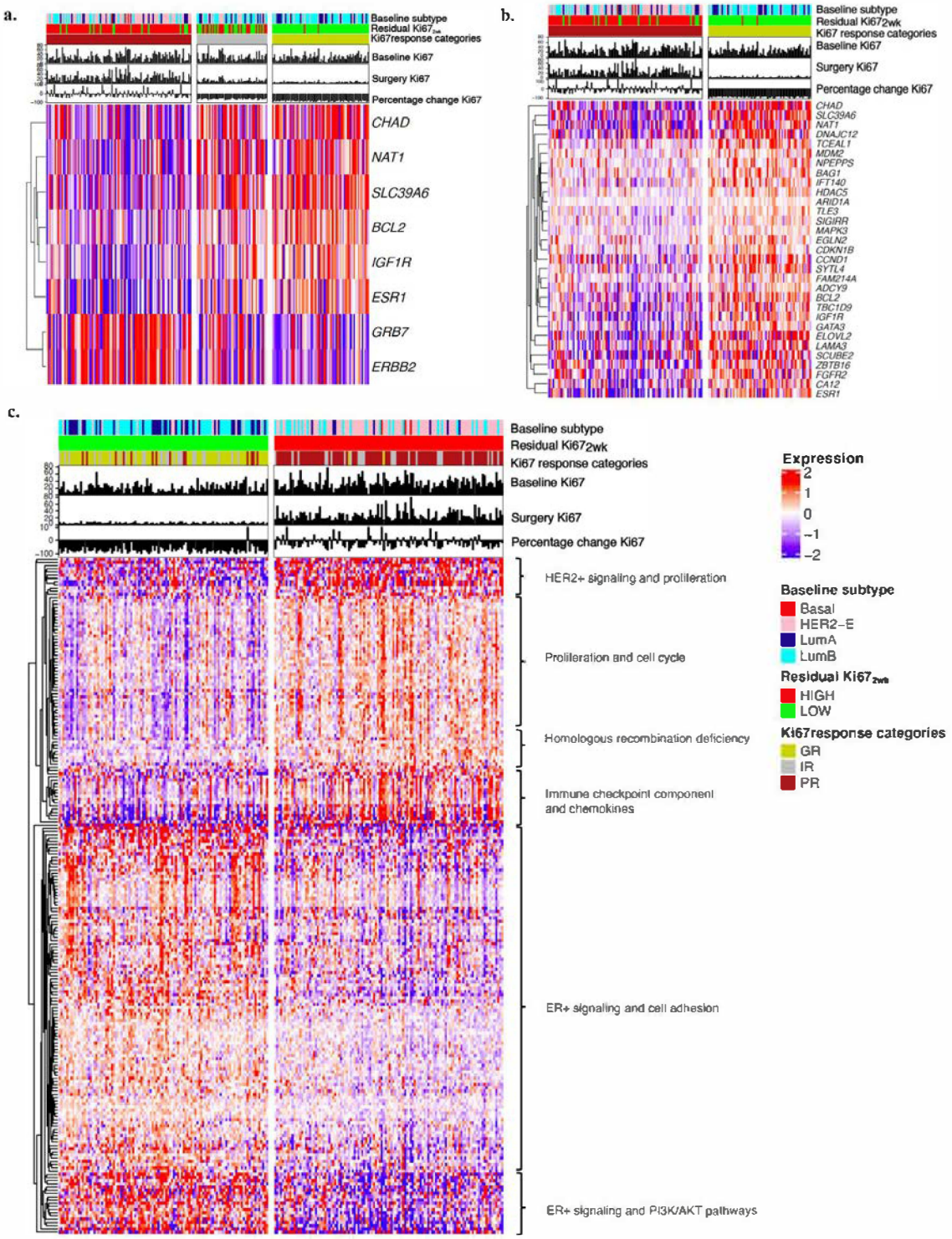


Figure 3. Hierarchical clustering using significant genes from SAM analysis of single gene expression for the different Ki67 endpoints in treated patients. a. Supervised hierarchical clustering of the multiclass SAM analysis for the three Ki67 response categories (GR/IR/PR) (*n*=226). b. Supervised hierarchical clustering of the two unpaired class SAM analysis (*n*=226). c. Supervised hierarchical clustering of the genes selected by two class unpaired SAM analysis for $Ki67_{2wks}$ categories: High vs Low (*n*=227): 211 significant genes, 128 associate with low and 83 with high residual Ki67. Abbreviations: HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype; $Ki67_{2wk}$, Ki67 at 2 weeks timepoint.

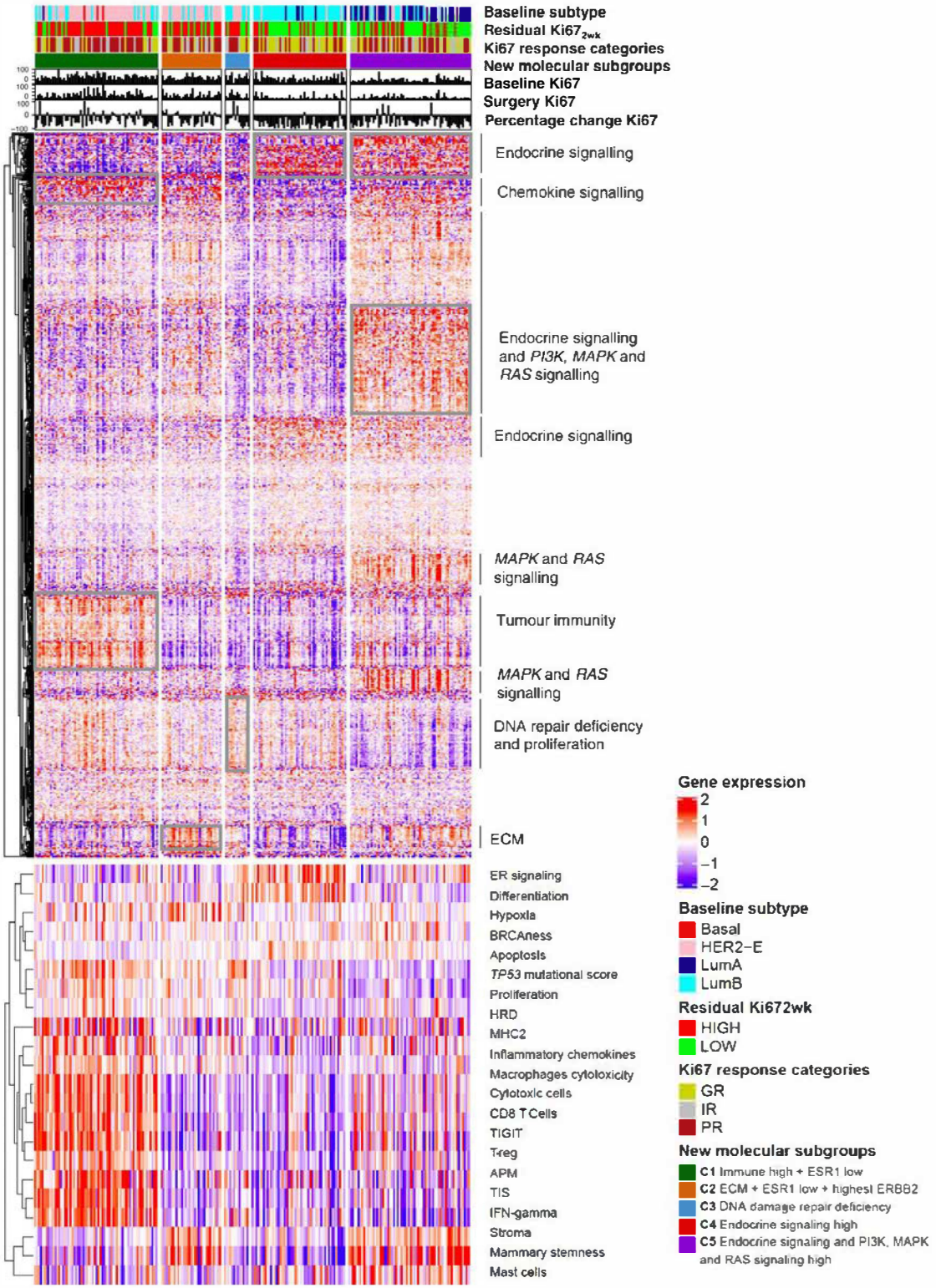


Figure 4. Five new molecular subgroups were identified based on consensus clustering of tumours using expression of 758 genes. Top panel illustrates single gene expression of all genes in treated patients with Ki67 data only (*n*=226), separated by the 5 novel molecular subgroups. Clusters of genes are annotated according to their functionality. Bottom panel illustrates the expression levels

squared values between them to assess the added value of the different models (Supplementary Table S6). HER2-E subtype remained as an independent predictor of higher risk of relapse (HR 2.55, 95% CI 1·14–5·69; p=0·022, multivariable regression model). C2 was also an independent predictor of shorter TTR compared to C1, indicating that this model adds significant value beyond intrinsic subtypes (Likelihood ratio test, p= 0.0025). (Supplementary Figure S5).

To explore further the role of adjuvant HER2-targeted treatment and chemotherapy on survival, we replicated the same analysis but separately for those patients that had received both of these treatments and those that did not (Supplementary Figure S6). Noteworthy, among patients who had received adjuvant trastuzumab plus chemotherapy, there were no significant differences in TTR observed between HER2-E and other subtypes (Supplementary Figure S6a). However, among patients who had not received trastuzumab and chemotherapy, HER2-E was still significantly associated with worse outcome compared to non-HER2-E (Supplementary Figure S6a). For the new molecular subgroups, the survival outcome within the new molecular subgroup 2 was worse compared to the others in those patients receiving adjuvant anti-HER2 treatment. In addition, in the multivariable models, the new molecular subgroups remained as independent predictors of TTR restricting to patients who received trastuzumab plus chemotherapy (HR 3.95; 95% CI 1·04–14·94; p=0·0043, multivariate regression model).

Finally, a series of multivariable analyses for TTR of signature expression adjusted for the clinicopathological factors showed that several immune related signatures (HR 0.50–0·78, nominal p <0·0001–0·0430, multivariable regression model) as well as the apoptosis signature (HR 0·36, 95% CI 0·15–0·38; p=0·024, multivariable regression model) were independent predictors of better TTR. By contrast, claudin-low signature showed independent worse prognostic value (HR 1.26, 95% CI 1·06–1·56; p=0·031, multivariable regression model) (Supplementary Table S7). A final model including all identified features was not performed due to the high collinearity amongst the main significant signatures (Supplementary Figure S7).

Discussion

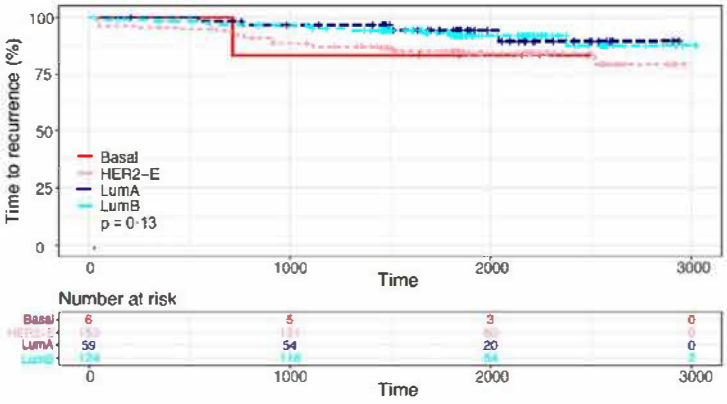
AI treatment is the standard of care and most effective therapy for post-menopausal women with early ER+ BC. However, ER+ tumours that also over-express HER2, show limited response to ET and thus, are at a higher risk of recurrence.[12] Most studies performed in ER+/HER2+ BC have focused on resistance to anti-HER2 targeted therapy while mechanisms of resistance to ET are not well understood within this subgroup. This study investigated the predictive and prognostic value of molecular features at baseline in ER+/HER2+ BC treated with perioperative AI.

Prior studies have revealed that HER2-E subtype is a predictor of higher sensitivity to anti-HER2 targeted therapy but worse outcome than other subtypes such as luminal tumours.[5,17–19] Nevertheless, the role of the intrinsic subtypes in response to ET has not been well established yet. A prior single-arm, multicentric study (PerELISA) included 65 postmenopausal patients with HR+/HER2+ operable patients receiving 2 weeks of letrozole and then undergoing to re-biopsy for Ki67 evaluation.[17] This study reported the association of PAM50 intrinsic subtyping with molecular responders (Ki67 relative reduction >20% from baseline). 92% of responders were luminal A and B versus 44% HER2-E and basal-like (P < 0·001). These results were aligned with ours; however, this is a small, single arm, non-randomised study, while POETIC was a randomised trial, enabling us to evaluate the predictive value of the intrinsic subtyping). Within PerELISA trial patients classified as molecular responders continued letrozole and started trastuzumab-pertuzumab for five cycles. They also reported that the pCR rate was significantly higher in HER2-E than in other subtypes (45·5% versus 13·8%, P=0·042). There was no association test of survival outcome reported, nevertheless, these results are of clinical significance as this provide a potential strategy for de-escaling the treatment management

Our results identified HER2-E subtype as a predictive biomarker of resistance to AI in ER+/HER2+ BC with an additional higher risk of relapse.[20,21] These findings highlight two aspects. First, they suggest the potential need of treatment intensification for HER2-E ER+/HER2 + BC tumours with intensive anti-HER2 targeted therapy. Second, the higher sensitivity to AI and good prognosis associated with luminal tumours, in particular with Luminal A, provides a rationale for testing de-escalation approaches, such as the reduction of anti-HER2 blockade duration or avoiding chemotherapy, previously suggested.[22] A related finding was published in 2021 as part of the ADAPT HER2+ trial in which the pooled TDM1 arm of patients with HER2-E subtype had a higher pCR, which was not observed in the trastuzumab arm.[23]

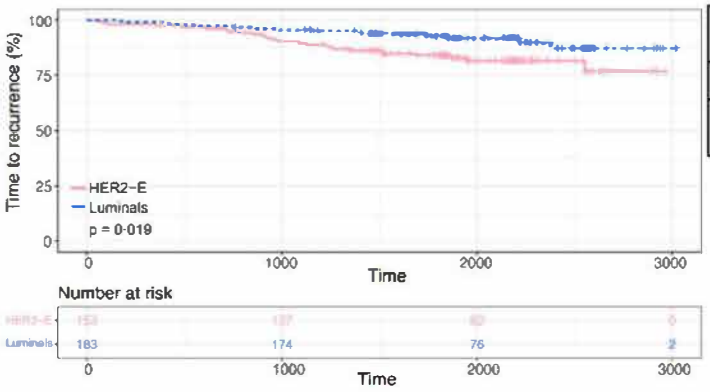
of the 21 multigene signatures, ordered according to the new 5 molecular subgroups. Abbreviations: HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype; GR, Good responders; IR, Intermediate responders, PR, Poor Responders; $Ki67_{2wk}$, Ki67 at 2 weeks timepoint, ER, Oestrogen receptor; HRD, Homologous recombination deficiency ECM, extracellular matrix; TIS, Tumour Inflammation Signature; TIGIT, T cell immunoreceptor with Ig and ITIM domain; MHC, Major Histocompatibility complex APM, Antigen Processing Machinery, IFN-gamma, interferon gamma.

a. Kaplan Meier curves according to all intrinsic subtype at baseline.



