Circulating biomarkers and resistance to endocrine therapy in metastatic breast cancers: correlative results from AZD9496 oral SERD Phase I trial

Authors: Costanza Paoletti[1*§], Gaia Schiavon[2*], Emily M. Dolce[1], Elizabeth P. Darga[1], T Hedley Carr[2], Joseph Geradts[2,3], Matthias Hoch[2], Teresa Klinowska[2], Justin Lindemann[2], Gayle Marshall[2], Shethah Morgan[2], Parul Patel[2], Vicky Rowlands[2], Nitharsan Sathiyayogan[2], Kimberly Aung[1], Erika Hamilton[4], Manish Patel[5], Anne Armstrong[6], Komal Jhaveri[7], Seock-Ah Im[8], Nadia Iqbal[9], Fouziah Butt[9], Caroline Dive[9], Elizabeth A. Harrington[2], J Carl Barrett[2], Richard Baird[10], Daniel F. Hayes[1]

* C. Paoletti and G. Schiavon contributed equally to this article

Affiliation:

[1] University of Michigan Rogel Cancer Center and the Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA;[2]IMED Biotech Unit, AstraZeneca, Cambridge, UK;[3]Current affiliation: City of Hope National Medical Center, Duarte, CA; [4]Sarah Cannon Research Institute/Tennessee Oncology, PLLC, Nashville, TN, USA;[5]Sarah Cannon Research Institute/Florida Cancer Specialists, Sarasota, FL, USA; [6]The Christie NHS Foundation Trust and the University of Manchester, UK; [7]Memorial Sloan Kettering Cancer Center, New York, NY, USA; [8]Seoul National University Hospital, Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea; [9]Cancer Research UK Manchester Institute, UK; [10]Cancer Research UK Cambridge Centre, UK.

[§]To whom correspondence should be addressed:

Costanza Paoletti, MD

7130 Cancer Center University of Michigan

1500 Medical Center Drive Ann Arbor, MI 48109 USA.

Telephone: +1-734-709-1598

Email: pcostanz@umich.edu

Word Count: Abstract: 253 words (as 05.02.2018); Text: 6,188

Figures: 5

Tables: 1

Supplementary Figures: 2

Supplementary Tables: 4

References: 37

Running Title: Circulating biomarkers in metastatic breast cancer patients enrolled in Phase I study of AZD9496.

Key Words: ER positive, metastatic breast cancer, oral selective estrogen receptor degrader, circulating tumor cells; circulating tumor DNA; *ESR1* mutations.

Abbreviations: AIs, aromatase inhibitors; BCL-2, B-cell lymphoma 2; ctDNA, circulating tumor DNA; CTC, circulating tumor cells; ddPCR, droplet digital PCR; ET(s), endocrine therapies; ER, estrogen receptor; *ESR1*, Estrogen Receptor alpha gene; FFPE, formalin-fixed paraffin embedded; HER2, human epidermal growth factor receptor type 2; H&E, hematoxylin and eosin; HR, Hormone Receptor; LBD, ligand binding domain; IHC, immunohistochemistry; MBC, metastatic breast cancer; PD, pharmacodynamics; PFS, progression free survival; PgR, Progesterone receptor; SERD, selective estrogen receptor degrader; WB, whole blood; WT, wild-type.

Additional information

Financial support

This study was sponsored by AstraZeneca.

Conflicts of interest disclosure

G.S., T.H.C., J.G., M.H., T.K., J.L., G.M., S.M., P.P., V.R., A.R., N.S., E.A.H., J.C.B. are, or were, employees of AstraZeneca at the time the study was conducted. CP received research funding from AstraZeneca and Pfizer. D.F.H. received support from Janssen Diagnostics, LLC, the commercial vendor of CellSearch®, for clinical and laboratory research. He is the inventor named on a patent held by the University of Michigan regarding CTC-Endocrine Therapy Index (CTC-ETI) and receives annual royalties from the commercial manufacturer of CellSearch® (previously Janssen LLC, now Menarini Silicon Biosystems, S.p.A.); He receives research funding from AstraZeneca, Puma, Pfizer, Merrimack, Eli Lilly. He is an advisor/board member of Pfizer, has stock options in InBiomotion and OncImmune, and received an honorarium. F.B. N.I. were funded via a translational research grant from AstraZeneca awarded to C.D. S-A. I. received research fund from AstraZeneca and worked as advisory board member for AstraZeneca, Hammi corp, Novartis, Pfizer, and Roche without compensation.

All other authors have no conflicts of interest to disclose (E.M.D., E.P.D., K.A., E.H., R.B., M.P., A.A., K.J.).

Presentation/Publications

Data from this work have been partially reported at San Antonio Breast Cancer Symposium 2016 meeting (poster # P6-12-03).

ABSTRACT (250 words) (as 05.02.18 word N= 253)

BACKGROUND: Common resistance mechanisms to endocrine therapy (ET) in estrogen receptor (ER) positive metastatic breast cancers include, among others, ER loss and acquired activating mutations in the ligand-binding domain of the ER gene (*ESR1*$_{LBD}$*m*). *ESR1* mutational mediated resistance may be overcome by selective ER degraders (SERDs). During the first-in-human study of oral SERD AZD9496, early changes in circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) were explored as potential non-invasive tools, alongside paired tumor biopsies, to assess pharmacodynamics and early efficacy.

METHODS: CTC were enumerated/phenotyped for ER and Ki67 using CellSearch® in serial blood draws. ctDNA was assessed for the most common *ESR1*$_{LBD}$*m* by droplet digital PCR (BioRad).

RESULTS: Before starting AZD9496, 11/43 (25%) patients had ≥5CTC/7.5mL whole blood (WB), none of whom underwent reduction to <5CTC/7.5mL WB on C1D15. 5/11 patients had baseline CTC-ER+, 2 of whom had CTC-ER+ reduction. CTC-Ki67 status did not change appreciably. Patients with ≥5CTC/7.5mL WB pre-treatment had worse progression-free survival (PFS) than patients with <5CTC (p=0.0003). Fourteen of 45 (31%) patients had *ESR1*$_{LBD}$*m*+ ctDNA at baseline, 5 of whom had ≥2 unique mutations. Baseline *ESR1*$_{LBD}$*m* status was not prognostic. Patients with persistently elevated CTC and/or *ESR1*$_{LBD}$*m*+ ctDNA at C1D15 had worse PFS than patients who did not (p=0.0007).

CONCLUSIONS: Elevated CTC at baseline was a strong prognostic factor in this cohort. Early on-treatment changes were observed in CTC-ER+ and *ESR1*$_{LBD}$*m*+ ctDNA, but not in overall

CTC number. Integrating multiple biomarkers in prospective trials may improve outcome prediction and ET resistance mechanisms' identification over a single biomarker.

Statement of translational relevance (120-150 words) (as 05.02.18 words= 149)

Determination of the molecular status of a cancer by evaluating circulating biomarkers has been designated as a 'liquid biopsy'. Liquid biopsies offer the possibility of serial non-invasive monitoring of the overall tumor burden as opposed to traditional research tissue biopsies, which assess a single site of disease. In patients with metastatic breast cancer (MBC), elevated circulating tumor cells (CTC) or *ESR1* mutations assessed in circulating tumor DNA (ctDNA) have been previously associated with worse prognosis. In this work, we used these circulating markers to assess pharmacodynamic (PD) changes in patients with ER positive/HER2 negative MBC participating in a first in human study of the oral selective estrogen receptor degrader (SERD), AZD9496. By integrating CTC and ctDNA, we were also able to explore the potential complementary utility of these circulating markers to detect more general mechanisms of resistance to ET non-invasively within a phase I trial of an oral SERD.

INTRODUCTION

Endocrine (or anti-estrogen) therapies (ETs) are the favored initial choice of treatment for most patients with estrogen receptor (ER) positive metastatic breast cancer (MBC). Commonly used ETs include the selective estrogen receptor modulator (SERM) tamoxifen, third-generation aromatase inhibitors (AIs) and the selective estrogen receptor degrader (SERD) fulvestrant (1).

Resistance to ET can occur *de novo* or be acquired during the course of therapy (2). Absence of ER expression is the single most potent mechanism of ET resistance (3). Other mechanisms of ET resistance include de-regulation of the ER pathway, for example by phosphorylation of factors downstream of ER, and/or activation of alternative pro-survival or proliferative pathways (4).

Several investigators have reported activating mutations in the ligand binding domain (LBD) of *ESR1*, the gene encoding ER. These mutations induce ligand-independent ER activity, leading to apparent resistance to AIs and other estrogen depletion strategies (5-8). The potential impact of $ESR1_{LBD}$ mutations ($ESR1_{LBD}m$) on clinical outcomes together with implications of sub-clonality of these mutations have not been fully elucidated. However, pre-clinical and independent exploratory clinical studies have suggested that patients with $ESR1_{LBD}m$ have shorter progression free survival (PFS) on subsequent AI therapy vs patients with $ESR1_{LBD}$ wild-type (WT) cancers and yet may retain relative (possibly dose-dependent) sensitivity to fulvestrant (5, 9-12). Increased dosing of fulvestrant might further improve its efficacy, but the bioavailability and pharmacokinetic (PK) limitations of this drug restrict the maximum feasible dose (MFD) to 500 mg once monthly intramuscular injection and steady state plasma concentrations are not reached until 3 to 6 months after first administration.

The development of an oral SERD with satisfactory bioavailability that could be given at higher relative doses than fulvestrant is highly desirable. AZD9496 (13) is one of several oral SERDs

that are being tested in clinical trials such as RAD1901 (NCT02338349) (14), GDC-9545 (NCT03332797); LSZ102 (NCT02734615); SAR439859 (NCT03284957).

