OPPORTUNE: Randomised phase II window study of short-term preoperative treatment with the PI3K inhibitor GDC-0941 plus Anastrozole versus Anastrozole alone in patients with ER-positive primary breast cancer.

Clinical Study Report

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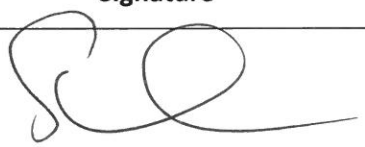
Prof Peter Schmid	Chief Investigator		17/12/19

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1 INTRODUCTION

1.1 Endocrine therapy and PI3K inhibitors in breast cancer

Breast cancer is the most common malignancy affecting women in northern Europe and North America, corresponding to an age-corrected annual incidence of 100 to 120 per 100000 females. Approximately 20-30% of all patients treated with curative intent will develop metastatic disease. Perioperative systemic treatment has made a major impact on relapse-free and overall survival of women with early-stage breast cancer [1, 2] with therapeutic strategies being based on the endocrine responsiveness and the estimated risk of relapse defined by tumour size, axillary lymph node involvement, histological and nuclear grade, lymphatic and/or vascular invasion, HER2/neu-overexpression and age [3].

Oestrogen and the oestrogen receptor (ER) play an important role in the development and progression of breast cancers. Therapeutic strategies directed at inhibiting the action of ER using selective ER modulators (SERMs), withdrawing oestrogen by surgical (oophorectomy) or medical (luteinizing hormone agonists) ovarian ablation or by aromatase inhibitors (AIs), or targeting ER for degradation with selective ER downregulators (SERDs) represent highly successful examples of targeted therapy for clinical breast cancer. Given that approximately 80% of invasive breast tumours diagnosed in postmenopausal women are ER- and/or progesterone receptor (PR)-positive, advances in endocrine therapy have the potential to result in dramatic reductions in breast cancer mortality.

Until recently, the standard of care for most postmenopausal women with ER-positive, invasive breast cancer was 5 years treatment with tamoxifen. Meta-analyses by the Early Breast Cancer Trialists' Collaborative Group demonstrate that tamoxifen results in a 47% relative reduction in the risk of recurrence and a 34% relative reduction in the risk of death in women with ER-positive tumours [4]. More recently however, the pre-eminence of tamoxifen has been challenged by the AIs. Reports from several groups showed that 5 years of adjuvant therapy with an AI alone improved disease-free survival as compared with 5 years of tamoxifen therapy [5-8]. Other large studies showed that switching to an AI after initial treatment with tamoxifen also improved survival compared to tamoxifen alone [9-15]. A meta-analysis of trials of initial and sequential strategies supported the recommendation in guidelines that an AI should be included in adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer[16].

Despite all this progress in endocrine treatment, it is still only a subgroup of patients that will derive the optimal therapeutic benefit, whereas other patients have refractory disease or will develop drug resistance during treatment. Over the last years, a great deal of basic and translational research has been directed at elucidating the processes of resistance and several studies indicate that acquired resistance to endocrine therapy is a progressive, step-wise phenomenon induced by the selective pressure of hormonal agents, which leads breast cancer cells from an oestrogen-dependent phenotype, that is responsive to endocrine manipulation, to a non-responsive phenotype, and eventually to an oestrogen-independent phenotype. Several different mechanisms have been hypothesized to be involved in developing resistance of breast cancer cells to hormonal therapy including molecular cross talk between ER, PR, and growth factor–receptor signalling pathways, oestrogen hypersensitivity associated with increased transcriptional activity of ER〈 and the relationship between the classical and non- classical, non-genomic effects of ER in breast cancer cells.

Abnormal activation of the phosphoinositide 3-kinase (PI3K) pathway in cancer, either via genetic alterations in PI3K pathway constituents [PI3K-activating mutations or genetic amplification, loss of the antagonistic tumour suppressor Phosphatase and tensin homolog (PTEN)] or via the transduction of aberrant receptor tyrosine kinase (RTK) signals, is a common finding in a variety of tumour types. Aberrant PI3K pathway activation frequently occurs in breast cancer, most commonly through activating mutations of the PI3K catalytic subunit (PI3KCA) or inactivation of the negative regulator PTEN [17-20]. Approximately one-third of HR-positive breast cancer patients have mutations in PIK3CA, the alpha subunit of PI3K (or p110a), and an additional one-fifth have loss of PTEN protein expression (PTEN null). Mutations in these two pathway components are generally mutually exclusive in breast cancer tumour samples. Both

alterations result in up-regulation of the PI3K pathway [21, 22] and make human breast cancer a rational target for PI3K inhibitors.

Activation of the PI3K pathway has been associated with poor prognosis and resistance to endocrine therapy in ER-positive tumours [21, 23-27]. Multiple lines of investigation have furthermore demonstrated that downregulation or inhibition of PI3K activity can overcome endocrine resistance [28-30]. Importantly, estradiol can suppress apoptosis induced by PI3K knockdown or inhibition in ER-positive breast cancer, suggesting independent PI3K-dependent and estradiol-dependent cell survival mechanisms [31]. Preclinical studies demonstrate synthetic lethality of PIK3CA&B inhibition and oestrogen deprivation, providing a strong rationale for the combination of PI3K inhibitors and endocrine therapy [31]. It has furthermore been shown that PI3K wild-type or mutant tumours equally benefit from combined PI3K and endocrine therapy, suggesting that eligibility should not be restricted by PIK3CA mutation status [31]. This combined with the association of therapeutic resistance with increased PI3K pathway signalling suggests that inhibition of PI3K signalling could have broad applications in the treatment of breast cancer.