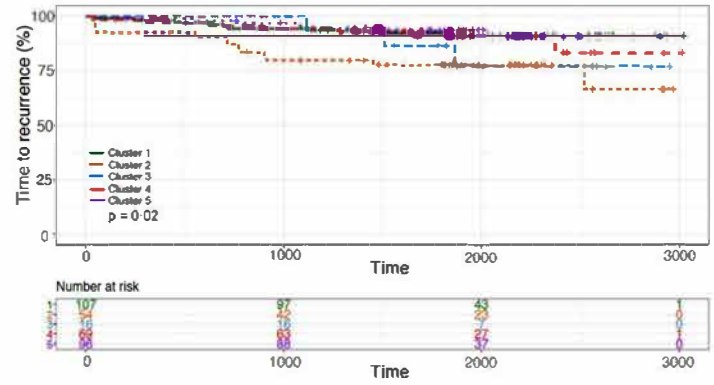
	HR	Lower CI 95%	Upper CI 95%	p-value
Basal-like	2·53	0·28	22·61	0·41
HER2-E	2·40	0·83	6·92	0·11
LumB	1·17	0·37	3·73	0·79
Reference LumA				

b. Kaplan Meier curves according to intrinsic subtype at baseline: HER2-E vs Luminals (A and B), excluding Basal-like tumours



	HR	Lower CI 95%	Upper CI 95%	p-value
HER2-E	2·14	1·11	4.17	**0·022**
Reference Luminals				

c. Kaplan Meier curves according to the 5 new molecular subgroups



	HR	Lower CI 95%	Upper CI 95%	p-value
C2	3·05	1·31	7·15	**0·01**
C3	1·97	0·53	7·30	0·31
C4	1·21	0·45	3·24	0·71
C5	0·88	0·33	2·35	0·80
Reference C1				

Figure 5. Kaplan Meier curves for TTR according to a. all PAM50 intrinsic subtype at baseline; b. HER2-E vs luminals and c. the new molecular subgroups. Abbreviations: TTR: Time to recurrence; HER2-E, HER2-enriched; LumB; Luminal B.

Nowadays, the biggest question is how to select patients for escalation and de-escalation strategies. Recent "chemotherapy-free" neoadjuvant studies have shown that the combination of HER2-E subtype with high *ERBB2* mRNA expression may identify patients with HER2+ early BC with higher sensitivity to double HER2-blockade.[18] However, only one third of ER+/HER + BC patients are HER2-E/*ERBB2* high and their role as prognostic biomarkers is still unknown. Using consensus clustering, we identified new molecular subgroups based on single gene expression at a higher risk of relapse beyond HER2-E subtype. Results from the stratified analysis by adjuvant treatment (chemotherapy plus anti-HER2 treatment yes vs no) showed similar TTR despite intrinsic subtype in those patients receiving trastuzumab plus chemotherapy. This highlights the hypothesised notion that patients with HER2-E subtype benefit the most from anti-HER2 treatment. The independent prognostic value for the new molecular subgroups for TTR in patients who received trastuzumab plus chemotherapy suggests the potential clinical utility of these new molecular subgroups to identify a set of ER+/HER2+ patients, beyond intrinsic subtypes, who may benefit from emerging treatment such as CDK4/6 inhibitors.

Noteworthy, tumours characterised by an enrichment of immune features showed the lowest risk of recurrence despite immune features being predictive markers of resistance to AI and poor outcome in ER +/HER2- BC.[24,25] Due to its intrinsic good prognosis, BC patients within this molecular subgroup showing an enrichment of immune characteristics could potentially benefit the most from a de-escalating approach, using for example 6 month of trastuzumab plus AI without chemotherapy followed by 5 years of AI treatment alone. Several trials in HER2+ BC have shown that tumours with higher baseline tumour infiltrating lymphocytes (TILs) and other immune features achieve higher pathological complete response rates and improved event-free survival.[26] Our study confirms the association of higher expression of immune-features with a very low risk of recurrence in ER+/HER2+ BC. By contrast, tumours with higher levels of *ERBB2* and lower associated immunity had a significantly higher risk of relapse, indicating that these patients may benefit from an intensified anti-HER2 treatment, using for example double anti-HER2 blockade or adjuvant TDM1.[27,28] Therefore, these new molecular subgroups might be essential to identify candidates for new escalating and de-escalating strategies.

The role of tumour immunity in response to ET has been mainly studied in ER+/HER2- BC disease, with higher expression of genes involved in immune enrichment and targetable immune checkpoint components being correlated with higher risk such as Luminal B tumours.[24,25] Our study shows that higher tumour immunity might also be a key driver of early resistance to ET in ER+/HER2+ BC. However, in this subgroup the association of higher expression of immune-features and lower risk of recurrence indicates the different role of tumour immunity between HER2+ and HER2- disease and may suggest a de-escalation approach.

Furthermore, our study shows other striking molecular associations such as high expression of HRD and *TP53* signature predicting poor response to AI, and higher apoptosis signaling being associated with good response and survival. The association of DNA damage repair defects with resistance to ET has been previously reported.[29] In addition, the inhibition of poly (ADP-ribose) polymerase-1 (PARP), has shown anti-tumour effects with a strong synergism and good tolerance in combination with anti-HER2 targeted therapy or ET *in vitro* and in phase I/II trials independently of DNA repair deficiency.[30] *TP53* mutational status has also been previously linked with poor survival[31] and overexpression of *HER2*,[32] although its role in response to AI is still unclear. Previous data has shown that some molecular features related to apoptosis can be predictive of adjuvant benefit from ET.[33] Based on our results, the association of high expression of some of the signatures reported above such as immune-related signatures and *TP53* mutational score with poor AI response could be explained by the high correlation with the HER2-E subtype.

Our study has two main limitations. Firstly, we only analysed gene expression for 758 genes and intrinsic subtypes profiles using the BC360 platform and other important pathways may have been missed. Secondly, clinical practice is currently different to that implemented in the recruited POETIC patients as high-risk tumours would be receiving additional pertuzumab and further anti-HER2 agents such as TDM1. It is noteworthy that one third of the patients in our cohort did not receive any adjuvant treatment apart from ET due to their advanced age, but we adjusted the survival analysis for age as a main surrogate of treatment choice. The major strengths of our study are that to our knowledge this is the largest cohort investigating response to presurgical AI in ER+/HER2+ subgroup in a real-world cohort which has a unique value to assess global gene expression data at baseline as defined in the clinical practice. Lastly, we were able to analyse both the molecular features associated to mechanisms of resistance and their prognostic value.

In conclusion, HER2-E subtype and *ERBB2* play a crucial role in ER+/HER2+ BC, driving resistance to ET and a higher risk of recurrence. Beyond them, new molecular subgroups enable the identification of patients at a higher risk of relapse. Altogether, the combination of these biomarkers would lead to a better tailoring of treatment strategies, including escalation and de-escalation approaches, to improve resistance to treatment in early BC. The appropriate new strategies need to be addressed in prospective clinical trials.

Contributors

MB and ELK designed, conducted part of the research work, analysed and verified the data and wrote the original draft of the manuscript. GM conducted part of the research work. HT did data analysis and interpretation. LK participated in study design, data collection and data interpretation. ES supervised some of the analysis of the data. AA and MH conducted part of the research work. HX participated in data analysis for manuscript revision. CH recruited patients to the POETIC trial and provided tissue. AS participated in POETIC study design, POETIC trial management group and clinical recruitment. JFR participated in POETIC trial design and reviewing the manuscript. IS participated in the design of original POETIC trial and was chief investigator of it. JMB has provided oversight and guidance for trial management, statistics and data interpretation throughout the trial including insights into the translational research project presented here. She reviewed and contributed to the manuscript. MD was involved in the investigation and visualisation of the study. MCUC designed and led the study. She also led the conceptualisation, methodology, resources and funding acquisition, data curation and statistical analysis plan, supervision. All authors made substantial contributions to the manuscript, revised the manuscript critically, gave their final approval and had full access to all the data and accept responsibility to submit for publication.

Data sharing statement

De-identified data will be made available to other researchers on request, subject to approval of a formal data access request in accordance with the ICR-CTSU data and sample access policy. Gene expression data is available on request by contacting poetic-icrctsu@icr.ac.uk. The ICR-CTSU supports the wider dissemination of information from the research it does, and increased cooperation between investigators. Trial data is collected, managed, stored, shared, and archived according to ICR-CTSU Standard Operating Procedures in order to ensure the enduring quality, integrity, and utility of the data. Formal requests for data sharing are considered in line with the Institute of Cancer Research Clinical Trials and Statistics Unit (ICR-CTSU) procedures with due regard given to funder and sponsor guidelines. Requests are via a standard proforma describing the nature of the proposed research and extent of data requirements. Data recipients are required to enter a formal data sharing agreement which describes the conditions for release and requirements for data transfer, storage, archiving, publication and intellectual property. Requests are reviewed by the Trial Management Group (TMG) in terms of scientific merit and ethical considerations including patient consent. Data sharing is allowed if proposed projects have a sound scientific or patient benefit rationale as agreed by the TMG and approved by the Trial Steering Committee as required. Restrictions relating to patient confidentiality and consent will be limited by aggregating and anonymising identifiable patient data.

Declaration of interests

MB reports grants from Fundacion Alonso Martin Escudero and has a patent filed for the novel molecular subgroups identified in this paper. ELK has a patent filed for the novel molecular subgroups identified in this paper. HT and LK report grants from Cancer Research UK, during the conduct of the study. JMB reports grants from Cancer Research UK, during the conduct of the study; grants and non-financial support from AstraZeneca, Merck Sharp & Dohme, Puma Biotechnology, Clovis Oncology, Pfizer, Janssen-Cilag, Novartis, Eli Lilly, and Roche, outside the submitted work. MD receives consulting fees from AstraZeneca, Lilly, Roche, Radius, H3 Biomedicine and G1. He also receives honoraria from Nanostring. M.C.U.C. has a patent for Breast Cancer Classifier: US Patent No. 9,631,239 with royalties paid and receive research funding from NanoString Technologies and Veracyte advisory role. And has a patent filed for the novel molecular subgroups identified in this paper. All other authors declare no competing interests. The information in this manuscript is subject to a pending patent application.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104205.

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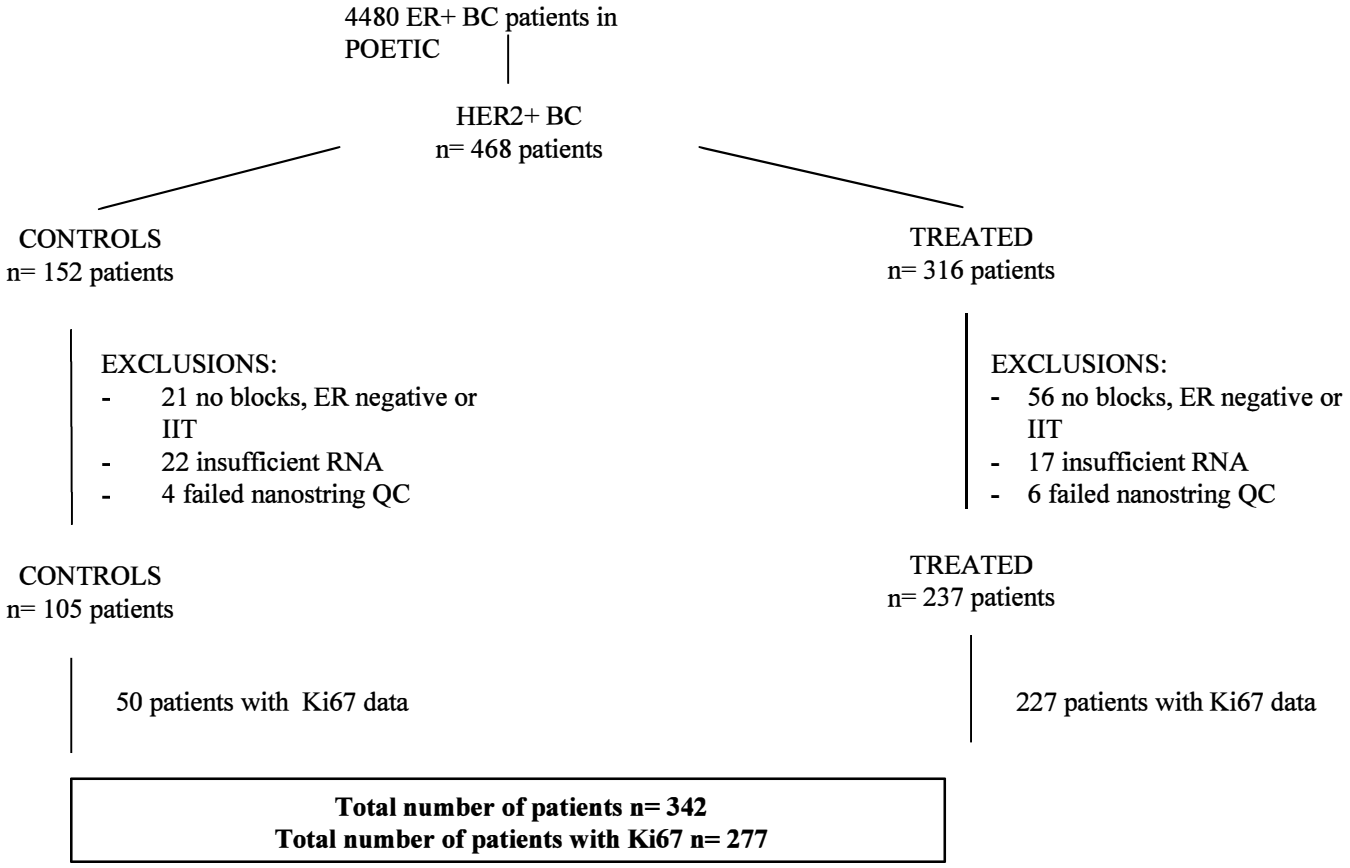
4.2.1 Supplementary materials from manuscript 3

Supplementary figure S1. Consort diagram of the study
Abbreviations: ER+, estrogen receptor positive; BC, Breast Cancer; IIT, insufficient infiltrating tumour·