In this first in human study, the PK of AZD9496 was characterized by a rapid absorption (median t_{max}: 1.55-3.00 h) and fast biphasic decline after reaching the maximum concentration with a mean terminal half-life (t½) of 1.4–5.7 h (13). Following multiple doses of AZD9496 the exposures of AZD9496 were consistently and dose-dependently lower than for a single AZD9496 dose, presumably due to auto-induction of cytochrome P450 (CYP) isoenzymes (13).

Ideally, pharmacodynamic (PD) markers should be incorporated into early therapeutic development trials to determine if the drug is reaching and affecting its drug target. However, serial metastatic tissue biopsies are invasive(15) and expensive. Conversely, 'liquid biopsies', such as assays for circulating tumor cells (CTC) or cell free tumor DNA (ctDNA), are relatively non-invasive and allow longitudinal assessment of circulating biomarkers (16). These markers may allow monitoring of PD changes, as well as identification of genetic and non-genetic determinants of drug response and development of drug resistance. We and others have reported the ability to accurately and reproducibly measure ER and Ki67 levels in CTC as well as $ESR1_{LBD}m$ from ctDNA ($ESR1_{LBD}m$ ctDNA) (11, 17-19).

Here, we report the results of correlative studies of CTC-phenotype, $ESR1_{LBD}m$ status in ctDNA, and PD analyses of paired biopsies in blood and tissue samples collected from patients with ER positive/HER2 negative MBC who participated in the first in human Phase I dose escalation trial of the oral SERD AZD9496 (13).

MATERIALS AND METHODS

Study Design and objectives

This correlative study was performed using specimens prospectively collected from patients who participated in a Phase I, open-label, multicenter trial (NCT02248090 in clinicaltrials.gov), designed to investigate the safety and tolerability and pharmacokinetics of increasing doses of the oral SERD AZD9496 (13). The main study was carried out in accordance with the principles of the International Conference on Harmonization guidelines for Good Clinical Practice, the Declaration of Helsinki, and all applicable laws. All subjects provided written informed consent approved by their local Institutional Review Board for collection of serial tissue biopsies and serial blood draws as part of their participation in the clinical trial.

The primary objectives of this correlative trial were to investigate: 1) the prognostic role of CTC enumeration and characterization prior to treatment and whether CTC enumeration, CTC-ER status and CTC-Ki67 status could be used as a PD biomarker of AZD9496 activity; 2) the prognostic role of baseline $ESR1_{LBD}$ mutational status in ctDNA and early changes in mutational levels during treatment as potential PD biomarker and/or for early prediction of response; 3) changes in ERα, progesterone receptor (PR), and Ki67 expression in optional paired tumor biopsies to assess the PD activity of AZD9496. Moreover, baseline variability of CTC and ctDNA was investigated.

Patient staging and follow-up

Details of eligibility, accrual, and conduct of the clinical trial have been reported in Hamilton et al (13). Briefly, eligibility was limited to patients with ER positive/HER2 negative metastatic or

locoregionally recurrent disease, not amenable to treatment with curative intent, who had progressed after ≥6 months of ET for ER positive breast cancer (before protocol amendment August 21 2015 must have spent ≥6 months on a line of endocrine therapy in the advanced setting). Pre- or peri-menopausal women must have started luteinizing hormone-releasing hormone (LHRH) agonist treatment at least 4 weeks before study treatment, and must have continued this treatment throughout the study. Sex hormone containing drugs such as hormone-replacement therapy (HRT), dehydroepiandrosterone (DHEA), other androgens (e.g., oxandrolone), selective estrogen receptor modulators (SERMs e.g. raloxifene), megestrol acetate were not permitted during the study. Of note, a wash out from any cytotoxic chemotherapy, investigational agents or other anti-cancer drugs for the treatment of advanced breast cancer from a previous treatment regimen or clinical study within 14 days of the first dose of study treatment was required. Although a fulvestrant-specific 'wash-out' period was not defined, for the purpose of CTC analysis in this study we defined fulvestrant 'wash-out' as 120 days (~3.3 half-lives).

Assessment of tumor response by RECIST 1.1 was performed every 8 weeks after the start of treatment for 24 weeks, and thereafter every 12 weeks (± 1 week) until objective disease progression, as defined by RECIST 1.1, even if a patient discontinued treatment prior to progression. A 'rolling 6' design was employed, in which each cohort at least three and up to six patients received AZD9496 at escalating dose. Dosing began at 20 mg once-daily up to 600 mg twice-daily, which was regarded as the maximum feasible dose on the basis of the number of tablets required for each dose.

Collection of CTC, plasma for ctDNA analysis, and paired biopsies

Overall timeline of sample collection is shown in Supplementary Table 1. Blood samples were drawn twice prior to starting therapy: a screen time point (-28 to -1 days prior to treatment initiation) and a second sample on the day of treatment initiation (C1D1). The duration of a cycle was 4 weeks for the first 6 cycles and 6 weeks thereafter. In one patient, Cycle 1, Day 2 (C1D2) sample was used as a surrogate sample for $ESR1_{LBD}m$ assessment due to missed screening/C1D1 samples. Collectively, these specimens are designated as 'baseline' but results from individual timepoints are reported separately as stated throughout this report.

CTC

For CTC evaluation, whole blood (WB) was collected into CellSave preservative tubes (Menarini Silicon Biosystems, Inc., San Diego) during a screening window [-28 to -1 days prior to C1D1], and subsequently on C1D1, Cycle 1, Day 15 (C1D15), and at treatment discontinuation. Three CellSave tubes were pooled and divided into 3 aliquots for CTC assessment (one to test CTC-ER status, one to test CTC-Ki67 status and one to perform RNA extraction from CTC [data not reported], respectively). Blood samples were maintained and shipped at room temperature to one of two central laboratories (University of Michigan or Cancer Research UK Manchester Institute) where they were processed within a maximum of 96 hours after blood draw.

ctDNA

For ctDNA analysis, blood was drawn into 10 mL Streck Cell-Free DNA BCT® (STRECK, INC, La Vista, NE) tubes at screening, C1D1, C1D2, C1D15, C3D1, Day 1 of every alternate cycle after C3D1, and at treatment discontinuation. Screening or C1D1 samples were used for baseline

determination of *ESR1*$_{LBD}$*m* status (mutation detected at any of the 2 timepoints), but the C1D2 sample was used as a surrogate in case of missing screening/C1D1 samples. For determination of changes of *ESR1*$_{LBD}$*m* levels at C1D15 versus baseline, C1D1 (when available) was used as baseline levels, otherwise screening was used. One tube of WB per timepoint was collected for ctDNA and shipped to a central lab (Covance) at ambient temperature range (6-37°C) for further processing.

Tissue

Consent for paired research tumor biopsies was optional for patients enrolled in this study. The pre-treatment samples were collected during screening or at disease progression on the therapy administered prior to study. Sites were requested to obtain the on-treatment sample on C2D1 +/- 7 days, but the sample could be taken outside this time window if agreed with the sponsor (before protocol amendment August 21, 2015, the on-treatment sample was scheduled at C1D15, however anytime between C1D7 and C1D28 was acceptable). Sites were instructed to collect the on-treatment biopsy between 2-12 hours after the latest dose of AZD9496. Patients could also consent to a tumor biopsy at disease progression, if clinically feasible.

Sample processing and assay methods

Blinding

Laboratory personnel were not blinded to trial subject number, dose group and timepoint of CTC and ctDNA samples. For the paired tumor tissue samples, the laboratory personnel and the pathologist were blinded to the timepoint, but not to trial subject number and dose group. Laboratory personnel for all samples types were blinded to the clinical outcomes.

CTC

Enrichment, enumeration, and characterization

Blood specimens were analyzed for CTC enumeration and semi-quantitative analysis of expression of ER and Ki67 using the CXC CellSearch® Kit and CellSearch® system (Menarini Silicon Biosystems, Inc., San Diego), as previously reported (17, 19). CTC were considered elevated if the aliquot contained ≥5 CTC/7.5 mL of WB according to previous literature (20). If an aliquot had elevated CTC, CTC-staining was expressed on an arbitrary scale of 0-3+, as described previously (19). For each marker, 0 and 1+ was considered negative and 2-3+ was considered positive (19). CTC enumeration, CTC-ER status and CTC-Ki67 status were determined by two independent reviewers (E.M.D.; E.P.D.; K.A.; C.P.; F.B.; N.I.). Discordant results were reconciled by joint readings. Quality control between the two sites was performed every 3-4 weeks to ensure reading methods were concordant. CTC response was defined as a reduction to <5 CTC/7.5 mL WB at C1D15 as previously described (21, 22).

CtDNA and tissue: molecular analyses

ddPCR analyses: tumor tissue DNA and circulating free DNA (cfDNA) extraction

DNA was extracted from FFPE sections using the QIAamp DNA FFPE Tissue Kit (QIAGEN©, Düsseldorf, Germany) according to the manufacturer's protocol, buffer ATE was applied to each column and incubated for 5 minutes at room temperature prior to elution into Eppendorf LoBind Microcentrifuge tubes. Blood samples were processed on day of arrival in two steps. In the first step, WB was centrifuged at approximately 2,000G for 10 mins using a pre-chilled centrifuge set to 4°C, and plasma was separated by pipette and transferred to a 15 mL Falcon tube. In the second step, plasma was centrifuged again at approximately 12,000G or higher for 10 mins using a pre-chilled centrifuge set to 4°C, separated by pipette, aliquoted and frozen at minus 80°C until

extraction. ctDNA was extracted from 2 mL plasma using the QIAamp Circulating Nucleic Acid Kit with the QIAvac 24 Plus vacuum manifold (QIAGEN©, Düsseldorf, Germany) as previously described (23). Samples were stored at –20°C prior to ddPCR analysis.