Supplementary table S1. Breast Cancer 360 Biological signatures

PATHWAYS	SIGNATURES	SUMMARY
Breast Cancer Receptors	*AR* expression	Gene encoding *AR* gene
	ER expression	Gene encoding the estrogen receptor
	HER2 expression	Gene encoding *ERBB2*
	PR expression	Gene encoding the progesterone receptor
Breast Cancer Signaling Pathways	*CDK4* expression	Gene encoding *CDK4*
	CDK6 expression	Gene encoding *CDK6*
	ER Signaling	Measures ER mediated signaling
	PTEN expression	Gene encoding *PTEN*
Breast Cancer Subtyping	Basal-Like Correlation	Correlation Coefficient for Basal-Like subtype
	Claudin-Low	Molecular subtype characterized by low level of luminal markers, high EMT
	HER2-Enriched Correlation	Correlation Coefficient for HER2-Enriched subtype
	Luminal A Correlation	Correlation Coefficient for LuminalA subtype
	Luminal B Correlation	Correlation Coefficient for LuminalB subtype
	PAM50 Subtypes	50-gene signature classifying breast cancer into 4 distinct subtypes
	TNBC Subtypes	Signature identifies 4 distinct TNBC subtypes
Breast Cancer Prognosis	Genomic Risk of Recurrence	Uses Subtype correlation and PAM50 proliferation score to estimate genomic risk of distant recurrence
Immune Cell Population Abundance	CD8 T-cells	Measures the abundance of CD8+ T cells
	Cytotoxic Cells	Measures the abundance of cytotoxic cells in tumour microenvironment
	Macrophace Abundance	Measures the abundance of macrophages
	Mast Cells	Measures the abundance of mast cells
	Treg abundance	Measures Treg abundance by measuresing FOXP3
Inhibitory Metabolism	Hypoxia	Measures genes associated with reduced oxygenation in the tumour
Inhibitory Tumour Mechanisms	*B7-H3*	Gene encoding *B7-H3*
	IDO1 expression	Gene encoding Indoleamine 2,3 dioxygenase 1
	PD-L1	Gene encoding Program cell death ligand-1
	TGF-Beta expression	Gene encoding *TGF-Beta*
Inhibitory Tumour Signaling	Inflammatory Chemokines	Measures chemokines that recruit myeloid and lymphoid populations to tumour
	PD-1	Gene encoding *PD-1*
	PD-L2	Gene encoding Program cell death ligand-2
	TIGIT expression	Gene encoding T cell immunocreceptor and Ig and ITIMS domains
Stromal Factors	Endothelial cells	Measures genes associated with vascular tissue and angiogenesis
	Stroma	Measures stromal components in the tumour microenvironment
Tumour Immunogenicity	APM	Measures abundance of genes in MHC Class I antigen presentation pathway
Anti-Tumour Immune Activity	Cytotoxicity	Measures molecules used by natural killer and CD8+ T cells
	Interferon gamma signalling	Tracks the canonical response to I*FN gamma*
	MHC class II antigen presentation	Measures the major human leukocyte antigens involved in MHC Class II antigen presentation
	TIS	Measures the abundance of a peripherally suppressed adaptive immune response
Tumour Mutational Response	*P53* mutational score	It is a surrogate of T*P53* status: higher scores refer to a mutant-like status and lower scores to a wild-type status·
	BRCAness	Informs about defects in DNA damage repair-genes BRCA1 and 2
	HRD	It functionally asseses HR repair status· Higher score refers to higher damage repair deficiency·
Tumour Regulation	Apoptosis	Captures genes associated with apoptotic processes, pro- and antiapoptotic genes
	Proliferation	Measures genes involved in tumour proliferation
	Cell Adhesion	Scores samples for downregulation in tight junction genes
	Differentiation Score	Assigns a score of differentiation to the sample·
	FOXA1 expression	Gene enconding *FOXA1* transcription factor
	Mammary Stemness	Measures a cluster of EMT genes upregulated in stem-cell-like tumours
	Rb1 Expression	Gene encoding retinoblastoma gene
	SOX2 Expression	Gene encoding SRY-box2

Supplementary table S2. Demography of the study population
Abbreviations: n: number; G: Grade; ILC: Invasive lobular carcinoma; IDC: Invasive ductal carcinoma; ER, Estrogen receptor; PgR, Progesterone receptor, ET, Endocrine Therapy.

	ALL (n=342)	TREATED (n=237)	CONTROL (n=105)
Age of randomisation (years)			
50-59	62 (18·1%)	40 (16·9%)	22 (21·0%)
60-69	159 (46·5%)	110 (46·4%)	49 (46·7%)
70-79	87 (25·5%)	63 (26·6%)	24 (22·9%)
≥80	34 (9·9%)	24 (10·1%)	10 (9·50%)
Median	66 (50-91)	66 (50-91)	66 (51-90)
Surgery Tumour size (cm)			
≤2	137 (40·0%)	90 (38·0%)	47 (44·7%)
>2 & ≤5	187 (54·7%)	137 (57·8%)	50 (47·7%)
Median	2·3 (0·6-16·5)	2·35 (0·6-8·40)	2·20 (0·8-16·5)
Surgery Nodal Status			
Positive	163 (47·7%)	105 (44·3%)	58 (55·2%)
Negative	179 (52·3%)	132 (45·7%)	47 (44·8%)
Pre-surgical Tumour Grade			
G1	12 (3·50%)	10 (4·20%)	2 (1·90%)
G2	165 (48·2%)	118 (49·8%)	47 (44·8%)
G3	131 (38·3%)	85 (35·9%)	46 (43·8%)
Unknown	34 (10·0%)	24 (10·1%)	10 (9·50%)
Histological Type			
ILC	21 (6·10%)	15 (6·30%)	6 (5·70%)
IDC	319 (93·3%)	220 (92·8%)	99 (94·3%)
Mucinous	2 (0·60%)	2 (0·90%)	0 (0·0%)
Unknown	0 (0·0%)	0 (0·0%)	0 (0·0%)
ER status			
Positive	342 (100·0%)	237 (100·0%)	105 (100%)
Negative	0 (0·0%)	0 (0·0%)	0 (0%)
PgR status			
Positive	159 (46·5%)	111 (46·8%)	48 (45·7%)
Negative	83 (24·3%)	70 (29·6%)	27 (25·7%)
Unknown	100 (29·2%)	56 (23·6%)	30 (28·6%)
Presurgical ET			
Letrozole	153 (44·7%)	153 (64·6%)	0 (0·0%)
Anastrozole	84 (24·6%)	84 (35·4%)	0 (0·0%)
No treatment	105 (30·7%)	0 (0·0%)	105 (100%)
Adjuvant treatment therapy			
Trastuzumab + chemotherapy	207 (60·5%)	135 (57·0%)	72 (68·5%)
Chemotherapy alone	23 (6·70%)	16 (6·80%)	7 (6·7%)
No chemotherapy or trastuzumab	112 (32·7%)	86 (36·2%)	26 (24·8%)
Adjuvant ET			
Yes	336 (98·2%)	233 (98·4%)	103 (98·1%)
Letrozole	174 (50·9%)	123 (51·9%)	51 (48·6%)
Anastrozole	139 (40·6%)	91 (38·4%)	48 (45·7%)
Exemestane	1 (0·30%)	1 (0·40%)	0 (0·0%)
Tamoxifen	7 (2·0%)	7 (3·10%)	0 (0·0%)
Sequential AI and tamoxifen	15 (4·40%)	11 (4·60%)	4 (3·8%)
No adjuvant endocrine therapy	4 (1·20%)	2 (0·80%)	2 (1·9%)
Unknown	2 (0·60%)	2 (0·80%)	0 (0%)

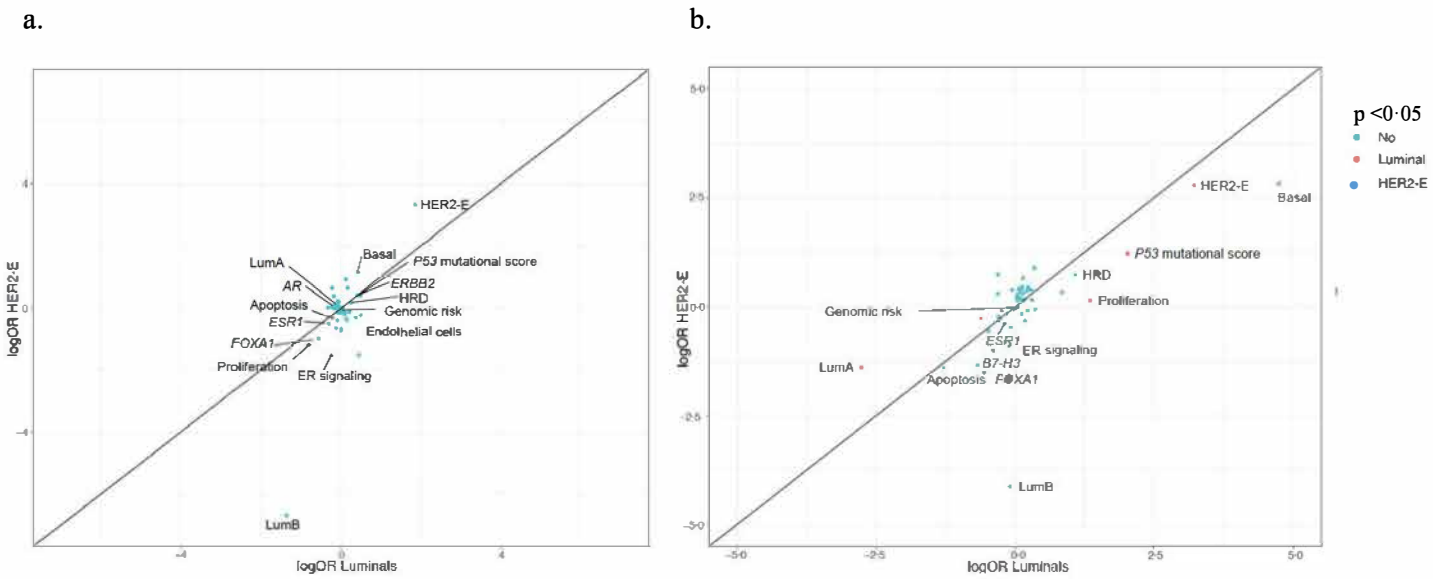
Supplementary table S3. Association analysis of signature expression and response to AI in treated patients (n=227) for the three response categories using ordinal logistic regression models with OR towards poor response and for the two $Ki67_{2wks}$ categories: high vs low, using univariate logistic regression models with OR towards high· In bold FDR values are the significant signatures. **Abbreviations**: *ESR1*, Estrogen Receptor 1; *ERBB2*, gene HER2; *FOXA1*, Forkhead BoxA1; HRD, Homologous Recombination Deficiency; *PGR*: Progesterone Receptor; *PD-L1*, Programmed death-ligand 1; Treg, Regulatory T cells; *PTEN*, Phosphatase and tensin homolog; *IDO1*, Indoleamine 2,3-dioxygenase; AR, Androgen Receptor, IFN Gamma, Interferon Gamma; TIS, Tumour Inflammation Signature; *PD-L2*, Programmed death-ligand 2; TIGIT, T cell immunoreceptor with Ig and ITIM domain; APM, Antigen Processing Machinery; BRCAness, Breast Cancer Gene deficiency; *CDK6*, Cyclin-dependant kinase 6; *TGFB*, Transforming growth Factor Beta; *Rb1*, Retinoblastoma; *SOX2*, SRY(Sex determining region Y)-box2; MHC2, major histocompatibility complex 2; *PD1*, Programmed cell death protein 1; *CDK4*, Cyclin-dependant kinase 4; CD8 T cells, Cytotoxic T lymphocytes; HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype; OR, Odds Ratio; FDR, False discovery rate, CI, Confidence Interval, $Ki67_{2w,}$ Ki67 at 2 weeks timepoint.

Signatures	$Ki67_{2wks}$					Ki67 response categories				
	OR	Lower 95%CI	Upper 95%CI	p-value	FDR	OR	Lower 95%CI	Upper 95%CI	p-value	FDR
HER2-E score	120·53	34·88	481·42	<0·0001	**<0·0001**	12·31	5·02	31·43	<0·0001	**<0·0001**
ESR1	0·57	0·46	0·69	<0·0001	**<0·0001**	0·65	0·55	0·75	<0·0001	**<0·0001**
ER-Signaling	0·38	0·26	0·55	<0·0001	**<0·0001**	0·44	0·31	0·60	<0·0001	**<0·0001**
ERBB2	1·59	1·33	1·93	<0·0001	**<0·0001**	1·52	1·29	1·80	<0·0001	**<0·0001**
LumB score	0·06	0·02	0·20	<0·0001	**<0·0001**	0·05	0·01	0·16	<0·0001	**<0·0001**
P53 mutational score	11·11	5·98	22·46	<0·0001	**<0·0001**	2·65	1·78	4·04	<0·0001	**<0·0001**
Basal score	460·36	93·08	2819·38	<0·0001	**<0·0001**	8·08	3·07	22·62	<0·0001	**0·00026**
FOXA1	0·28	0·16	0·46	<0·0001	**<0·0001**	0·42	0·26	0·63	<0·0001	**0·00046**
LumA score	0·02	0·01	0·06	<0·0001	**<0·0001**	0·25	0·11	0·53	0·00037	**0·0017**
Apoptosis	0·15	0·06	0·35	<0·0001	**<0·0001**	0·26	0·12	0·54	0·00034	**0·0017**
HRD	6·56	3·57	12·83	<0·0001	**<0·0001**	2·33	1·45	3·83	0·00061	**0·0026**
PGR	0·74	0·64	0·85	<0·0001	**<0·0001**	0·82	0·72	0·92	0·0015	**0·0057**
Hypoxia	2·84	1·67	5·03	0·00019	**0·00050**	2·18	1·36	3·60	0·0017	**0·0059**
Proliferation	4·30	2·61	7·45	<0·0001	**<0·0001**	1·39	0·94	2·07	0·10	0·25
Genomic Risk	1·06	1·04	1·08	<0·0001	**<0·0001**	1·02	1·00	1·03	0·04	0·12
IDO1	1·67	1·36	2·08	<0·0001	**<0·0001**	1·19	1·01	1·42	0·04	0·12
IFN Gamma	1·76	1·37	2·29	<0·0001	**<0·0001**	1·14	0·91	1·42	0·26	0·42
AR	0·58	0·43	0·77	0·00022	**0·00056**	0·80	0·63	1·01	0·07	0·17
PD-L1	1·88	1·35	2·67	0·00029	**0·00071**	1·28	0·95	1·74	0·11	0·25
TIS	1·64	1·26	2·16	0·00032	**0·00073**	1·12	0·88	1·42	0·35	0·56
Endothelial Cells	0·37	0·21	0·63	0·00036	**0·00080**	0·71	0·43	1·14	0·16	0·30
PD-L2	1·85	1·31	2·65	0·0006	**0·0012**	1·15	0·85	1·57	0·37	0·57
Treg	1·73	1·26	2·43	0·0010	**0·0020**	1·24	0·93	1·65	0·14	0·28
Macrophages	1·92	1·26	2·99	0·0029	**0·0053**	1·10	0·74	1·62	0·64	0·72
APM	1·42	1·13	1·80	0·0029	**0·0053**	1·03	0·83	1·26	0·81	0·83
Cytotoxicity	1·52	1·16	2·03	0·0032	**0·0056**	1·10	0·86	1·41	0·43	0·61
TIGIT	1·41	1·12	1·80	0·0045	**0·0077**	1·06	0·86	1·32	0·58	0·70
Inflammatory Chemokines	1·56	1·15	2·15	0·0051	**0·0083**	1·22	0·92	1·62	0·18	0·33
Cytotoxic Cells	1·49	1·13	1·98	0·0053	**0·0084**	1·09	0·85	1·40	0·48	0·63
PTEN	0·45	0·24	0·85	0·016	**0·024**	0·63	0·34	1·15	0·13	0·28
SOX2	1·25	1·01	1·56	0·041	0·061	1·08	0·89	1·32	0·44	0·61
Mast Cells	0·78	0·61	0·99	0·045	0·065	0·92	0·73	1·15	0·45	0·61
CD8-T-Cells	1·31	1·00	1·73	0·049	0·069	1·06	0·83	1·36	0·63	0·72
BRCAness	1·75	1·00	3·14	0·055	0·072	1·82	1·08	3·15	0·03	0·09
CDK6 expression	1·47	1·00	2·21	0·056	0·072	1·33	0·92	1·94	0·14	0·28
Claudin Low	0·79	0·61	0·99	0·054	0·072	0·94	0·76	1·17	0·59	0·70
B7-H3	0·63	0·38	1·02	0·066	0·082	0·92	0·59	1·43	0·71	0·76
TGF-Beta	0·67	0·42	1·08	0·10	0·12	0·75	0·48	1·17	0·21	0·36
Stroma	0·75	0·53	1·06	0·11	0·13	0·94	0·68	1·30	0·71	0·76
Mammary Stemness	0·87	0·71	1·05	0·15	0·18	0·99	0·82	1·19	0·89	0·89
PD-1	1·26	0·91	1·77	0·17	0·19	1·09	0·80	1·49	0·58	0·70
CDK4 expression	1·50	0·80	3·04	0·23	0·25	1·19	0·67	2·19	0·55	0·70
Rb1	0·70	0·36	1·33	0·28	0·30	0·69	0·37	1·28	0·25	0·42
Differentiation	0·87	0·59	1·26	0·45	0·48	0·70	0·48	1·02	0·07	0·17
MHC2	1·05	0·82	1·35	0·67	0·69	1·10	0·87	1·38	0·43	0·61
Cell Adhesion	0·96	0·77	1·19	0·71	0·71	1·03	0·84	1·26	0·76	0·80

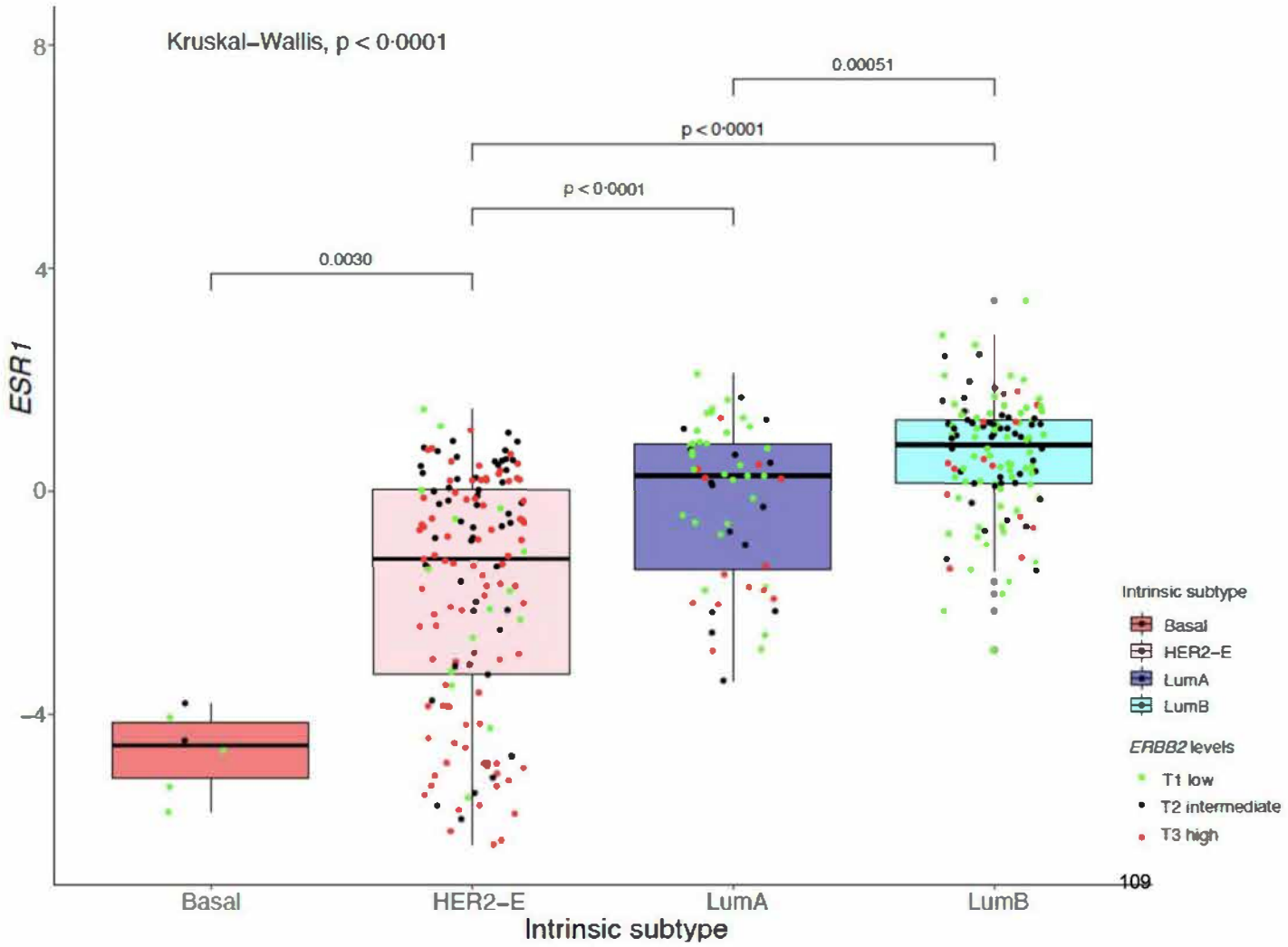
Supplementary table S4. Association analysis of signature expression and response to AI in treated patients (n=227) for the two Ki67 response categories: >20% vs <20%, using univariate logistic regression models with OR towards high. **Abbreviations**: *ESR1*, Estrogen Receptor 1; *ERBB2*, gene HER2; *FOXA1*, Forkhead BoxA1; HRD, Homologous Recombination Deficiency; *PGR*: Progesterone Receptor; *PD-L1*, Programmed death-ligand 1; Treg, Regulatory T cells; *PTEN*, Phosphatase and tensin homolog; *IDO1*, Indoleamine 2,3-dioxygenase; AR, Androgen Receptor, IFN Gamma, Interferon Gamma; TIS, Tumour Inflammation Signature; *PD-L2*, Programmed death-ligand 2; TIGIT, T cell immunoreceptor with Ig and ITIM domain; APM, Antigen Processing Machinery; BRCAness, Breast Cancer Gene deficiency; *CDK6*, Cyclin-dependant kinase 6; *TGFB*, Transforming growth Factor Beta; *Rb1*, Retinoblastoma; *SOX2*, SRY(Sex determining region Y)-box2; MHC2, major histocompatibility complex 2; *PD1*, Programmed cell death protein 1; *CDK4*, Cyclin-dependant kinase 4; CD8 T cells, Cytotoxic T lymphocytes; HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype; OR, Odds Ratio; FDR, False discovery rate, CI, Confidence Interval, $Ki67_{2w,}$ Ki67 at 2 weeks timepoint.

Signature	OR	Lower 95%CI	Upper 95%CI	p-value	FDR
ER Signaling	4·06	2·56	6·77	<0·0001	<0·0001
ESR1	1·61	1·37	1·92	<0·0001	<0·0001
LumB	40·76	10·86	173·92	<0·0001	<0·0001
ERBB2	0·56	0·44	0·70	<0·0001	<0·0001
HER2E	0·04	0·01	0·13	<0·0001	<0·0001
Basal	0·07	0·02	0·23	<0·0001	<0·0001
Hypoxia	0·29	0·16	0·51	<0·0001	<0·0001
PGR	1·46	1·23	1·77	<0·0001	<0·0001
LumA	6·41	2·47	17·76	<0·0001	0·0011
P53 mutational score	11·11	0·26	0·68	<0·0001	0·0018
BRCAness	0·39	0·20	0·74	0·0046	0·019
CDK6 Expression	0·51	0·31	0·81	0·0056	0·020
HRD	0·44	0·24	0·78	0·0057	0·020
FOXA1	1·79	1·20	2·88	0·010	0·034
Mast Cells	1·43	1·08	1·91	0·013	0·039
Apoptosis	2·71	1·14	6·71	0·027	0·077
IDO1	0·80	0·65	0·98	0·037	0·100
Proliferation	0·61	0·37	0·99	0·051	0·13
CDK4 Expression	2·18	0·97	5·44	0·079	0·19
PTEN	1·86	0·91	3·90	0·093	0·21
Stroma	1·38	0·94	2·02	0·10	0·22
Genomic Risk	0·98	0·96	1·00	0·11	0·22
Differentiation	1·36	0·87	2·16	0·18	0·36
APM	0·85	0·66	1·10	0·22	0·42
Treg	0·81	0·56	1·16	0·25	0·45
Cytotoxicity	0·84	0·62	1·13	0·26	0·45
PD-L1	0·81	0·56	1·17	0·27	0·46
Rb1	1·46	0·70	3·09	0·31	0·51
AR	1·15	0·87	1·50	0·32	0·51
Cytotoxic Cells	0·87	0·64	1·17	0·36	0·54
TIS	0·88	0·65	1·17	0·36	0·54
TIGIT	0·89	0·68	1·16	0·38	0·54
Claudin-Low	1·13	0·87	1·55	0·39	0·54
IFN Gamma	0·89	0·68	1·17	0·40	0·55
Mammary Stemness	1·09	0·87	1·37	0·43	0·57
SOX2	0·91	0·72	1·16	0·46	0·58
TGF-Beta	1·20	0·71	2·01	0·50	0·62
Inflammatory Chemokines	0·90	0·64	1·26	0·52	0·63
PD-L2	0·90	0·62	1·30	0·57	0·64
PD-1	0·90	0·62	1·31	0·57	0·64
Endothelial Cells	1·18	0·66	2·13	0·57	0·64
B7-H3	1·15	0·66	2·03	0·62	0·67
MHC2	0·93	0·70	1·24	0·63	0·67
Cell Adhesion	1·06	0·83	1·33	0·64	0·67
CD8 T-Cells	0·95	0·70	1·29	0·75	0·77
Macrophages	0·94	0·59	1·50	0·80	0·80

Supplementary figure S2. Differential signature expression by subtypes including HER2-E BC (n=95) and Luminal BC tumours (n=128)· The scatterplots show the logORs from the logistic regression models calculated for the expression of the 46 signatures for a. Ki67 response categories and b. Ki67 2 weeks categories. **Abbreviations**: HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A; OR, Odds Ratio.



Supplementary figure S3· *ESR1* gene expression levels by PAM50 intrinsic subtype and coloured by different levels (tertiles) of *ERBB2* gene expression. **Abbreviations:** HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype; T, Tertiles.

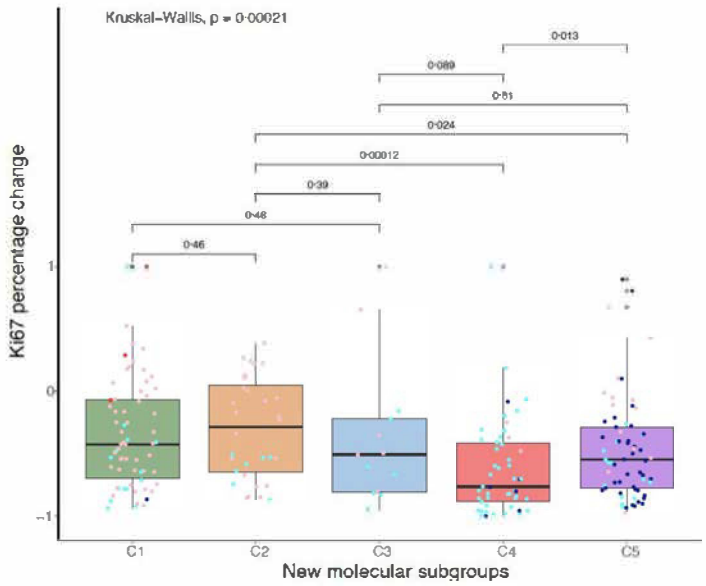


Supplementary table S5. Association analysis of the new molecular subgroups based on single gene expression and response to AI by different endpoints. **Abbreviations:** GR, Good Response, IR: Intermediate Response, PR: Poor response.