ESR1 mutations analyses by ddPCR

Droplet digital PCR (ddPCR) was performed using the QX200 AutoDG Droplet Digital PCR System (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. Custom assays were designed by IDT (Coralville, IA, USA), incorporating locked nucleic acid (LNA) bases into each probe to increase discrimination. Probes were from IDT and primers (SePOP desalted) from Eurogentec (Liège, Belgium) (Supplementary Table 2). The D538G, E380Q, Y537C, Y537N, and Y537S *ESR1* assays were run as singleplex assays. Multiplexing three assays (V534E, L536Q and L536R) was achieved by modifying both probe concentrations and the concentration of FAM and HEX fluorescent labels. In any cases where the *ESR1* multiplex result was not conclusive, ddPCR was repeated with relevant singleplex assays. If the baseline sample was negative for E380Q and the multiplex assay, no samples of subsequent timepoints were tested due to limited sample material. For the remainder of the assays (D538G, Y537C, Y537N and Y537S), every time point was tested regardless of status at baseline. Each 20 µL ddPCR reaction contained 5 µL of cfDNA or formalin-fixed paraffin embedded (FFPE)-extracted DNA. Positive and negative controls were run in triplicate or quadruplicate on each plate. 150 bp gBlocks (IDT) or sheared plasmids (AZ Bioscience) containing the mutation of interest were used as positive controls. Human genomic DNA (male) was used as wild-type control (Promega, Madison, WI, USA). Appropriate elution buffer and water served as negative controls. Thresholds were manually set for each sample using acceptance criteria defined during the optimization of each assay. QuantaSoft software (version 1.7.4; BioRad, Hercules, CA USA) was used to assign

positive/negative droplets and convert counts into mutant copies/mL (23). Mutation levels were expressed as mutant DNA copies/mL plasma. '*ESR1*$_{LBD}$*m+*' ctDNA indicates the presence of at least one *ESR1*$_{LBD}$*m* (i.e. definite positive by the pre-defined acceptance criteria) in the ctDNA (at baseline or at other time-point, as specified throughout the report). 'Borderline' cases (i.e. <3 positive droplets, which is the pre-defined threshold of positivity) were considered "not detected" (or "negative") unless otherwise specified. A 'ctDNA response' was defined as ≥50% decline in mutational levels of the dominant mutation at C1D15 versus baseline.

Tissue

Immunohistochemistry analyses

Tumor biopsies were processed into FFPE blocks prior to immunohistochemistry (IHC) analysis at the participants' institutions. IHC analysis was performed at AstraZeneca for semi-quantitative ER, progesterone receptor (PR), and Ki67 (24). See Supplementary Table 3 for antibody details. IHC staining was performed using a LabVision™ Autostainer (Thermo Fisher Scientific, Inc, Massachusetts, USA), following optimal pretreatment using a HistoS-3 microwave pressure retrieval system. Antibody-specific staining for ER and PR was scored manually by a board certified pathologist at AstraZeneca (J.G.) and/or at a contracted external GCP accredited laboratory (Source BioScience, Nottingham, United Kingdom). For both hormone receptors, the percentage of tumor cells was assessed with an intensity of negative, weak, moderate, and strong staining (0, negative; 1, weak; 2, moderate; 3, strong). Results for ER and PgR were recorded as an H score (sum of [1 x percentage weak] + [2 x percentage moderate] + [3 x percentage strong]) to a maximum of 300. Ki67 index was expressed as average % positive cells (count 300 cells).

Pharmacokinetics

The exploratory PK/PD analysis involved visual evaluation of changes in CTC count and CTC-ER status, CTC-Ki67 status, and *ESR1*$_{LBD}$*m* ctDNA levels over treatment time in relation to AZD9496 dose and PK parameters (AUC, C_{max}), assessed by standard non-compartmental methods. Absolute changes in biomarker at C1D15 (compared with baseline) were used to perform the assessment. The CA15-3 time-series biomarker data (assessed by local laboratories) was assessed through visual evaluation and model based assessment using mixed effects modelling in R software v3.3.1. In the initial modelling step, linear and nonlinear models were investigated within the R software package 'nlme' to describe the time course of the biomarker data. Subsequently, model-based evaluations were performed to test the statistical significance of AZD9496 dose or PK endpoints as explanatory covariates for the rate of change of CA15-3 over the time course of the study. Model selection was based on the likelihood ratio test (p=0.01). The PK/PD relationship analysis with tissue markers was not performed.

Statistical analysis

All statistical analyses were performed with GraphPad Prism version 7.04 (GraphPad Software, La Jolla, CA, USA) or Microsoft Excel. Association of CTC levels and ctDNA with PFS was assessed using logrank test. Progression free survival was defined as the time from start of treatment until objective disease progression as defined by RECIST 1.1 or death (by any cause in the absence of progression) regardless of whether the patient withdrew from study treatment or received another anti-cancer therapy prior to progression. Patients who had not progressed or died at the end of the study were censored at the time of their last evaluable RECIST assessment. Unless stated otherwise, p values were two tailed and considered significant if $p<0.05$. Treatment

was ongoing in six patients (13.3%) at the data cut-off of January 31, 2017. The study is reported according to the REMARK guidelines (25).

RESULTS

Patients

All 45 patients enrolled in the Phase I trial were also enrolled in this correlative study. Each subject had at least one blood sample collected at any time point for CTC and ctDNA assessment (Figure 1). In particular, 43 patients had CTC collected at screening and/or C1D1, whereas 2 patients only had CTC collected at time of study discontinuation. In addition, 44 patients had ctDNA collected at screening and/or C1D1. For the patient, who did not have ctDNA collected at screening and C1D1, the sample from C1D2 was considered as baseline. Patient and disease characteristics were previously described (13). Data cut-off was on 31 January 2017.

CTC enumeration and $\mathit{ESR1}_{LBD}\mathit{m}+$ ctDNA at baseline

Of the 43 patients for whom CTC were collected at screening and/or C1D1, 23 (53.5%) and 11 (25%) had ≥1 and ≥5 CTC/7.5 mL WB at one or the other, or both, baseline blood draws (screening or C1D1), respectively (Table 1; Figure 2A). Fourteen of 45 (31%) patients had $\mathit{ESR1}_{LBD}\mathit{m}+$ ctDNA at baseline (screening or C1D1 sample for 44 patients and C1D2 for 1 patient). Five of these 14 patients (36%) had more than one $\mathit{ESR1}_{LBD}\mathit{m}$ identified in the ctDNA in the same blood draw (Figure 2B), all of which featured a D538G clone (5/5). For this report, the 'dominant mutation' was considered the mutation with the highest mutational levels at baseline. When considering all $\mathit{ESR1}_{LBD}\mathit{m}$ detected at baseline, the most common mutations were D538G (6/14), followed by Y537S (5/14) and Y537N (5/14). Overall, of the 43 patients who had both CTC and ctDNA assessed, 23 (53.5%) patients had either elevated CTC numbers or $\mathit{ESR1}_{LBD}\mathit{m}+$ ctDNA. In particular, 11 patients (26%) had elevated CTC (≥5 CTC/7.5 mL WB), and 12 (28%) had $\mathit{ESR1}_{LBD}\mathit{m}+$ ctDNA at baseline (Table 1). Only four (9%) patients had both elevated CTC and $\mathit{ESR1}_{LBD}\mathit{m}+$ ctDNA. Eight of the 32 (25%) patients with <5 CTC/7.5 mL WB had elevated

ESR1$_{LBD}$m+ ctDNA, whereas 7/31 (23%) patients with '*ESR1$_{LBD}$m* not detected' (*ESR*1$_{LBD}$*m*– ctDNA) had elevated CTC levels (Table 1).

CTC count, ER positivity on CTC [(CTC-ER positive), % ER positive CTC], and *ESR1* mutational levels tended to remain stable in absence of treatment (screening vs C1D1, with a median interval period of 9 days, range 3-21) (paired T test) (Figure 2C, D). One patient had a dramatic reduction and one patient had a substantial increase in *ESR1$_{LBD}$m* ctDNA levels (Figure 2D). Variation in CTC number and/or ER positivity was observed in three cases. As illustrated in Figure 3A, patient #36 had 12.5% of CTC that were ER positive on 16 CTC at screening, which decreased to 0% on 13 CTC at C1D1. Similarly, patient #6 had 7.7% ER positivity on 13 CTC at screening which decreased to 0% on 12 CTC at C1D1. Likewise, patient #27 had 21.2% CTC-ER positive on 74 CTC at screening which reduced to 4.3% on 208 CTC at C1D1.

Early changes in CTC levels, CTC-ER status, and *ESR1* mutational levels in ctDNA during treatment with AZD9496

CTC

None of the 11 patients with ≥5 CTC/7.5 mL WB at baseline experienced a CTC response defined as a reduction to <5 CTC/7.5 mL WB (Figure 3A). Of the 10 patients for whom CTC-ER analysis was available at baseline, five had CTC with some degree of ER positivity (2+ and 3+) prior to treatment (Figure 3A). In two of these five patients (patients #30 and #27) treated at the dose of 150 mg twice daily (BID) and 250 mg BID, respectively, CTC-ER positivity was reduced in the subsequent sample (C1D15) compared to baseline (Figure 3A).