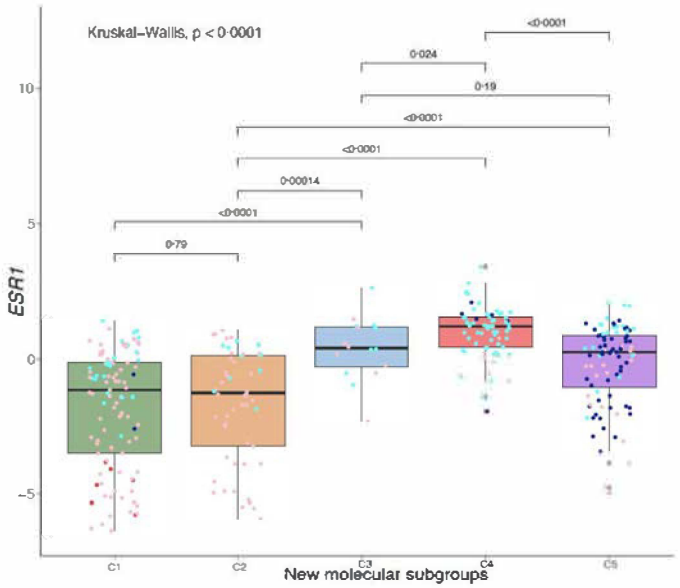
	Single gene based clusters				
	C1	**C2**	**C3**	**C4**	**C5**
Response Categories					
GR	15 (22·7%)	6 (18·8%)	4 (30·7%)	26 (52·0%)	19 (29·2%)
IR	14 (21·3%)	9 (28·1%)	3 (9·4%)	8 (16·0%)	17 (26·2%)
PR	37 (56·0%)	17 (53·1%)	6 (46·2%)	16 (32·0%)	29 (44·6%)
	Chi-squared statistics 15·9263, p-value 0·043				
$Ki67_{2wks}$					
LOW	15 (23·7%)	9 (29·1%)	5 (38·5%)	35 (70·0%)	44 (67·7%)
HIGH	51 (77·3%)	23 (71·9%)	8 (61·5%)	15 (30·0%)	21 (32·3%)
	Chi-squared statistics 42·23, p-value <0·0001				

Supplementary figure S4. Differential expression of **A.** Ki67 percentage changes **B.** *ERBB2* and **C.** *ESR1* by the 5 new subgroups in the entire cohort. **Abbreviations**: C, Cluster; HER2-E, HER2-Enriched Subtype; LumB, Luminal B subtype; LumA, Luminal A subtype.

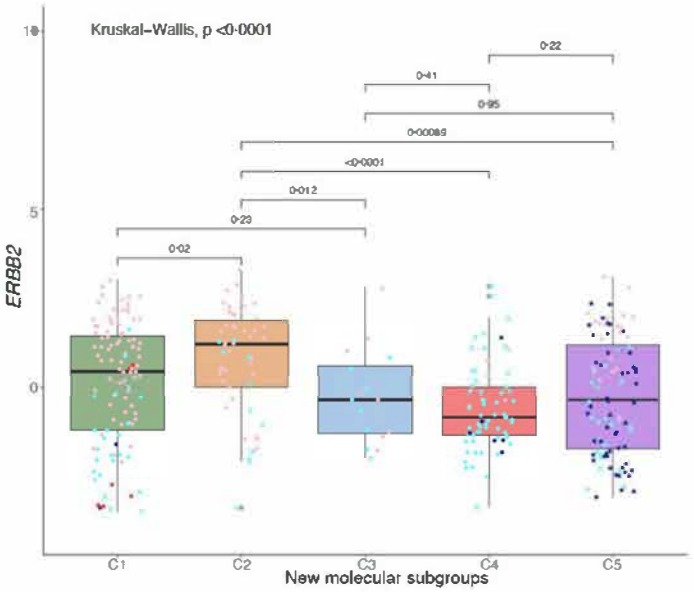
A·

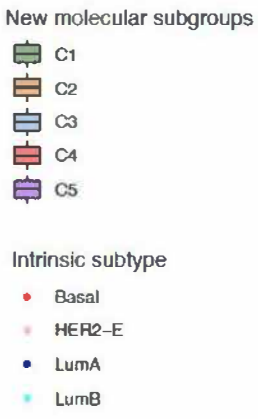


B·



C·

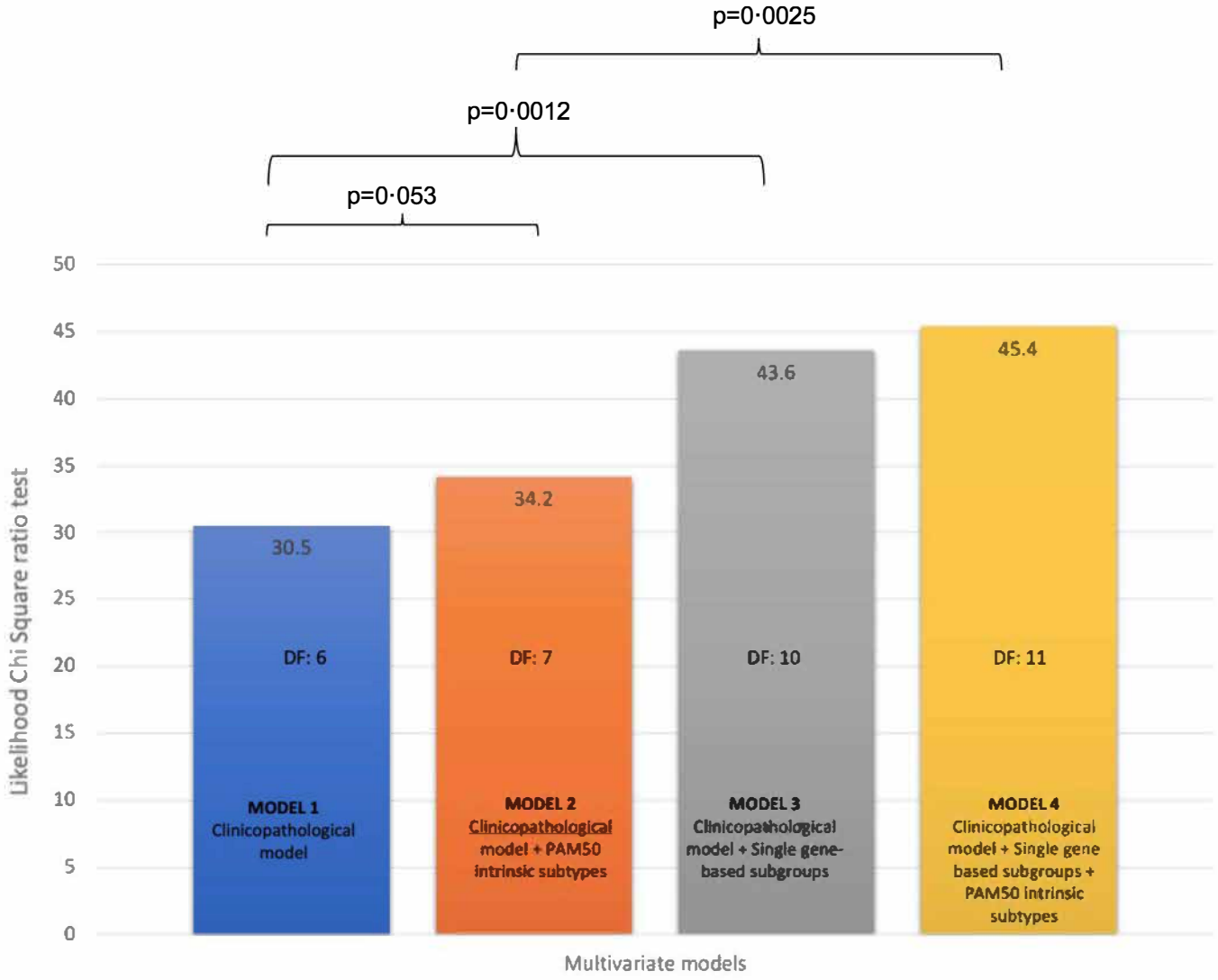




Supplementary table S6. Multivariable cox regression analysis for TTR for selected prognosis factors and other variables driving differential adjuvant chemotherapy and HER2 treatment choice. **Abbreviations:** HR, Hazard Ratio; FDR, False discovery rate, CI: Confidence Interval; N, nodal; EMC: Extra celular matrix, HER2-E, HER2-enriched.

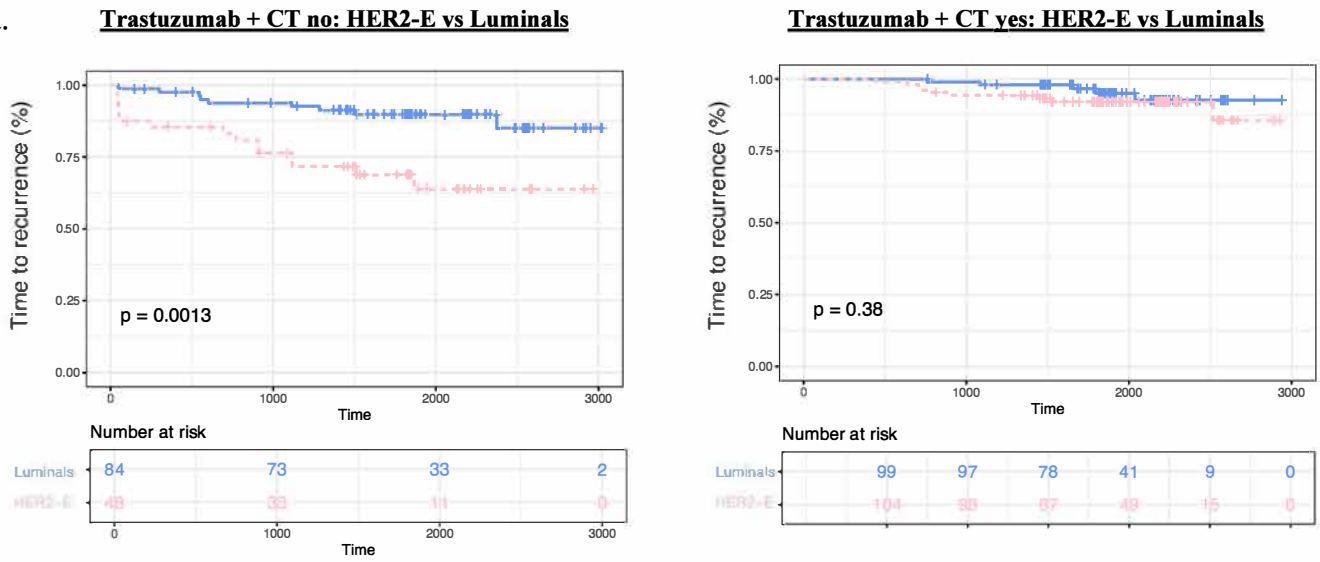
		HR	Lower CI 95%	Upper CI 95%	p-value	Significance
MODEL 1	Age >70	2·29	1·17	4·49	0·016	*
	Nodal status N1-3	1·14	0·48	2·74	0·765	
	Nodal status N4+	2·75	1·27	5·95	0·01	*
	Post tumour size	1·13	1	1·28	0·052	·
	Post tumour grade 1	0	0	Inf	0·99	
	Post tumour grade 3	1·86	0·95	3·65	0·071	·
	Likekihood Chi square ratio test: 30·48 on 6 df, p<0·0001					
MODEL 2		HR	Lower CI 95%	Upper CI 95%	p-value	Significance
	Age >70	2·43	1·23	4·79	0·011	*
	Nodal status N1-3	1·15	0·48	2·76	0·76	
	Nodal status N4+	2·68	1·24	5·77	0·012	*
	Post tumour size	1·16	1·02	1·33	0·024	*
	Post tumour grade 1	0	0	Inf	0·99	
	Post tumour grade 3	1·52	0·75	3·06	0·24	
	Intrinsic subtype: HER2-E vs Luminals	1·98	0·97	4·02	0·059	·
	Likekihood Chi square ratio test: 34·2 on 7 df, p<0·0001					
MODEL 3	Age >70	2·24	1·14	4·41	0·019	*
	Nodal status N1-3	1·55	0·63	3·82	0·35	
	Nodal status N4+	3·89	1·76	8·59	0·00078	***
	Post tumour size	1·14	1	1·3	0·042	*
	Post tumour grade 1	0	0	Inf	1	
	Post tumour grade 3	1·85	0·91	3·77	0·089	·
	Cluster 2 EMC + highest ERBB2	4·82	1·93	12·05	0·00075	***
	Cluster 3 DNA-damage repair deficiency	2·29	0·59	8·85	0·23	
	Cluster 4 Endocrine signalling	1·29	0·45	3·71	0·64	
	Cluster 5 Endocrine signaling + PI3K, MAPK and RAS signalling	1·35	0·47	3·91	0·58	
	Likekihood Chi square ratio test: 43·58 on 10 df, p<0·0001					
MODEL 4	Age >70	2·36	1·19	4·68	0·014	*
	Nodal status N1-3	1·53	0·62	3·8	0·36	
	Nodal status N4+	3·65	1·65	8·05	0·0014	**
	Post tumour size	1·16	1·02	1·32	0·028	*
	Post tumour grade 1	0	0	Inf	1	
	Post tumour grade 3	1·65	0·8	3·41	0·18	
	Intrinsic subtype: HER2-E vs Luminals	1·76	0·76	4·06	0·19	
	Cluster 2 EMC + highest ERBB2	4·6	1·84	11·46	0·0011	**
	Cluster 3 DNA-damage repair deficiency	2·39	0·62	9·21	0·21	
	Cluster 4 Endocrine signalling	1·75	0·56	5·52	0·34	
	Cluster 5 Endocrine signaling + PI3K, MAPK and RAS signalling	1·71	0·56	5·16	0·35	
	Likekihood Chi square ratio test: 45·39 on 11 df, p<0·0001					

Supplementary figure S5. Barplots comparing the multivariate chi-square likelihood test for each multivariable model.
Abbreviations: p: p-value; DF: degrees of freedom.

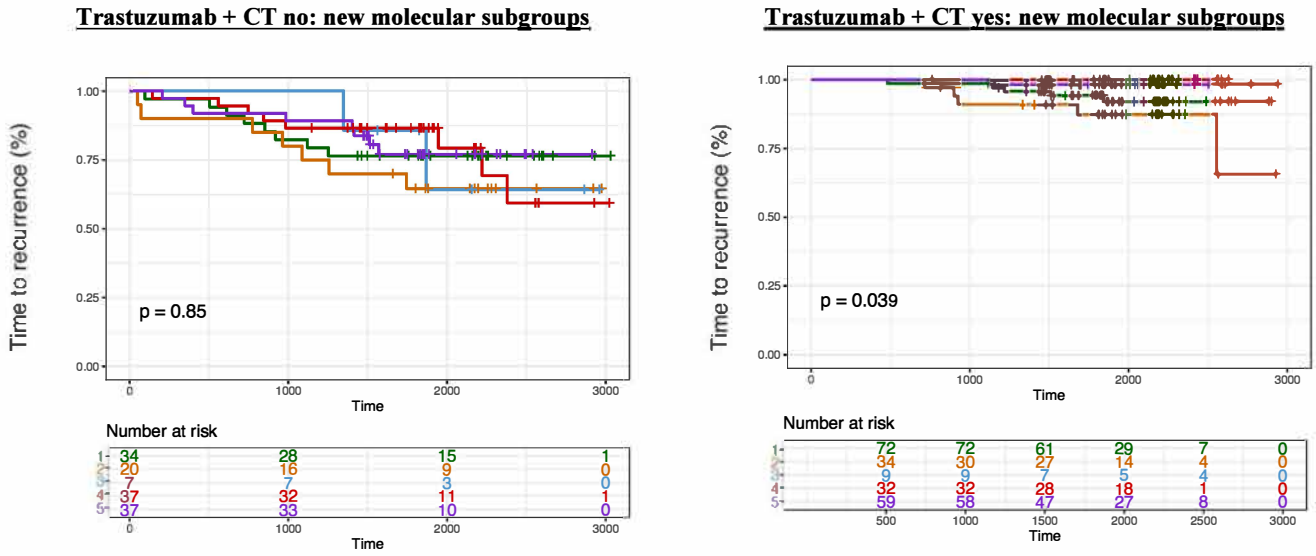


Supplementary figure S6. Kaplan Meier curves stratified by adjuvant treatment (trastuzumab + chemotherapy yes vs no) for TTR according to a. PAM50 Intrinsic Subtypes and b. to the new molecular subgroups. **Abbreviations:** HER2-E: HER2-enriched, CT:Chemotherapy, TTR: Time To Recurrence.

a.