CTC-ER status was negative in the remaining five patients with ≥5 CTC/7.5 mL WB prior to treatment (Figure 3A). Samples from two patients (patients #44 and #26) were drawn

within the ‘wash-out’ period of fulvestrant and therefore, the CTC may have exhibited downregulated-negative ER. The remaining three cases (patients #35, #13, and #17), who were outside the fulvestrant ‘wash-out’ period, had CTC-ER negative at all time points with an increase in CTC number on treatment. All but two patients with ≥1 CTC/7.5 mL WB, from whom blood specimens were drawn during the wash-out period of fulvestrant, had CTC-ER negative, except patient #15 and #26, who had one and two CTC-ER positive, respectively (Supplementary Table 4). The mean Ki67 positivity on CTC (CTC-Ki67 positivity) was 11.7% (range 0-21%) in all the patients at baseline and did not change appreciably over time [mean at C1D15 was 15.2% (range 0-45%)] (Figure 3B). There was no apparent correlation between changes in CTC-Ki67 status and CTC-ER status.

ESR1 mutational status in ctDNA

$ESR1_{LBD}$ mutations exhibit variable dynamics during AZD9496 therapy (Figure 4A). A decline of $ESR1_{LBD}m$ mutational levels (dominant mutation) of ≥50% at C1D15 vs baseline was observed in 8 of the 14 (57%) patients with detectable $ESR1_{LBD}m$ at baseline (Figure 4B), without apparent dose-response relationship (Figure 4B). In 3 out of these 8 patients, the $ESR1_{LBD}m$+ ctDNA became $ESR1_{LBD}m$- ctDNA at C1D15. In patient #1, $ESR1_{LBD}m$+ ctDNA was detected again at C3D1 when she discontinued treatment, whereas, in patients #29 and #21, clearance was durable until the last on-treatment sample tested (C7D1 and C9D1), respectively. Within the blood specimens from the five patients with multiple *ESR1* mutations, serial levels demonstrated both convergent and divergent evolution of the different sub-clones during treatment with AZD9496 (Figure 4C).

Three patients (patients #31, #37, #12) who had *ESR1*$_{LBD}$*m–* ctDNA at baseline had *ESR1*$_{LBD}$*m+* ctDNA at a later time point. The immediate prior therapy for these 3 patients was everolimus plus exemestane (patient #31; wash-out 35 days), fulvestrant +/- BYL719 (patient #37; wash-out 35 days), and letrozole plus palbociclib (patient #12; wash-out 20 days). Overall, when considering any given timepoint, 17/45 (38%) patients had *ESR1*$_{LBD}$*m+* ctDNA (Figure 4A).

Clinical Outcomes associated with CTC and ctDNA analyses

The median duration on treatment with AZD9496 was 64 days (range 22 to 643 days, across the wide range of doses examined) (13) (Supplementary Figure 1) and 6 patients (13.3%) were still on treatment up to the data cut-off of January 31, 2017. Of the 39 patients who discontinued treatment, 38 discontinued due to progressive disease or death, one due to an adverse event.

The prognostic effect of the baseline circulating biomarkers was investigated. Consistent with prior reports (21, 22, 26), patients with ≥5 CTC/7.5 mL WB at baseline had significantly worse PFS than those with <5 CTC/7.5 mL WB (median PFS of 54 vs 164 days; logrank p=0.0003) (Figure 5A). In contrast, *ESR1*$_{LBD}$ mutational status at baseline was not predictive of outcome on AZD9496 in all patients (Figure 5B) and when considering only the subgroup of patients with <5 CTC/7.5 mL WB (Figure 5C).

As noted, none of the 11 patients with elevated CTC at baseline experienced a decline to <5 CTC/7.5 mL WB at C1D15. Using relative changes in CTC enumeration from baseline to C1D15 (+/-50% from baseline), numerically similar median PFS (mPFS) was seen between patients who had an increase (changes ≥50%) in CTC number (N=5; mPFS of 45 days), stable (changes <50%) CTC number (N= 3; mPFS of 56 days), or a decrease (changes ≥50%) in CTC

number (N=3; mPFS of 54 days). Six of the seven patients with available CTC enumeration at treatment discontinuation had a ≥50% increase in CTC at C1D15 (range from 195% to 7,538%). By protocol, if a patient had <5 CTC/7.5 mL WB at screening/C1D1, subsequent blood draws were not collected for CTC analysis and therefore, we could not address whether these patients had developed ≥5 CTC/7.5 mL WB on C1D15.

Patients with $ESR1_{LBD}m$- at baseline or decline of ≥50% at C1D15, had a trend towards longer PFS compared to those with residual $ESR1_{LBD}m$+ ctDNA at C1D15, when the analysis included all patients (N=38; mPFS=111 days vs. N=6; mPFS=54 days; logrank p=0.055; Figure 5D). The subgroup of patients with <5 CTC/7.5 mL WB showed a similar trend (N=28 mPFS=166 days vs N=3; mPFS= 109 days), but numbers were too small to draw meaningful statistical conclusion (Figure 5E). At C1D15, patients with either persistently elevated CTC or $ESR1_{LBD}m$+ ctDNA had a worse PFS (N=15; mPFS= 55 days) compared to patients with neither biomarker elevated (N=30; mPFS= 166 days) (logrank p=0.0007) (Figure 5F).

Of note, one patient (#43) achieved partial and durable response on AZD9496 accompanied by sustained reduction of the tumor marker CA15-3 (13). She had $ESR1_{LBD}m$– *ctDNA* at baseline and during treatment, and 0 CTC/7.5 mL WB at baseline (Supplementary Fig 1). The patient was treated with 250 mg BID AZD9496 and stopped treatment due to progressive disease after approximately 26.4 months on therapy.

Tissue markers

Paired biopsies from 5 patients were considered evaluable for PD analysis (Figure 1; Supplementary Figure 2). Timing of the on-treatment samples and number of hours post-last dose of AZD9496 are shown in Supplementary Figure 2A. These 5 patients were treated at various AZD9496 doses ranging from 40 to 400 mg BID.

We identified significant limitations (i.e. sample characteristics and/or time point of collection) associated with the paired tumor samples collected from metastatic lesions in this trial. A reduction in ER H score was detected in paired biopsies from two subjects (31% and 47%), of whom the first one was accompanied by reduction in Ki67 (52%). Details are described in Supplementary Figure 2B.

Overall, variable and inconsistent changes were seen in biomarker expression (Supplementary Figure 2B) without any clear dose-response. Taken together, this limited sample set did not allow a robust assessment of the PD activity of AZD9496, speaking to the importance of circulating tumor biomarker evaluation.

PK/PD relationship analysis

We examined whether there was a correlation between doses of AZD9496 and the PK parameters (AUC, C_{max}) for AZD9496 and the changes over time for CTC, *ESR1*$_{LBD}$*m* ctDNA levels, and CA15-3. No graphical correlation was found between the dose nor the PK parameters of AZD9496 and CTC number or status, *ESR1*$_{LBD}$*m* ctDNA levels, or CA15-3 at any time point studied. With respect to model based assessments of CA15-3, no significant relationships between dose or PK parameters (AUC, C_{max}) and change in CA15-3 (linear slope parameter) could be detected over the course of the study at any time point.

DISCUSSION

In this correlative study of a prospective first-in-patient Phase I trial, we have investigated the PD activity of an oral SERD, AZD9496, through blood-borne and tumor tissue biomarkers. We have also explored potential predictive markers of outcome on AZD9496 in ER positive MBC patients.

As expected, pre-treatment CTC levels (≥5 CTC/7.5 mL WB) were a strong prognostic marker in this cohort of MBC, as seen in prior studies (21, 22). Twenty-five percent of patients had ≥5 CTC/7.5 mL WB at baseline, but none had a reduction of CTC numbers to <5 CTC/7.5 mL WB on treatment (C1D15). The lack of reduction suggests that, at least in this patient group, the agent failed to induce an early 'CTC response', defined as a reduction to <5 CTC/7.5 mL WB. It remains unclear whether this is due to the insufficient target inhibition of the agent or due to the C1D15 timepoint's being too early to assess changes in CTC during ET monotherapy. Additional data regarding CTC numbers and ET at different timepoints are under investigation within a prospective Phase 2 clinical trial (COMETI P2 NCT01701050) (27) .

Of the 10 patients for whom CTC-ER status was available at baseline, only five had pre-treatment CTC-ER positivity, while the others with CTC-ER negative were not informative for PD. In two of the five patients there was a reduction of CTC-ER positivity at C1D15 versus baseline, although it is difficult to discern if this observation was due to a true PD effect or due to analytic variability, since we did not have an untreated patient group as control.

The other five patients with ≥5 CTC/7.5 mL WB at baseline had CTC-ER negative (Figure 3A). We speculate that two of these cases may have represented 'downregulated' CTC-ER, since they had received recent fulvestrant treatment with possible impact on ER expression in CTC due to its mechanism of action. Three cases, which were not recently treated with or

were beyond the fulvestrant 'wash-out' period, had CTC-ER negative at all time points, and all three experienced an increase in CTC number during treatment with AZD9496. We speculate that in these patients ER negative clones may have emerged, and therefore endocrine independent metastases, even though their primary cancers were ER positive. We have previously reported data regarding CTC enumeration and CTC characterization of ER and BCL2 in patients progressing on fulvestrant and have shown potential ability for CTC to provide insights into the potential mechanisms of resistance to this drug (17).

Because serial tissue Ki67 reduction is associated with benefit from neoadjuvant ET (28), we explored whether serial Ki67 values on CTC would reflect response to AZD9496. Proliferative status of CTC using Ki67 expression has been investigated in several diseases including breast cancer (19, 29-32). However, in this study, the percentage of CTC-Ki67 positivity within each patient at baseline was relatively low (<21%) and did not change substantially in any patient. Although we only collected 5 paired biopsies, of interest, a paired liver biopsy was obtained from patient #30 at roughly the same time as CTC collection, and tissue Ki67 decreased by ~50% in a set of paired baseline-follow-up biopsies. This observation suggests that CTC-Ki67 status may not reflect tissue Ki67. We speculate, but would require further study to demonstrate, that CTC may stop proliferating when they are in circulation.

$ESR1_{LBD}m+$ ctDNA was detected in 31% of patients at baseline, and about a third of these patients had at least two different *$ESR1_{LBD}$* mutations. Ninety percent of all patients had received prior AI therapy, consistent with the evidence that profound estrogen depletion may select for *ESR1m* (9). We observed that neither CTC enumeration, CTC-ER status, nor *$ESR1_{LBD}$* mutational levels changed significantly between screening and treatment initiation (C1D1), confirming the analytic validity of serial assays for these markers in the absence of intervening

therapy in this disease and setting. To our knowledge this is the first study reporting 'double baseline' results on ctDNA mutational levels and CTC.

The presence or absence of *ESR1*$_{LBD}$*m*+ ctDNA at baseline was not associated with outcome in this study. Serial tracking of *ESR1*$_{LBD}$*m* showed variable patterns of dynamics during AZD9496 therapy. A 'ctDNA response' (decline in *ESR1*$_{LBD}$*m* levels of ≥50% at C1D15 compared to baseline) was observed in 57% of patients (8 of 14) who harboured *ESR1*$_{LBD}$*m* at baseline, of whom 3 had a complete and durable clearance of the dominant mutation. Patients with lack of ctDNA response had shorter time of study versus patients with ctDNA response or *ESR1*$_{LBD}$*m*– ctDNA. There was no apparent relationship between high mutational levels at baseline and later ctDNA response. In patients with "ctDNA response" at C1D15, *ESR1*$_{LBD}$*m* levels increased at subsequent timepoint in some cases, while some others maintained suppressed *ESR1*$_{LBD}$*m* levels throughout treatment, even at the time of progression. While early changes of *ESR1* mutational levels in ctDNA might be a valid tool to assess PD of a SERD, tracking *ESR1*$_{LBD}$*m*+ ctDNA levels to anticipate relapse could be of limited value in some cases, possibly due to the fact that *ESR1*$_{LBD}$*m* are frequently subclonal (i.e. they represent a subclone in a substantial fraction of patients, poorly representing the cancer overall). This observation is supported by recent findings by O'Leary et al. who investigated the dynamics of *PIK3CAm* and *ESR1*$_{LBD}$*m* during treatment with fulvestrant plus palbociclib/placebo and described that *ESR1*$_{LBD}$*m* can be lost even when patients are experiencing disease progression and may not determine clinical outcome (33). Taken together, these results indicate that genetic heterogeneity might be itself another mechanism of resistance (34). Among the *ESR1*$_{LBD}$*m*+ cases, five had multiple *ESR1*$_{LBD}$*m* in ctDNA and mutation tracking revealed that both convergent and divergent evolution of different sub-clones is possible. It is uncertain whether this is dependent on varying

affinity of AZD9496 to the different mutant receptors or other factors associated with clonal abundance of the concurrent $ESR1_{LBD}$ mutations. Of note, we do not report here on the more comprehensive assessment of tumor genetics. However, investigating the dynamics of tumor mutational burden or truncal mutations during treatment with AZD9496 and their correlation with clinical outcomes warrants further exploration. Additional genomic analyses of DNA derived from CTC would also help to clarify if these mutations are polyclonal or if they are present in the same DNA sequence. Prior studies suggest that both circumstances are possible (11, 35, 36).

When we correlated the blood-borne biomarkers with clinical outcome, we found that the presence of elevated CTC (≥5 CTC/7.5 mL WB) at baseline was a strong prognostic factor in this cohort (logrank p=0.0003). In contrast, $ESR1_{LBD}m$ status at baseline was not prognostic in all the patients or in those with <5 CTC/7.5 mL WB. Interestingly, patients with either persistently elevated CTC number or $ESR1_{LBD}m+$ ctDNA at C1D15 compared to baseline had a worse PFS than patients with neither biomarker elevated (logrank p=0.0007). Although the small sample size limits the opportunity for a meaningful multivariate analysis, there was little overlap in the patient populations with respect to persistent elevations of CTC number and $ESR1_{LBD}m+$ at C1D15. Only 2 of 15 patients in this analysis had both persistently elevated CTC number and $ESR1_{LBD}m$ status, and persistent elevations of both factors appeared to be independently prognostic. Of note, none of the patients with elevated CTC at baseline converted to non-elevated and residual *ESR1m* at C1D15 was prognostic (Figure 5D).

Limitations of our exploratory study include the small sample size, the fact that patients were treated at different doses of AZD9496 and without a comparator arm, and the lack of conclusive data on the pharmacodynamically and clinically effective dose of AZD9496.

Additional limitations are that we only assessed the most common *ESR1* LBD mutations and we tested relatively limited volumes of plasma. None of the PD markers early changes correlated with the AZD9496 dose or PK parameters. It remains uncertain whether C1D15 is a too early timepoint to assess PD changes during ET monotherapy. Only 11% of the patients (5/45) provided evaluable paired biopsies, preventing any conclusive data on the PD effect of AZD9496. These results illustrate the challenge of collecting serial tissue specimens from advanced cancer patients to enable a thorough assessment of proof of mechanism in early phase trials.

Despite these limitations, >95% of patients enrolled in this Phase I trial provided blood samples for circulating biomarkers, whereas collection of tissue biopsies was limited to only few participants, emphasizing one of the potential advantages of liquid biopsies. In addition, we observed several potential general mechanisms of resistance to ET (37). The first potential resistance mechanism observed was the loss of ER expression on CTC in ER positive MBC. In particular, of the 10 patients who had ≥5 CTC/7.5 mL WB at baseline, three patients had CTC-ER negativity after excluding the 2 patients who were within the fulvestrant ‘wash-out’ period. A second potential mechanism was the identification of a mutation of the *ESR1* gene either by ctDNA at baseline or in tissue, with five patients harboring >1 $ESR1_{LBD}m+$ ctDNA. We could also hypothesize that additional mechanisms may be present when CTC-ER positivity is still present along with $ESR1_{LBD}m–$ ctDNA, but these mechanisms were not assessed in this current trial. It should be noted that, although one patient treated with 250 mg BID AZD9496 achieved a confirmed and durable partial response (13), there is still uncertainty on the dose-response relationship for AZD9496. Moreover, the impact of the PK characteristics of AZD9496 (a relatively short half-life [alpha half-life of 1-2 h] and a dose-dependent decrease in exposure

upon multiple doses, presumably due to CYP auto-induction) on the PD activity is unclear, limiting our ability to be conclusive on the correlation between these biomarker findings and benefit from AZD9496. A pre-surgical window of opportunity study (NCT03236974) is currently ongoing and is aimed to compare the PD effects of AZD9496 with those of fulvestrant in women with ER positive early breast cancer awaiting surgery with curative intent, and to characterize the PK/PD relationship. In summary, possible mechanisms of ET resistance may differ for patients with CTC-ER positive vs. CTC-ER negative. For the ER positive component, failure to downregulate ER may be due to $ESR1_{LBD}$ mutations or because they received a non-efficacious dose of AZD9496. In the CTC-ER negative component, it may be due the emergence of ER negative/endocrine independent clones, or perhaps due to a downregulation of CTC-ER positive accompanied by a concomitant upregulation of other secondary pathways.

Taken together, these results suggest that complementary and comprehensive characterization of CTC and ctDNA may be required for noninvasive early prediction of outcome during ET. Once validated, these tools have the potential to be used in prospective trials to select optimal therapy upfront, or after short exposure to treatment. The latter could involve switching to different ET agent, continuing the same ET and adding other targeted treatment such as mTOR or CDK4/6 inhibitors, or switching to non-endocrine treatment.

ACKNOWLEDGEMENTS

We would like to thank all the investigators and site staff, with special thanks to the patients and families. We also wish to thank the following contributors to this study: Stuart Findlay, Graham Fisher, Catherine Geh, Amandine Gojon, William Howat, Audrey Lawrence, Grace Lu, Tony Nash, Sabina Patel, Shyamprasad Perisetla, Alison Pritchard, Andrzej Rutkowski, Claire Smith, and Jonathan Tugwood. We also acknowledge Cancer Research UK, ECMC and NIHR/BRC infrastructure funding for the Cambridge Cancer Centre.