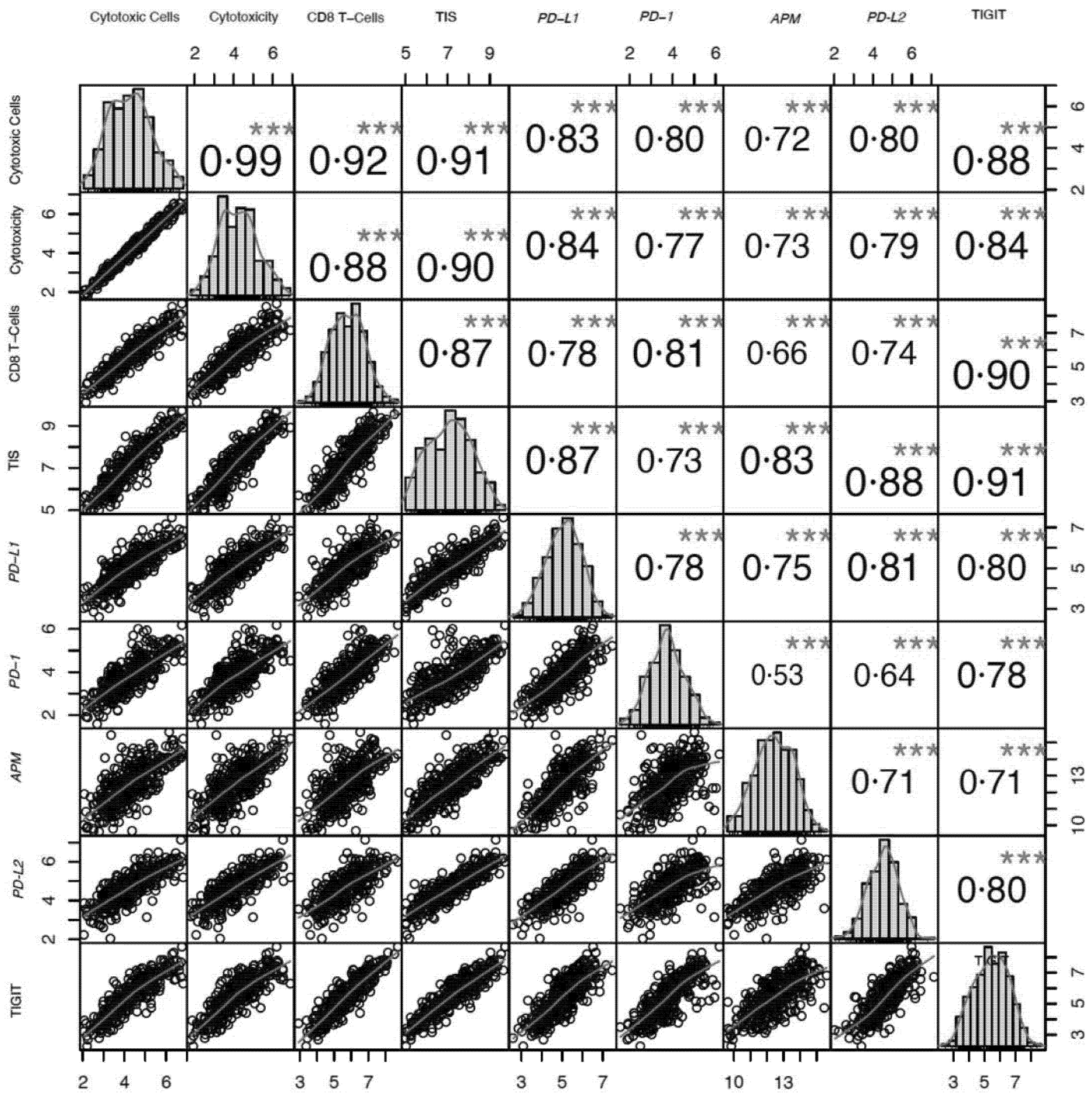
b.



Supplementary table S7. Series of multivariable analysis for TTR of signature expression adjusted by the basic clinicopathological factors (age, nodal status, post tumour size, post tumour grade). **Abbreviations**: *ESR1*, Estrogen Receptor 1; *ERBB2*, gene HER2; *FOXA1*, Forkhead BoxA1; HRD, Homologous Recombination Deficiency; *PGR*: Progesterone Receptor, BC: Breast Cancer Signature; *PD-L1*, Programmed death-ligand 1; Treg, Regulatory T cells; *PTEN*, Phosphatase and tensin homolog; *IDO1*, Indoleamine 2,3-dioxygenase; *AR*, Androgen Receptor, IFN Gamma, Interferon Gamma; TIS, Tumour Inflammation Signature; *PD-L2*, Programmed death-ligand 2; TIGIT, T cell immunoreceptor with Ig and ITIM domain; APM, Antigen Processing Machinery; BRCAness: Breast Cancer Gene deficiency; *CDK6,* Cyclin-dependant kinase 6; *TGFB*: Transforming growth Factor Beta; *Rb1*, Retinoblastoma; *SOX2*, SRY(Sex determining region Y)-box2; MHC2, major histocompatibility complex 2; *PD1*, Programmed cell death protein 1; *CDK4*, Cyclin-dependant kinase 4; CD8 T cells, Cytotoxic T lymphocytes; HR, Hazard Ratio; FDR, False discovery rate, CI: Confidence Interval.

Signatures	HR	Lower 95%CI	Upper 95%CI	p value	FDR
Cytotoxic cells	0·50	0·36	0·70	<0·0001	0·0014
Cytotoxicity	0·50	0·36	0·71	<0·0001	0·0014
CD8 T-Cells	0·53	0·39	0·73	<0·0001	0·0014
TIS	0·60	0·44	0·82	0·0011	0·013
PD-L1	0·58	0·41	0·82	0·0020	0·019
PD-1	0·58	0·39	0·86	0·0070	0·052
APM	0·71	0·55	0·92	0·0096	0·052
PD-L2	0·60	0·41	0·89	0·0099	0·052
TIGIT	0·70	0·54	0·92	0·010	0·052
IFN Gamma	0·71	0·53	0·94	0·019	0·087
Apoptosis	0·36	0·15	0·88	0·024	0·10
Claudin Low	1·26	1·02	1·56	0·031	0·11
IDO1	0·78	0·62	0·98	0·032	0·11
MHC2	0·73	0·54	0·99	0·043	0·14
Inflammatory Chemokines	0·70	0·48	1·02	0·066	0·20
Macrophages	0·64	0·39	1·06	0·085	0·24
HER2-E score	3·11	0·80	12·15	0·10	0·26
LumB score	0·43	0·15	1·20	0·11	0·26
Hypoxia	1·59	0·90	2·81	0·11	0·26
ER signaling	0·76	0·54	1·07	0·12	0·28
ERBB2	1·17	0·95	1·44	0·13	0·29
AR	0·82	0·64	1·07	0·14	0·30
SOX2	1·18	0·94	1·48	0·15	0·30
Stroma	1·39	0·87	2·23	0·17	0·31
P53 mutational score	1·43	0·86	2·37	0·17	0·31
Mammary Stemness	1·19	0·91	1·56	0·21	0·36
FOXA1	0·85	0·66	1·10	0·21	0·36
CDK6 expression	0·76	0·49	1·18	0·22	0·36
Treg	0·81	0·57	1·15	0·24	0·38
Endothelial Cells	0·67	0·34	1·33	0·25	0·38
Mast Cells	0·87	0·65	1·16	0·34	0·50
TGF Beta	0·78	0·44	1·38	0·39	0·57
PTEN	1·31	0·64	2·66	0·46	0·64
CDK4 expression	1·24	0·64	2·39	0·52	0·71
Rb1	0·80	0·38	1·69	0·56	0·73
Basal score	1·24	0·41	3·79	0·70	0·87
Differentiation	0·92	0·58	1·46	0·72	0·87
B7-H3	1·12	0·60	2·07	0·73	0·87
LumA score	0·83	0·27	2·56	0·75	0·87
ESR1	0·98	0·84	1·13	0·75	0·87
PGR	0·98	0·82	1·16	0·78	0·87
HRD	1·08	0·56	2·10	0·82	0·89
BRCAness	1·07	0·56	2·02	0·84	0·90
Genomic Risk	1·00	0·97	1·02	0·87	0·91
Proliferation	0·96	0·53	1·74	0·90	0·92
Cell adhesion	1·01	0·79	1·28	0·96	0·96

Supplementary figure S7. Spearman correlation tests of the significant signatures by nominal p-value in the series of multivariablee analysis using each of the signatures in the baseline clinicopathological model.



4.2.2 Additional information on new molecular subgroups based on BC360 signature expression (manuscript 3)

In addition to the new molecular subgroups based on single gene expression, we identified four new molecular subgroups based on the nCounter Breast Cancer 360 panel. This work was not included in the final manuscript due to lack of space and some overlap with single gene expression signatures. However, we presented this data at the San Antonio BC Symposium 2021 and I was awarded the Coltman Scholar Award for it. The work was highly rated by the committee.

Identification of new molecular subgroups predicting response to aromatase inhibitors

Using consensus clustering, we also identified four novel molecular subgroups based on signature expression predicting significantly different responses to AI in tumour samples. These clusters were also associated with Ki67 at the two-week time point (**Table 1**). Interestingly, this set of clusters mainly divided HER2-E subtype samples with lower response to AI into three groups with differential expression of molecular features such as ERBB2, ER signalling, or immune-related pathways.

For the four new clusters based on signature expression (**Figure 7A**): Cluster 1 (41.8%) was characterised by overexpression of immune features and lower ER signalling; Cluster 2 (15.5%) by low immune but significantly higher levels of *ERBB2* expression regardless of subtype (**Figures 7B and 7C**); Cluster 3 (4.1%) was characterised by high *ESR1* and low *PgR* expression, and Cluster 4 (38.6%) by high endocrine signalling and the lowest *ERBB2* expression. PAM50 subtype distribution varied among these subgroups with enrichment of Cluster 1 with HER2-E (62.2%) and luminal B (26.6%), Cluster 2 HER2-E (73.6%), Cluster 3 luminal B (64.3%) and Cluster 4 Luminal B (55.3%) and luminal A (29.5%). The highest overlap between single gene and signature-based subgroups and the single gene molecular subgroups defined in the manuscript was in the single gene-based Cluster 1 (89.5%) and signature-based Cluster 1 (68.6%) (**Table 2)**, followed by signature-based Cluster 4, in which 77.2% of patients were included in single gene clusters 4 and 5.

To assess the independent prognostic value of these gene-expression-based variables with survival outcomes in terms of TTR, we performed a series of Cox regression models for multivariable survival analysis and compared the changes in chi-square values between them to assess the added value of the different models (**Table 3**). In the multivariable analysis

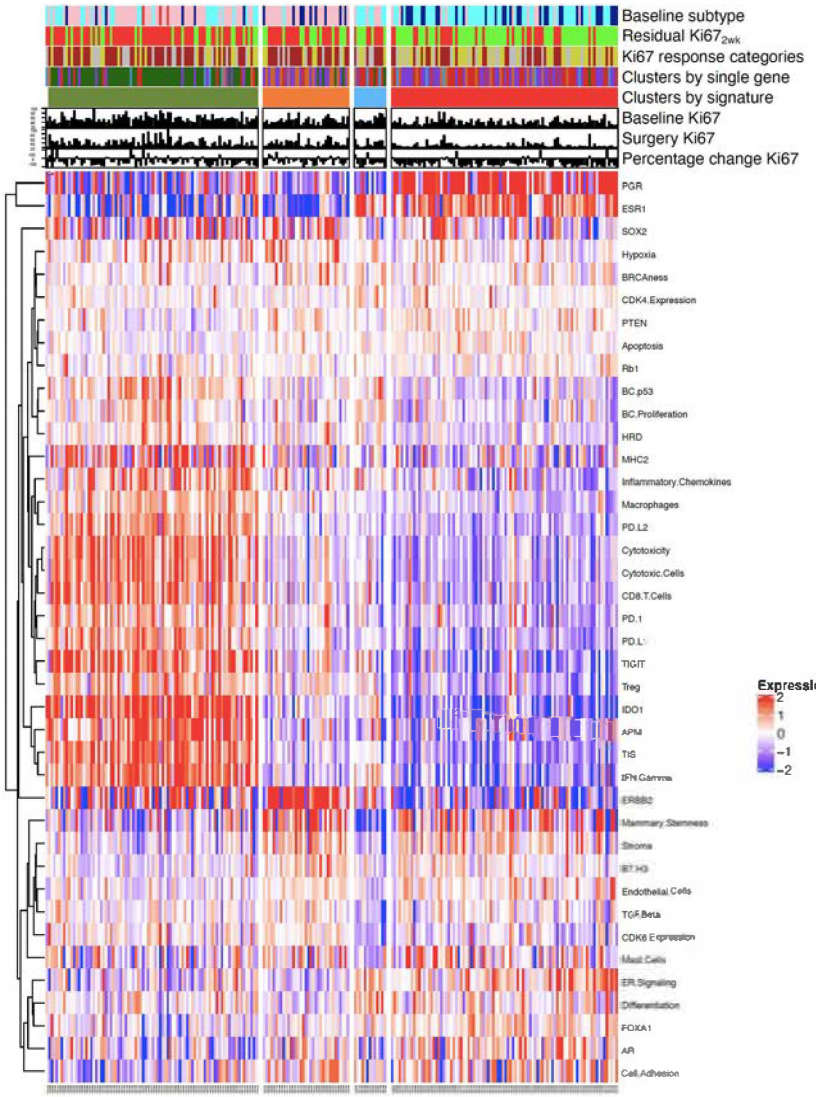
adjusted for post-surgery clinicopathological variables (histological grade, tumour size, nodal status, and age), clusters 2 and 4 were also independent predictors of shorter TTR compared with aingle gene based cluster 1, adding significant value beyond intrinsic subtypes (Likelihood ratio test, p<0.001). Two additional models: one including all clusters from both sets together and a second with all clusters and PAM50 did not add any additional value than when just considering one set of clusters and PAM50.