REFERENCES

1. Johnston SRD, Schiavon G. Treatment of Metastatic Breast Cancer: Endocrine. In: Harris JR, Lippman ME, Morrow M, Osborne CK, editors. Diseases of the Breast. Fifth Edition ed: Wolters Kluwer|Lippincott Williams & Wilkins, Philadelphia, 2014; 2014. p. 905-28.
2. Cardoso F, Costa A, Senkus E, Aapro M, Andre F, Barrios CH, et al. 3rd ESO-ESMO International Consensus Guidelines for Advanced Breast Cancer (ABC 3). Ann Oncol. 2017.
3. Osborne CK, Yochmowitz MG, Knight WA, 3rd, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer. 1980;46:2884-8.
4. Nardone A, De Angelis C, Trivedi MV, Osborne CK, Schiff R. The changing role of ER in endocrine resistance. Breast. 2015;24 Suppl 2:S60-6.
5. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet. 2013;45:1446-51.
6. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet. 2013;45:1439-45.
7. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, et al. D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. Cancer Res. 2013;73:6856-64.
8. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res. 2014;20:1757-67.
9. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, et al. Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. J Clin Oncol. 2016;34:2961-8.
10. Spoerke JM, Gendreau S, Walter K, Qiu J, Wilson TR, Savage H, et al. Heterogeneity and clinical significance of ESR1 mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. Nat Commun. 2016;7:11579.
11. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med. 2015;7:313ra182.
12. Chandarlapaty S, Chen D, He W, Sung P, Samoila A, You D, et al. Prevalence of ESR1 Mutations in Cell-Free DNA and Outcomes in Metastatic Breast Cancer: A Secondary Analysis of the BOLERO-2 Clinical Trial. JAMA Oncol. 2016;2:1310-5.
13. Hamilton EP, Patel MR, Armstrong AC, Baird RD, Jhaveri K, Hoch M, et al. A first in human study of the new oral selective estrogen receptor degrader AZD9496 for HR+/HER2- advanced breast cancer. Clin Cancer Res. 2018.
14. Bardia A, Kabos P, Wilks S, Richards D, Harb W, Elledge R, et al. Elacestrant, oral selective estrogen receptor degrader (SERD) in patients with ER positive (ER+)/HER2- advanced breast cancer: Updated phase 1 efficacy, safety and pharmacodynamic results. Abstract# PD5-08. SABCS; 2017; 2017.
15. Gupta S, Wallace MJ, Cardella JF, Kundu S, Miller DL, Rose SC, et al. Quality improvement guidelines for percutaneous needle biopsy. J Vasc Interv Radiol. 2010;21:969-75.
16. Alix-Panabieres C, Pantel K. Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy. Cancer Discov. 2016;6:479-91.
17. Paoletti C, Larios JM, Muniz MC, Aung K, Cannell EM, Darga EP, et al. Heterogeneous estrogen receptor expression in circulating tumor cells suggests diverse mechanisms of fulvestrant resistance. Mol Oncol. 2016.
18. Chu D, Paoletti C, Gersch C, VanDenBerg DA, Zabransky DJ, Cochran RL, et al. ESR1 Mutations in Circulating Plasma Tumor DNA from Metastatic Breast Cancer Patients. Clin Cancer Res. 2016;22:993-9.

19. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer. Clin Cancer Res. 2015;21:2487-98.
20. Cristofanilli M. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781-91.
21. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781-91.
22. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating Tumor Cells and Response to Chemotherapy in Metastatic Breast Cancer: SWOG S0500. J Clin Oncol. 2014.
23. Banerji U, Dean E, Perez-Fidalgo JA, Batist G, Bedard PL, You B, et al. A Phase 1 open-label study to identify a dosing regimen of the pan-AKT inhibitor AZD5363 for evaluation in solid tumors and in PIK3CA-mutated breast and gynecologic cancers. Clin Cancer Res. 2017.
24. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, et al. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. J Natl Cancer Inst. 2011;103:1656-64.
25. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst. 2005;97:1180-4.
26. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol. 2014;15:406-14.
27. Paoletti C, Regan MM, Liu MC, Marcom PK, Hart LL, Smith II JW, et al. Circulating tumor cell number and CTC-endocrine therapy index predict clinical outcomes in ER positive metastatic breast cancer patients: Results of the COMETI Phase 2 trial Abstract# P1-01-01. SABCS; 2016; San Antonio; 2016.
28. Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, Griffith C, et al. Short-term changes in Ki-67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. Clin Cancer Res. 2005;11:951s-8s.
29. Kallergi G, Konstantinidis G, Markomanolaki H, Papadaki MA, Mavroudis D, Stournaras C, et al. Apoptotic circulating tumor cells in early and metastatic breast cancer patients. Mol Cancer Ther. 2013;12:1886-95.
30. Muller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, et al. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res. 2005;11:3678-85.
31. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol. 2012;30:525-32.
32. Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. Proc Natl Acad Sci U S A. 2010;107:18392-7.
33. O'Leary B, Hrebien S, Morden JP, Beaney M, Fribbens C, Huang X, et al. Early circulating tumor DNA dynamics and clonal selection with palbociclib and fulvestrant for breast cancer. Nat Commun. 2018;9:896.
34. Fribbens C, Garcia Murillas I, Beaney M, Hrebien S, O'Leary B, Kilburn L, et al. Tracking evolution of aromatase inhibitor resistance with circulating tumour DNA analysis in metastatic breast cancer. Ann Oncol. 2018;29:145-53.
35. Paoletti C, Cani AK, Larios JM, Hovelson DH, Aung K, Darga EP, et al. Comprehensive mutation and copy number profiling in archived circulating breast cancer tumor cells documents heterogeneous resistance mechanisms. Cancer Res. 2017.

36. Pusztai L, Chung J, Young L, Schrock AB, Hartmaier R, Frampton GM, et al. Genomic profiling of circulating tumor DNA (ctDNA) from patients (pts) with metastatic breast cancer (mBC). Journal of Clinical Oncology. 2017;35:1016-.
37. Paoletti C, Aung K, Cannell EM, Darga EP, Chu D, Kidwell KM, et al. Molecular analysis of circulating tumor cells (CTC), cancer tissue, and cell-free plasma tumor DNA (ptDNA) suggests variable mechanisms of resistance to endocrine therapy (ET) in estrogen receptor (ER) positive metastatic breast cancer (MBC). San Antonio Breast Cancer Symposium; abstract # P3-05-01; 2015; 2015.

Figures Legend

Figure 1. Remark diagram for patient enrollment and distribution

Figure 2. CTC enumeration and *ESR1$_{LBD}$m* in ctDNA at baseline
2A. CTC enumeration for ER aliquot in log scale only for patients with ≥1 CTC/7.5 mL WB; The red horizontal line represents 5 CTC/7.5 mL WB. No bar represent patient with 1 CTC only since the graph is in log scale. *Enumeration assessed in Ki67 aliquot; 2 patients were not included because CTC were only drawn at the time of discontinuation and had ≥5 CTC/7.5 mL WB. **2B**. Prevalence and distribution of *ESR1$_{LBD}$m* in ctDNA by ddPCR at baseline; *all the cases with ≥2 *ESR1$_{LBD}$m* featured a D538G mutation. **2C.** CTC enumeration of ER aliquot at screening and C1D1 (paired T test) in patients with ≥5 CTC/7.5 mL WB; *Enumeration assessed in Ki67 aliquot. **2D.** *ESR1* mutational levels (copies/mL plasma) at screening and C1D1 (paired T test). For patients with ≥2 *ESR1$_{LBD}$m*, all mutations are reported. "Borderline" cases (i.e. <3 positive droplets, which is the pre-defined threshold of positivity) were included if at least one definite positive mutant result was detected at one of the 2 timepoints. *Abbreviations*: CTC: circulating tumor cells; ctDNA: circulating tumor DNA; C1D1: Cycle 1 Day 1; ER: estrogen receptor; *ESR1*, Estrogen Receptor alpha gene; LBD: ligand-binding domain; ND: not detected; WB: whole blood.

Figure 3. Serial changes in CTC enumeration and characterization during treatment with AZD9496 for patients with ≥5 CTC/7.5 mL WB (N=11)
CTC-biomarker enumeration and staining intensity for ER positivity (**3A**) and Ki67 positivity (**3B**). In both figures, the above bar graphs represent the number of CTC and each group of bars represents a different patient at different timepoints; in the below bar graph, the individual colors within each bar provide the percentage of CTC that stained 0 (■ blue), 1+ (■ red), 2+ (■ green), or 3+ (■ purple) for ER and Ki67 expression within each patient. ^patient within fulvestrant wash-out period at the time of trial enrollment; *patient was *ESR1* (D538G) mutant at discontinuation; **patient was *ESR1$_{LBD}$m*– in ctDNA, but *ESR1* (Y537C) mutant in tissue. For the remaining cases, *ESR1$_{LBD}$m* status in tissue was not available. *Abbreviations:* BID: twice-daily; CTC: circulating tumor cells; C1D1-pre: Cycle 1 Day 1 pre-dose; C1D15-post: Cycle 1 Day 15 post-dose; Disc.: discontinuation; ER: estrogen receptor; *ESR1*: Estrogen Receptor alpha gene; LBD: ligand-binding domain; OD: once daily; Screen: screening; WT: wild type.

Figure 4. Dynamics of *ESR1$_{LBD}$m* in ctDNA during treatment with AZD9496
4A. *ESR1* mutational levels tracking shown for 17 patients with longitudinal plasma collected throughout treatment and who were *ESR1$_{LBD}$m+* by droplet digital polymerase chain reaction analysis at any timepoint (14 patients with *ESR1$_{LBD}$m+* pre-treatment and 3 patients with *ESR1$_{LBD}$m* absent prior to treatment, but detected in on-treatment ctDNA samples). For patients with ≥2 *ESR1$_{LBD}$m*, the mutation with highest levels (copies/mL plasma) pre-treatment ("dominant mutation") is shown; **4B.** A decline of circulating *ESR1$_{LBD}$m* of ≥50% at C1D15 compared to treatment was evident in 8/14 (57%) of patients with no apparent dose-response relationship; **4C**. *ESR1$_{LBD}$m* tracking in five cases with ≥2 *ESR1$_{LBD}$m+* during treatment with AZD9496. In one case (patient #21) *ESR1* (D538G and Y537N) mutations were detected at screen but not at C1D1. **ESR1$_{LBD}$m* status was "borderline" (i.e. below pre-defined threshold of

positivity) at C1D1, but clearly detectable at another timepoint. [#]Still receiving treatment at data cut-off of January 31, 2017. *Abbreviations*: BID: twice a day; C1D15: Cycle 1 Day 15; C9D1: Cycle 9 Day 1; disc: discontinuation; *ESR1*: Estrogen Receptor alpha gene; LBD: ligand-binding domain; ND: not detected; OD: once daily.

Figure 5. Circulating biomarkers data and prediction of treatment outcome

Kaplan–Meier plots for: **5A.** Progression-free survival (PFS) of all patients according to CTC levels (<5 vs. ≥5 CTC/7.5 mL whole blood (WB)) at baseline. Red line, ≥5 CTC/7.5 mL WB; Black line, <5 CTC/7.5 mL WB; **5B.** PFS of all patients according to $ESR1_{LBD}m$ status ($ESR1_{LBD}m$ negative (-) vs. $ESR1_{LBD}m$ positive (+)) at baseline. Red line, $ESR1_{LBD}m$+;
Black line, $ESR1_{LBD}m$–. **5C.** PFS of patients with <5 CTC/7.5 mL WB according to *ESR1* status ($ESR1_{LBD}m$- vs. $ESR1_{LBD}m$+) at baseline. **5D**. PFS of all patients according to $ESR1_{LBD}m$ status (*ND=never detectable or "ctDNA response" defined as drop ≥50% at C1D15 in the dominant $ESR1_{LBD}m$ vs. residual $ESR1_{LBD}m$) at C1D15. Red line, residual $ESR1_{LBD}m$+; Black line, ND or ctDNA response. **5E.** PFS of patients with <5 CTC/7.5 mL WB according to $ESR1_{LBD}m$ status (*ND=never detectable or "ctDNA response" in the dominant $ESR1_{LBD}m$ vs. residual $ESR1_{LBD}m$) at C1D15. Red line, residual $ESR1_{LBD}m$+; Black line, ND or ctDNA response. **5F.** PFS of all the patients according to persistently elevated biomarker (**≥5 CTC/7.5 mL WB and/or lack of ctDNA response defined as a decline of <50% of $ESR_{LBD}1m$ level) at C1D15. Red line, persistently elevated biomarkers at C1D15; Black line, Other. *Abbreviations*: CTC: circulating tumor cells; ctDNA: circulating tumor DNA; C1D15: Cycle 1 Day 15; *ESR1*: Estrogen Receptor alpha gene; LBD: ligand-binding domain; ND: never detectable; WB: whole blood.

Figure 1

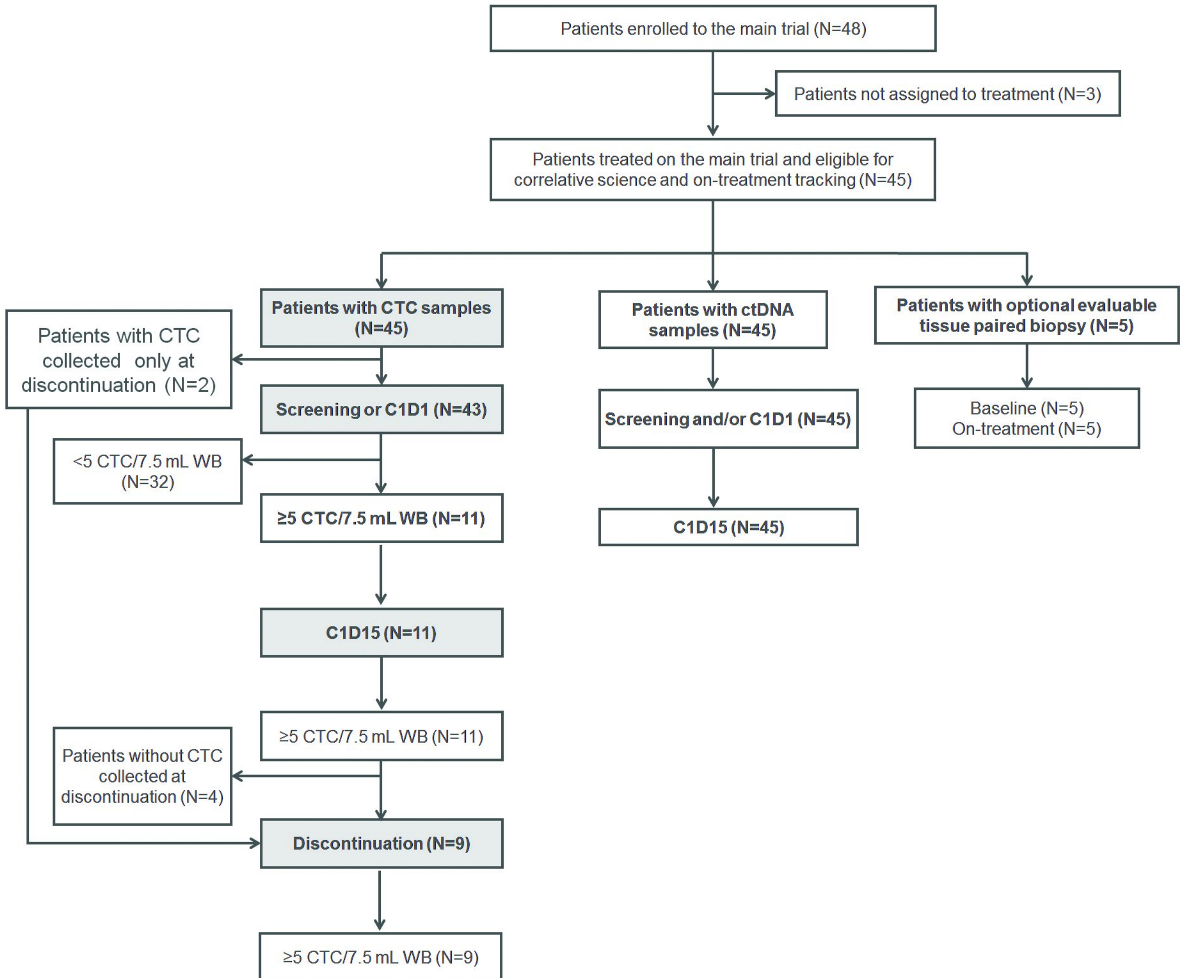
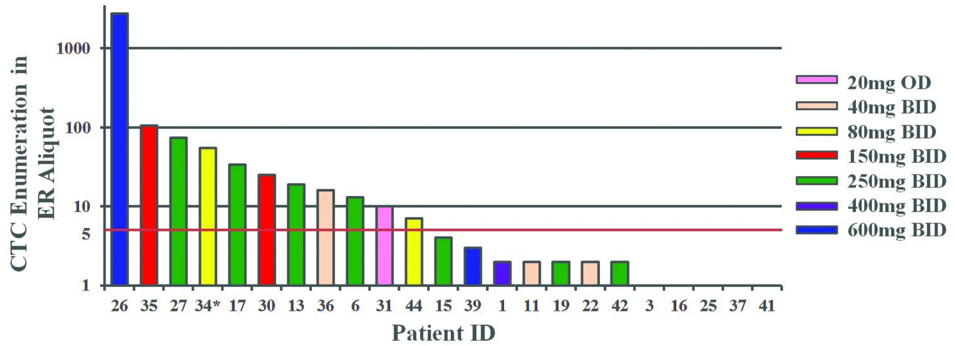
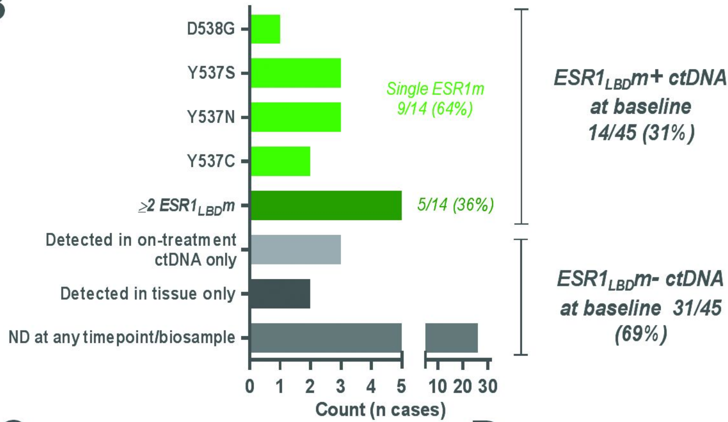


Figure 2

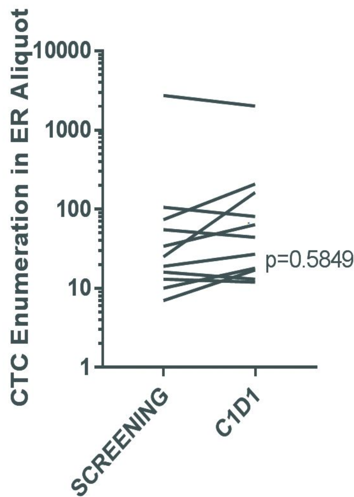
A



B



C



D

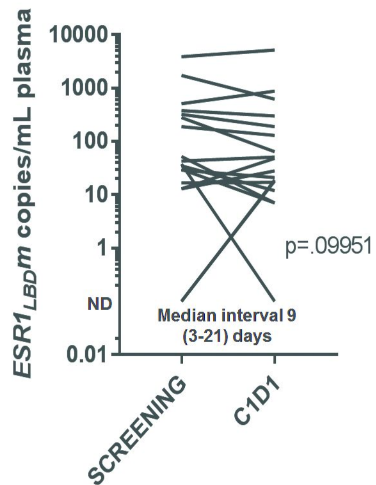


Figure 3

A

CTC Enumeration in ER Aliquot

10000
1000
100
10
1

ER CTC Status

100%
80%
60%
40%
20%
0%

Screen.
C1D1-pre
C1D15-post
Disc. (+46 days)
Disc. (+55 days)
Disc. (+169 days)
Disc. (+56 days)
Disc. (+56 days)
Disc. (+45 days)
Disc. (+56 days)
Disc. (+85 days)
Disc. (+28 days)
Disc. (+56 days)
Disc. (+49 days)