Table 1. Association analysis of the new molecular subgroups based on signature expression and response to aromatase inhibitors by different endpoints.
Abbreviations: GR: Good Response, IR: Intermediate Response, PR: Poor response.

	Signature-based new molecular subgroups			
	1	**2**	**3**	**4**
Response Categories				
GR	21 (24.4%)	7 (20%)	3 (23.1%)	39 (42.4%)
IR	19 (22.1%)	6 (17.1%)	4 (30.8%)	22 (23.9%)
PR	46 (53.5%)	22 (62.9%)	6 (46.2%)	31 (33.7%)
	Chi-square statistics 13.780. p-value is 0.032			
$Ki67_{2wks}$				
HIGH	61 (74.4%)	22 (62.9%)	7 (53.8%)	28 (30.4%)
LOW	25 (25.6%)	13 (27.1%)	6 (46.2%)	64 (69.6%)
	Chi-square statistics 31.1665. p-value is <0.0001			

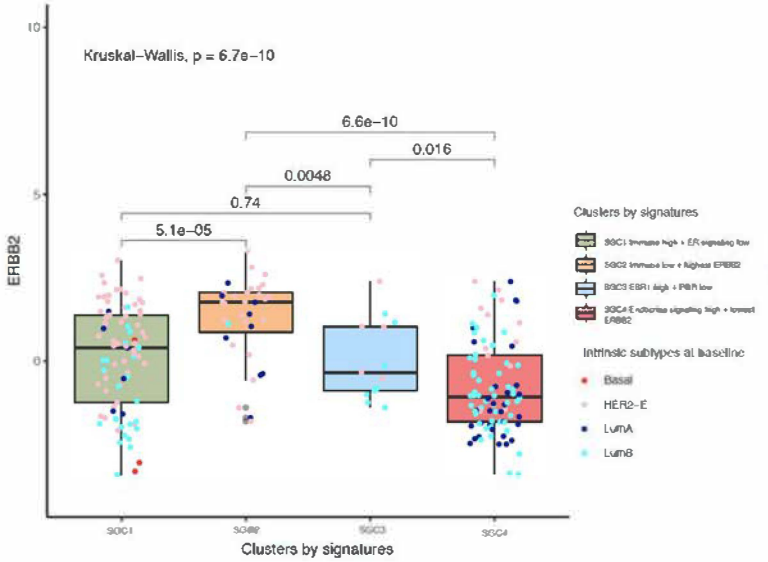
Figure 7. New molecular subgroups based on gene expression signatures**.** A. Consensus clustering using signature expression at baseline in all patients (treated and controls). The heatmap shows the single gene expression by the five new clusters in treated patients with Ki67 data only (n=226). B. Boxplots of differential *ERBB2* expression by new signature-based clusters in the entire cohort and C. in HER2E and Luminals separately.
Abbreviations: HER2-E: HER2-Enriched Subtype, lumB: luminal B subtype, lumA: Luminal A subtype, GR: Good responders, IR: Intermediate responders, PR: Poor Responders, $Ki67_{2wk}$: Ki67 at the two-week time point, HRD: Homologous Recombination Deficiency.

A.



Cluster 1- Immune high + ER signalling low
Cluster 2- Immune low + highest *ERBB2*
Cluster 3- *ESR1* high + *PGR* low
Cluster 4- Endocrine signalling + lowest *ERBB2*

B.



C.

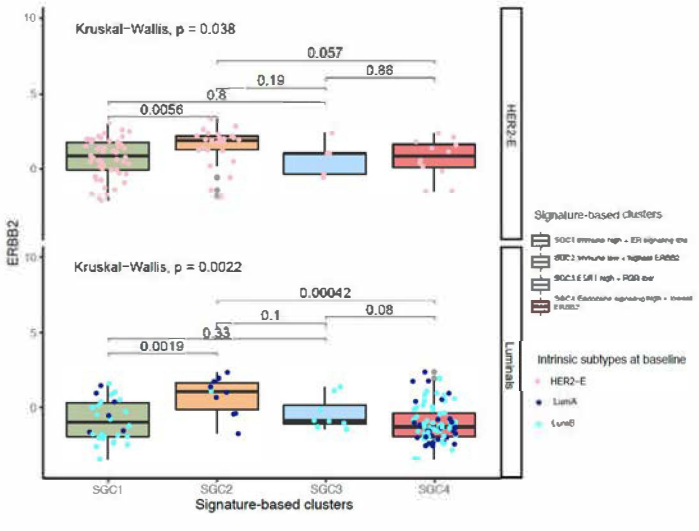


Table 2. Association analysis of the new molecular clusters based on single gene expression and signature expression and response to aromatase inhibitors by different endpoints.
Abbreviations: GR: Good Response, IR: Intermediate Response, PR: Poor response.

		5 single gene clusters				
4 cluster signatures		1- Immune high	2-EMC + *ERBB2* highest	3- DNA-damage repair deficiency	4- Endocrine signalling	5- Endocrine and PI3K, MAPK, and RAS signalling
	1- Immune high + ER signalling low	59 (89.4%) (68.6%)	3 (9.40%) (3.50%)	5 (38.5%) (5.80%)	8 (16.0%) (9.30%)	11 (16.9%) (12.8%)
	2- Immune low + *ERBB2* highest	4 (6.06%) (11.4%)	14 (43.8%) (40.0%)	0 (0.0%) (0.0%)	2 (4.0%) (5.70%)	15 (23.1%) (42.9%)
	3- *ESR1* high + *PGR* low	2 (3.04%) (15.4%)	1 (3.1%) (7.70%)	2 (15.4%) (15.4%)	7 (14.0%) (53.8%)	1 (1.50%) (7.70%)
	4- Endocrine signalling	1 (1.5%) (1.1%)	14 (43.7%) (15.2%)	6 (46.1%) (6.5%)	33 (66.0%) (35.9%)	38 (58.5%) (41.3%)

Table 3. Multivariable cox regression analysis for time to recurrence for selected prognosis factors and other variables driving differential adjuvant chemotherapy and HER2 treatment choice.
Abbreviations: HR: Hazard Ratio, FDR: False discovery rate, CI: Confidence Interval, N: nodal, EMC: Extracellular matrix, HER2-E: HER2-enriched, DF: degrees of freedom.

		HR	Lower CI 95%	Upper CI 95%	p-value	Significance
MODEL 4	Age >70	2.41	1.18	4.92	0.016	*
	Nodal status N1-3	1.3	0.53	3.16	0.562	
	Nodal status N4+	3.54	1.59	7.87	0.0019	**
	Post tumour size	1.12	0.98	1.28	0.096	.
	Post tumour grade 1	0	0	Inf	1	
	Post tumour grade 3	1.73	0.84	3.55	0.14	
	Cluster 2 Immune low + highest *ERBB2*	4.32	1.76	10.61	0.0014	**
	Cluster 3 *ESR1* high + *PGR* low	1.63	0.35	7.66	0.53	
	Cluster 4 Endocrine signalling high	3.46	1.43	8.4	0.0061	**
	Baseline-subtype: HER2-E vs. Luminals	2.55	1.14	5.69	0.022	*
	Likelihood Chi square ratio test: 47.37 on 10 df, p<0.0001					

4.2.3 Ethical approval

NHS
Health Research Authority

London - South East Research Ethics Committee

Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

25 April 2017

Professor Ian Smith
Consultant Medical Oncologist
The Royal Marsden NHS Foundation Trust
London and Surrey Breast Unit
The Royal Marsden NHS Foundation Trust
Fulham Road, London
SW3 6JJ

Dear Professor Smith

Study title:	**Trial of Perioperative Endocrine Therapy - Individualising Care (POETIC)**
REC reference:	**08/H1102/37**
Protocol number:	**CCR2973**
EudraCT number:	**2007-003877-21**
Amendment number:	**10**
Amendment date:	**09 February 2017**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper [Covering Letter]		09 February 2017
Notice of Substantial Amendment (CTIMP) [Substantial Amendment 10]		09 February 2017
Other [Clafification of anonymisation]		23 March 2017

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

08/H1102/37:	**Please quote this number on all correspondence**

Yours sincerely

pp
Professor David Caplin
Chair

E-mail: nrescommittee.london-southeast@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

London - South East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 01 May 2017

Committee Members:

Name	*Profession*	*Present*	*Notes*
Ms Stephanie Cooper	Retired Solicitor	Yes	
Dr Morven Leese	Reader in Biostatistics	Yes	

5. DISCUSSION

5.1 Summary of the main results

Around 70% of BCs are ER+ and the standard of care for patients with early-stage BC includes, among others, ET for 5-10 years depending on their risk of recurrence and tolerance. Although AI treatment is the most effective ET for postmenopausal women with early ER+ BC, 15-20% of them still recur (22). In particular, ER+ BC tumours that also overexpress HER2 show limited response to ET (19,88,105).

The search for predictive biomarkers in breast cancer has mainly been limited to baseline tumour characteristics. Baseline or pre-treatment gene expression profiles are useful to distinguish those tumours more likely to respond to neoadjuvant therapy from those that do not (85). However, biological changes that occur in the tumour and the tumour's microenvironment in response to therapy may identify new mechanisms of response and resistance to treatment, that ultimately may improve patient management and prognosis (118). Furthermore, the effect of different lengths of neoadjuvant ET on BC's molecular features is still unknown.

This Ph.D. project was designed to provide a comprehensive characterisation of RNA-based expression in ER+ early-stage BC treated with perioperative AI. This thesis consists of two main studies that investigate the predictive and prognostic value of baseline molecular features and the changes that occur under short and longer times of AI treatment exposure in both ER+/HER2- and ER+/HER2+ tumours. Molecular characterisation includes not only the intrinsic subtypes but also other key BC signalling pathways and compares the impact of different durations of neoadjuvant ET on BC characteristics. It also evaluates the association of those baseline characteristics and changes that occur under AI treatment with treatment resistance and patient outcome. To our knowledge, both studies included in this thesis are unique and include patients from the largest cohort to date investigating response to pre-surgical AI in ER+ BC.

In ER+/HER2- early BC, this work shows the similar impact on intrinsic subtype modulation following different lengths of AI treatment, but with more and larger molecular changes at the single gene expression and pathway levels with longer AI exposure. An artefactual effect based on the upregulation of *FOS* and *JUN* gene expression is also described in patients with

ER+/HER2- BC tumours following no treatment (control arm). Finally, some of the observed changes after AI treatment had an impact on BC patient outcomes.

In ER+/HER2+ BC, our results established the HER2-E subtype as the first standardised biomarker driving poor response to AI and worse outcome. Signature expression of DNA damage response, *TP53* mutational status, and immune-tumour tolerance are also predictive biomarkers of early resistance to AI in this setting. Lastly, novel molecular subgroups identify additional non-HER2-E tumours not responding to AI with an increased risk of relapse.

5.1.1 Mechanisms of resistance to aromatase inhibitors in ER+/HER2- breast cancer (manuscript 2)

The main observations from this study were:

5.1.1.1 Most AI-sensitive luminal B and HER2-E tumours change their intrinsic subtype within just two weeks of treatment, those changes being mainly from luminal B or HER2-E towards luminal A or Normal-like. These changes are similar between short and longer-term AI treatment, therefore, treatment length seems to not be associated with changes in intrinsic subtypes.

5.1.1.2 Longer AI treatment induces more and a larger magnitude of gene expression changes than two-week treatment only, involving some key BC pathways such as *PI3K/mTOR/AKT.*

5.1.1.3 There is an upregulation of *FOS* and *JUN* related genes and pathways from baseline to surgery in the control samples. These differences were observed in both treated and control samples, thus they might be caused by sample manipulation (i.e., time to formalin fixation) and not by AI treatment itself.

5.1.1.4 Changes from a "high-risk" intrinsic subtype to "low-risk" based on proliferation levels are associated with better response to AI compared with those remaining as high-risk. BC tumours showing early resistance to AI are characterised by a greater expression of immune-checkpoint component molecular features, immune-cell enrichment, and proliferation genes. These characteristics are increased by longer AI treatment.

5.1.1.5 Some of the significant changes that occur under AI treatment, particularly those associated with significantly different responses to AI, have an impact on TTR.

5.1.2 Mechanisms of resistance to aromatase inhibitors in ER+/HER2+ breast cancer (manuscript 3)

The main observations from this study were:

5.1.2.1 HER2-E is one of the main components driving resistance to AI and is associated with a higher risk of relapse in ER+/HER2+ BC.

5.1.2.2 Beyond the association of ER-related signature expressions with good response to AI and *ERBB2* with poor response, DNA damage response, the *p53* mutant surrogate signature and immune-tumour tolerance-related signatures are associated with resistance to AI. Similarly, genes involved in *PI3K/AKT, MAPK*, and oestrogen signalling are associated with good response to AI while genes involved in immune-checkpoint component, proliferation, and cell-cycle regulation are associated with poor response.

5.1.2.3 Five single gene-based new molecular subgroups can distinguish HER2-E and Luminal tumours responding or not to AI treatment but at a higher risk of relapse.

5.1.2.4 The combination of the new molecular subgroups and the intrinsic subtypes might be a key tool to distinguish patients with differential responses to AI therapy and at a higher risk of relapse. Interestingly, immune-related molecular features drive an intrinsic lower risk of relapse despite predicting poor response to AI, while higher levels of *ERBB2* lead to worse survival outcomes.

5.2 New findings

The predictive and prognostic value of changes in Ki67, measured by IHC after two weeks of AI treatment has been validated in the POETIC trial (83,119). In this study, Smith *et al.*, showed that patients whose Ki67 remains "HIGH" (≥ 10%) after two weeks of AI treatment have a substantially poorer prognosis than those with "HIGH" baseline Ki67 that is markedly reduced to "LOW" (<10%). Beyond its main objectives, the POETIC trial was designed to conduct multiple translational sub-studies to assess the differential gene expression between "H-H" and "H-L" response groups, aiming to distinguish those patients who might benefit the most from AI treatment from those who would not.