Patient ID	31	36	34	44^	30	35	6	13	17	27	26^
Dose (mg)	20 OD	40 BID	80 BID	80 BID	150 BID	150 BID	250 BID	250 BID	250 BID	250 BID	600 BID
ESR1$_{LBD}$ ctDNA status at C1D1	WT*	WT**	WT	WT	Y537C/ D538G/ E380Q	Y537S	Y537N	WT	WT	WT	Y537C

B

CTC Enumeration in Ki67 Aliquot

10000
1000
100
10
1

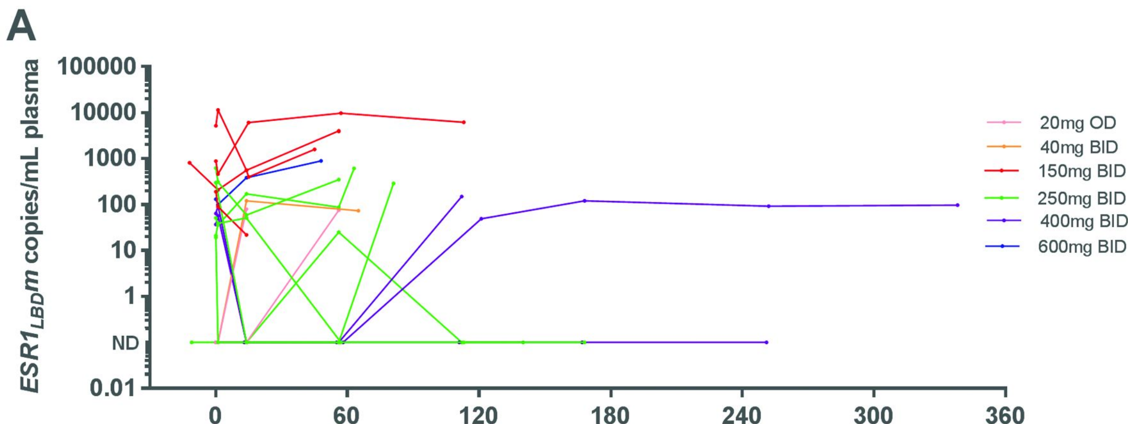
Ki67 CTC Status

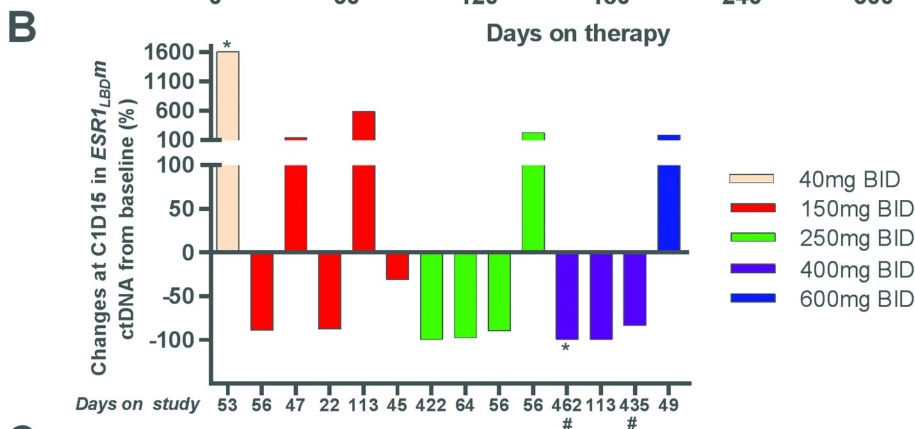
100%
80%
60%
40%
20%
0%

Screen.
C1D1-pre
C1D15-post
Disc. (+46 days)
Disc. (+55 days)
Disc. (+169 days)
Disc. (+56 days)
Disc. (+56 days)
Disc. (+45 days)
Disc. (+56 days)
Disc. (+85 days)
Disc. (+28 days)
Disc. (+56 days)
Disc. (+49 days)

Patient ID	31	36	34	44	30	35	6	13	17	27	26
Dose (mg)	20 OD	40 BID	80 BID	80 BID	150 BID	150 BID	250 BID	250 BID	250 BID	250 BID	600 BID

Figure 4





C

Patient #30 - 150mg BID

Patient #15 - 250mg BID

Patient #19 - 250mg BID

Patient #1 - 400mg BID

Patient #21# - 400mg BID

$ESR1_{LBD}m$ copies/mL plasma

Y537C, D538G, E380Q

Y537S, D538G, E380Q

Y537N, D538G, E380Q, L536R

Y537N, D538G

Days on therapy

disc

C9D1 ongoing

Figure 5

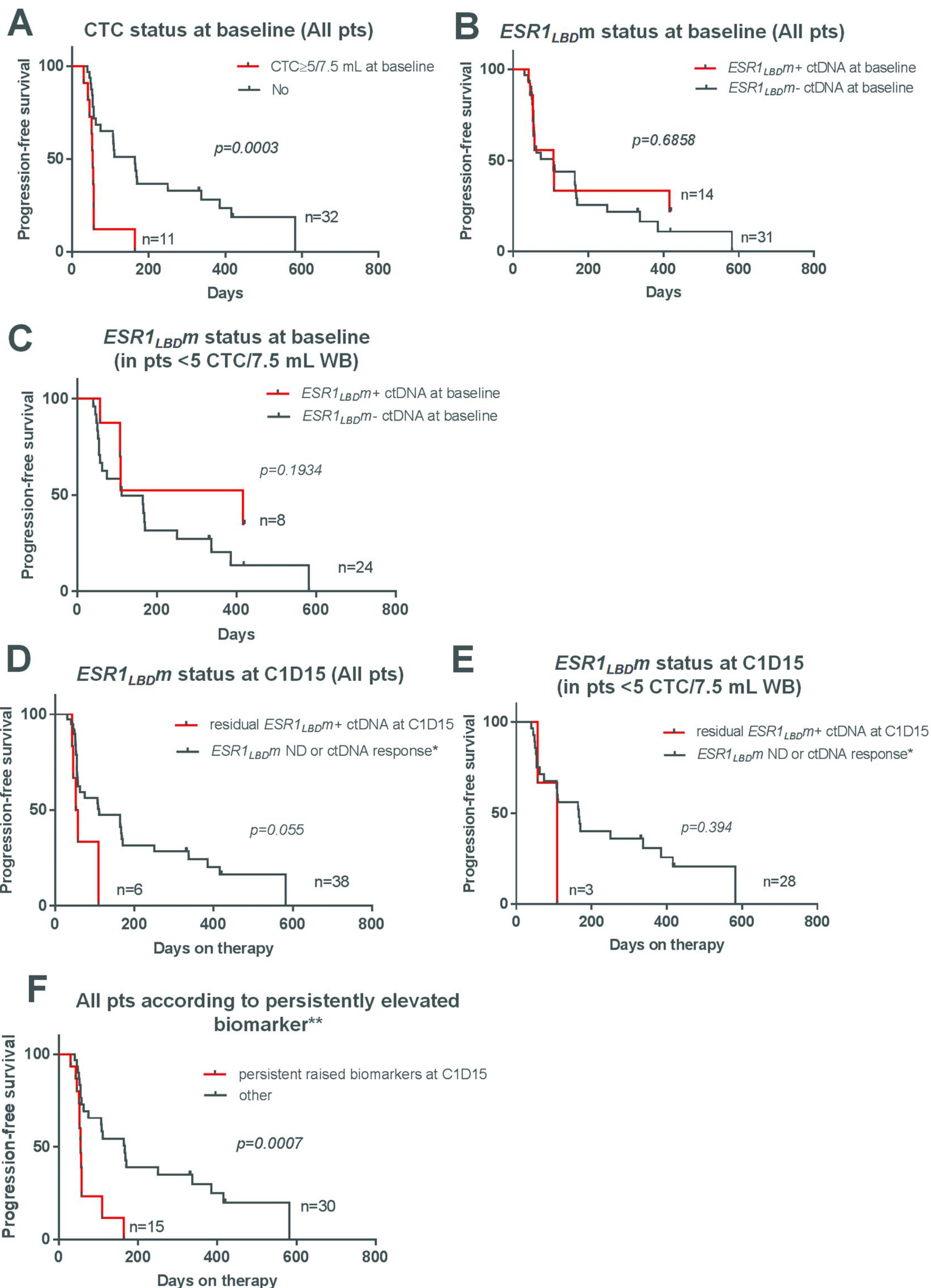


Table 1. CTC enumeration (<5 vs. ≥5 CTC/7.5 mL whole blood) and $ESR1_{\text{LBD}}m$ status ("$ESR1_{\text{LBD}}m$-" vs. "$ESR1_{\text{LBD}}m$+" [≥1]) in 43 patients who had both CTC and ctDNA assessed at baseline.

$ESR1_{\text{LBD}}m$ status at baseline	**CTC at baseline**		
	<5 CTC/7.5 mL WB	**≥5 CTC/7.5 mL WB**	**Total**
$ESR1_{\text{LBD}}m$+ ctDNA	8	4	12
$ESR1_{\text{LBD}}m$- ctDNA	24	7	31
Total	32	11	43[a]

Legend: CTC: circulating tumor cells; ctDNA: circulating tumor DNA; $ESR1_{LBD}m$+ : *ESR1* mutation detected; $ESR1_{LBD}m$- : *ESR1* mutation "not detected"; LBD: ligand-binding domain; WB: whole blood; [a]43/45 patients had both CTC and ctDNA at baseline (2 patients only had ctDNA, but not CTC assessed).