Decreased tumour cell expression of Ki67 and other clinical and pathological endpoints have often been used as a proxy for treatment response, including assessing the early effect of ET within pre-surgical windows of opportunity and neoadjuvant treatment trials (17,120,121). However, these clinical and pathological characteristics are not always sufficient to evaluate

the risk of recurrence and decide the patient's management (122). In the last decade, multi-parameter gene expression-based prognostic signatures have been developed to estimate the residual risk of recurrence after surgery in order to guide the patient's systemic adjuvant treatment. Amongst the most widely used prognostic signatures in ER+ breast cancer are the Oncotype DX Recurrence Score (RS), Mammaprint, EndoPredict (EP/EPclin), and Prosigna® Risk Of Recurrence score (ROR). Each one has been endorsed for prognostic use within authoritative guidelines (11,113,123,124). Another proposed approach was the combination of parameters such as the conversion from luminal B to luminal A type and/or the reduction of ROR scores as alternative measures of treatment activity in the CORALLEEN study (125). This study evaluated the activity of neoadjuvant treatment with either standard combination chemotherapy (doxorubicin and cyclophosphamide plus paclitaxel) or an investigational drug combination with letrozole plus ribociclib. The primary endpoint was to evaluate the proportion of patients with PAM50 low-risk-of-recurrence measured by the molecular downstaging of luminal B/ER+HER2- tumours (125). Although the use of this biological endpoint is supported by the assumption that luminal A subtypes have a good prognosis, approximately 20-30% of luminal/ER+HER2- can be classified as either A or B even at a boundary of luminal A/B subtype assignments, so the long-term effect of this biological conversion of luminal B to A remains to be validated before it can be widely adopted. In line with this approach, we evaluated the predictive and prognostic value of the changes in intrinsic subtypes after AI treatment and identified a high correlation between the conversion of "high proliferative intrinsic subtypes" such as luminal B or HER2-E to "lower proliferative" subtypes such as luminal A with a $Ki67_{2wk}$ endpoint <10% and better TTR.

Moreover, as mentioned before, most translational studies investigating biomarkers of response to ET have focused on the baseline characteristics after a pre-defined period. Little is known about molecular changes happening after different lengths of ET or whether pre-treatment or post-treatment characteristics are better predictors of prognosis. It has been suggested that reduced ER dependence and E2F-signalling activation after short- and long-term neoadjuvant AIs are associated with poor response to AI, respectively (126,127). However, an association was reported for an enrichment in *ESR1* mutations with long-term neoadjuvant AI in primary BC using a real-world cohort of patients treated at the Royal Marsden Hospital, London (127). Therefore, an in-depth characterisation of the effect of different lengths of neoadjuvant AI therapy on molecular features is necessary to elucidate the full impact on molecular alterations that might limit response and lead to clinical resistance.

Our study provides unique results from a direct comparison of the impact of different lengths of AI treatment on ER+ early BC molecular biology.

On the one hand, our study addresses the important clinical question of the differential effect of short- and long-term neoadjuvant treatment with ET on the molecular characteristics of ER+ BC and whether these changes would have an impact on patient outcomes. To our knowledge, this is the first study to directly compare the effect of different lengths of AI treatment. It used a new approach based on activity module scores covering different cancer pathways including immune-related features and advanced statistical analysis methods to identify differential gene expression according to response to AI. It provides evidence supporting that there is a similar impact on the changes of intrinsic subtype classification after short and longer-term treatment with AI, contrary to the conventional hypothesis. However, there is a greater impact on the transcriptional level of cancer-signalling pathways such as *MAPK* and *PI3K/AKT/mTOR* and immune response with a longer duration of neoadjuvant AI.

On the other hand, most studies performed in ER+/HER2+ BC have focused on resistance to anti-HER2 targeted therapy while mechanisms of resistance to ET are not well understood in this subgroup yet. We have investigated and characterised the predictive and prognostic value of molecular features at baseline in patients with ER+/HER2+ BC treated with peri-operative AI. To our knowledge this is the largest and first cohort investigating the response to pre-surgical AI in this BC subgroup, integrating comprehensive molecular profiles with short-term response to AI, and modelling these biological data with outcome. Beyond the expected association of the HER2-E subtype as the first biomarker of early resistance to AI, this study shows other interesting molecular associations, such as high expression of HRD and *TP53* mutant status surrogate signature predicting poor response to AI.

Furthermore, this thesis throws light on the role of tumour immunity on response to ET in ER+ early BC, in both HER2- and HER2+ populations. Recent data suggested that immune-enrichment and immune-checkpoint-related characteristics are upregulated in most luminal B tumours with poor response to ET as measured by higher Ki67 and poorer outcome (41,60). These studies have shown an association of high expression of several immune-related characteristics at baseline with early resistance to AI treatment measured by different Ki67 endpoints, including some features involved in immune-checkpoint component and immune-cell enrichment. Luminal tumours are usually known to be less immunogenic than other subtypes, but those with higher immunogenicity have been correlated with poorer prognosis or

response to ET therapy (60,128). Here we demonstrated that the enrichment of immune-related characteristics plays a key role in early resistance to AI in ER+ early BC, either HER2- or HER2+. Although the association of the high expression of these characteristics with survival in ER+/HER2- is not conclusive, our results suggest an association of high expression of these immune features with an intrinsic lower risk of recurrence in ER+/HER2+ early BC. These findings suggest a de-escalating approach in this setting and indicate the different roles of tumour immunity in HER2+ and HER2- disease.

Based on our results, the assessment of immune characteristics at baseline could be informative to detect mechanisms of resistance to AI.However, further investigations are still necessary to understand the utility of the analysis of immune-related characteristics in surgical samples of patients treated with long-term AI, and whether the enrichment of some immune-related signatures in AI sensitive BC tumours after longer AI treatment play a role in acquired resistance to ET. Further investigation on the use of immune-checkpoint component inhibition in this setting is also warranted. Taking together our results and those from the literature, a small subgroup of patients with ER+/HER2- and ER+/HER2- BCcould potentially benefit from immunotherapy-based treatments currently approved for a subset of triple-negative BC that is currently being tested in other BC subsets.

5.3 Impact and applicability of the results

Nowadays, the biggest question is how to select patients for escalation and de-escalation strategies. This work provides a rationale for the assessment of both baseline molecular characteristics and changes that happen under treatment to guide clinicians on the patient's treatment selection. We demonstrated that changes in the intrinsic subtype classification after just two weeks of neoadjuvant AI can serve as a biomarker of early resistance or efficacy to ET in ER+ early BC. Results also indicated the importance of BC molecular subtyping beyond the standard assays such as hormone receptor and HER2 to optimize treatment. For example, patients with ER+ HER2- and basal-like tumours by gene expression will obtain little or no benefit from neoadjuvant ET treatment.

The observed association of molecular changes (including changes in immune-checkpoint component and other immune-related pathways) with resistance to AI treatment and patient outcome, provides evidence for their evaluation as predictive and prognostic biomarkers in prospective clinical studies. Our results provide evidence that neoadjuvant AI therapy beyond

two weeks could be considered for high-risk ER+ BC, endocrine sensitive intrinsic subtypes. The molecular changes observed in ER+/HER2- BC might be crucial in the future to identify more vulnerable BC patients with a higher risk of relapse, who could benefit from additional treatments in combination with ET or the chance to include them in clinical trials.

Another main finding presented in this work is the identification of the HER2-E intrinsic subtype as the first predictive biomarker of resistance to AI in ER+/HER2+ BC with an additional higher risk of relapse. These findings highlight two aspects: firstly, the potential need for further anti-HER2 combinations to improve the outcome of patients with HER2-E ER+/HER2+ BC tumours, with additional anti-HER2 targeted therapy; secondly, the higher sensitivity to AI and good prognosis associated with luminal tumours, in particular with luminal A ER+/HER2+, provides rationale for the assessment of de-escalation approaches in this selected subgroup of patients (94). Recent "chemotherapy-free" neoadjuvant studies have shown that the combination of HER2-E subtype with high *ERBB2* mRNA expression may identify patients with HER2+ early BC with higher sensitivity to double HER2- blockade (94). However, only one-third of ER+/HER+ BC patients are HER2-E/ERBB2 high and their role as prognostic biomarkers is understudied.

Using consensus clustering, we have identified new molecular subgroups based on single gene expression at a higher risk of relapse beyond the HER2-E subtype. Noteworthy, in ER+/HER2- BC, tumours characterised by an enrichment of immune features presented the lowest risk of recurrence despite immune features associated with AI resistance and poor outcomes (41,60). Data showed that the higher expression of genes involved in immune cells and targetable immune-checkpoint components correlated with higher-risk tumours, such as luminal B tumours. In addition, several trials in HER2+ BC have shown that tumours with higher baseline tumour infiltrating lymphocytes and other immune features achieve higher pathological complete response rates and improved event-free survival (129). Our study confirms the association of higher expression of several immune features with resistance to AI but with a very low risk of recurrence in ER+/HER2+ BC. Due to its intrinsic good prognosis, BC patients belonging to this molecular subgroup, showing an enrichment of immune characteristics, could potentially benefit the most from a de-escalating approach using, for example, six months of trastuzumab plus AI without chemotherapy followed by five years of AI treatment alone (130). By contrast, tumours with higher levels of *ERBB2* and lower association of immunity have a significantly higher risk of relapse, indicating a need for intensified anti-HER2 treatment, such as dual-HER2 blockade plus chemotherapy (73,89). Other molecular subgroups characterised

by high ER signalling might also benefit from the addition of treatments that enhance the efficacy of ET, such as CDK4/6 inhibitors. Therefore, the new molecular subgroups identified in this work might be useful for the identification of candidates for escalating and de-escalating
strategies. Altogether, the combination of new biomarkers of early resistance to peri-operative AI with the intrinsic subtypes could be essential for individualizing therapy, including escalation and de-escalation treatment approaches, to improve treatment response and survival outcomes in early BC.

5.4. Future lines of research

To continue with the investigation iniciated in this doctoral project, we are currently developing a series of studies on the field:

5.4.1 Sub-study in extreme responder ER+/HER2+ breast cancer patients

Various studies using three different techniques are being carried out in a subset of patients with ER+/HER2+ disease from the POETIC study that are extraordinary responders based on the highest and lowest reductions of Ki67 after AI treatment (not all techniques overlap in the same samples):

5.4.1.1 Whole exome sequencing (WES) has been performed in **41 samples at baseline**. WES has been used from both selected baseline tumour samples and matched blood samples for technical validity and correlation. Our aim is to assess clonal heterogeneity and copy number alterations and correlate these molecular features with response to AI and survival.

5.4.1.2 RNA-sequencing in paired tumour samples (baseline and at surgery) from all extreme responders (n=41). We aim to assess copy number alterations, gene expression, and mutations and correlate them with response to AI and survival outcomes.

5.4.1.3 Immunohistology-phenotyping in 30 paired tumour samples (baseline and at surgery) from extreme responders (n=30). Our aim is to characterise and correlate immune cell type abundance based on four different immune markers (CD3, CD20, CD68, and FOXP3) stained on the same slide for ER+ and Ki67 and correlate these features with response and survival. We will associate the number of immune cells with Ki67 and the immune-related signatures assessed in the present work.

5.4.2 Gene expression changes in ER+/HER2+ breast cancer

A secondary study in the ER+/HER2+ BC patient cohort, from the POETIC study, focusing on gene expression changes that occur from baseline to surgery is being developed. We hypothesize that mechanisms of AI resistance and outcome are not only driven by baseline molecular alterations but also by changes that occur in response to therapy. We also hypothesize that gene expression changes are different between responders and non-responders and between those patients with a poorer and higher risk of relapse.

5.4.3 Deeper characterisation of baseline immune features in ER+/ HER2- early breast cancer

Additional whole transcriptome work in a much larger subset of the POETIC treatment arm is ongoing to better understand the diversity of intrinsic resistance mechanisms to AI treatment and to increase the power of our survival analyses. We are currently developing a more refined algorithm capturing the biological effect (conversion of subtypes).

5.4.4 Project on the investigation of genomic biomarkers of resistance to CDK4/6 inhibitors – Rio Hortega grant

I have been awarded with a Rio Hortega Grant to begin in 2022 with a project searching for genomic biomarkers of resistance to CDK4/6 inhibitors in advanced ER+ BC, in which we aim to investigate the role of different gene expression signatures in treatment efficacy with a focus on tumour immunity.

5.4.5 SEOM grant with a project on the characterisation of molecular differences between baseline and surgery seen in early breast cancer without treatment

I have been awarded with a grant to begin in 2022 funded by the Spanish Society of Oncology (SEOM). The project will consist in a study to further investigate and characterise the molecular differences observed in the control arm of the POETIC trial despite not having received any treatment – the artefactual effect – described in manuscript 1.

We aim to investigate the differences that occur between baseline and surgery samples in all clinical BC subgroups (ER+, triple negative (TN), and HER2+), and characterise and compare them.

We also aim to generate a hypothesis regarding the causes of these molecular changes (i.e., drugs administered before surgery, formalin/paraffination processes…) by exploring the differences in molecular characteristics across different types of sample collections and types of BC.

In this project, we will propose a method of handling this data artefact– i.e., adjustment. We aim to explore further differences/heterogeneity observed between baseline and surgery beyond gene expression (prognostic IHC factors). Based on these differences observed in our previous studies, we will also investigate whether it is more accurate to assess prognostic markers in the surgery excision instead of just in the baseline sample.

6. Conclusions

1. This work highlights the importance of the identification of strong biomarkers for patient selection for different treatments, including the newly emerging immune-therapeutic agents and the use of adequate endpoints, such as Ki67, as a surrogate marker of treatment response and survival benefit.

2. No single factor determines early resistance to ET, but diverse mechanisms including baseline characteristics and molecular changes that occur early on-treatment should be considered. Factors such as length of AI treatment and HER2 status also drive the differential response to AI.

3. In ER+/HER2- early BC, high expression of immune related genes are associated with early resistance to AI.

4. In ER+/HER2- early BC, longer AI treatment induces more and a larger magnitude of gene expression changes than two-week treatment only, involving some key BC pathways such as PI3K/mTOR/AKT. However, changes on intrinsic subtype are similar between short and longer AI treatment.

5. An upregulation of FOS and JUN related genes from baseline to surgery are observed in the control samples, despite no treatment being administered. These molecular differences might be caused by sample manipulation (i.e., time to formalin fixation) and not by AI treatment itself.

5. In ER+/HER2+ BC, the HER2-E subtype and high *ERBB2* gene expression, as well as proliferation-related pathways and high tumour immunity drive early resistance to AI.

6. Beyond HER2-E and proliferation pathways, the new molecular subgroups identified in early ER+/HER2+ BC enable the identification of patients at a higher risk of relapse beyond the HER2-E intrinsic subtypes.

7. The findings exposed in this work suggest a huge variety of mechanisms being involved in AI resistance at a gene expression level and differing across the different BC tumour type. It also highlights the important and differential role of immune-related features in ER+ BC in the response to AI, according to the different BC characteristics, for example, HER2 status.

9. The association of immune-related features with early resistance to AI suggests that high tumour immune-tolerance could be associated with early resistance to AI in both ER+/HER2- and ER+/HER2+ BC patients, however, the impact of these features on survival might differ amongst BC subtypes.

10. Overall, this project focused on the identification of strong molecular biomarkers that facilitate the identification and stratification of patients for the optimisation of different treatment options that may improve patient outcomes and their quality of life.

7. Bibliography

Bibliography included in each of the manuscripts can be found at the end of the correspondent article (sections 4.1, 4.2 and 5.4.1). The bibliography included in this section contains the manuscripts cited in the introduction and in the discussion.

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