1.2 GDC-0941

GDC-0941 is a potent, novel, selective, small-molecule inhibitor of Class I PI3K being developed by Genentech as an anti-cancer therapeutic. GDC-0941 is a potent inhibitor of the kinase activity of recombinant human p110a/p85a, with mean IC_{50} values of 8.0 and 3.4 nM by a fluorescence polarization competition assay and a scintillation proximity assay, respectively. In addition, GDC-0941 is equipotent for both H1047R and E545K p110a mutants and potently inhibits other members of the Class I PI3K family (p110b/p85a, p110d/p85a, and p110y), with IC_{50} values of <75 nM (scintillation proximity assay). It binds Classes II, III, and IV PI3K family members weakly or not at all, including DNA-dependent protein kinase (DNA-PK) and mammalian target of rapamycin (mTOR). Thus, these data suggest that GDC-0941 is a pan-inhibitor of the Class I PI3K family members.

Efficacy with GDC-0941 has been observed in multiple mouse xenograft models, including breast, prostate, and lung cancer cell models. Comparable in vivo efficacy at the maximum efficacious dose (MaxED) was observed when one-half of the daily dose was administered twice a day in the PC3-NCI and MCF7-neo/HER2 xenograft models, suggesting that efficacy is primarily driven by total exposure. In addition, correlative downstream PD markers of PI3K activity such as phosphorylated AKT (pAKT), phosphorylated S6 (pS6), and phosphorylated PRAS40 (pPRAS40) were suppressed for approximately 4−8 hours at doses that were consistent with efficacy in these xenograft models. Efficacy was also observed with GDC-0941 in combination with the novel targeted antibody-drug conjugate trastuzumab-MCC-DM1 (T-DM1), as well as with the established standard-of-care molecules such as erlotinib and of docetaxel, in mouse breast or lung cancer models.

As of November 2010, GDC-0941 has been studied clinically in 223 cancer patients and 71 healthy volunteers. There are currently two ongoing Phase Ia trials with single- agent GDC-0941 administered to cancer patients and five ongoing Phase Ib trials with GDC-0941 administered in combination with other anti-cancer therapies to cancer patients. There are two completed Phase I pharmacokinetic studies in healthy volunteers. Preliminary safety data are available for a total of 128 patients enrolled in the two open-label dose-escalation Phase Ia studies with GDC-0941 administered daily as a single agent to patients with advanced or metastatic solid tumour malignancies. The maximum tolerated dose (MTD) was exceeded at 450 mg QD in both studies with the following dose- limiting toxicities (DLTs): 3 patients with Grade 3 rash and 1 patient with an asymptomatic Grade 3 T- wave inversion finding on ECGs. The MTD was also exceeded at 450 mg total daily dose (TDD) with DLTs of Grade 3 thrombocytopenia and Grade 4 hyperglycaemia.

As of May 2011, new preliminary safety data (some of which have not been source-verified by Genentech) have been received that were not included in the most recent IB (Edition 4, dated 14 April 2011, data cut-off date of 8 November 2010). These clinical events, which occurred in patients enrolled in the two single agent GDC-0941 Phase I studies (GDC4254g and GDC4255g) were considered significant new information and are described below. Five patients have

received GDC-0941 at a dose of 400 mg on a 21-of-28 day schedule (Study GDC4255g) and one patient has received GDC-0941 at a dose of 400 mg on a 28-of-28 day schedule (Study GDC4254g). The MTD for the 21-of-28-day schedule was exceeded at 400 mg as two patients experienced the following DLTs: Grade 3 fatigue and myalgias in one patient and Grade 3 nausea and fatigue in another patient. Seven patients have received GDC-0941 at a dose of 330 mg on a 28-of-28 day schedule in Study GDC4255g. One DLT of Grade 4 elevation of troponin was reported among these patients. The only other Grade ≥3 adverse event (AE) assessed as related to GDC-0941 observed in these seven patients was Grade 3 hypokalaemia in one patient. Other drug-related AEs were Grades 1-2 and were consistent with the safety profile of GDC-0941 observed in other patients in Study GDC4255g. Non-DLT events of possible drug-related pneumonitis were also reported in one patient who received GDC-0941 at a dose of 330 mg (165 mg AM/165 mg PM) on a 21- of-28 day schedule, one patient who received GDC-0941 at a dose of 400 mg on a 21-of-28 day schedule, and one patient who received GDC-0941 at a dose of 400 mg on a 28-of-28-day schedule. These symptomatic pulmonary events were reversible and resolved within 7-10 days following discontinuation of GDC-0941 and the initiation of anti-inflammatory steroid therapy and/or antibiotics.

There have been three confirmed partial responses by Response Evaluation Criteria in Solid Tumours (RECIST) for single-agent GDC-0941: 1 patient with ER-positive, HER2-negative metastatic breast cancer treated at 130 mg QD; 1 patient with melanoma treated at 330 mg QD, and 1 patient with endocervical cancer treated at 330 mg TTD (165 mg BID). There have been no deaths that were evaluated as related to GDC-0941, and the studies are ongoing to further define the recommended Phase II dose.

There are five open-label Phase Ib, dose-escalation trials using a 3 + 3 design to assess GDC-0941 administered in combination with various anti-cancer therapies (Studies GDC4626g, GDC4627g, GDC4628g, GDC4629g, and MEK4752g). As of November 2010, preliminary safety data are available for 93 patients from these five studies.

1.3 Concept of short-term preoperative treatment

Short-term preoperative 'window' studies of 2-4 weeks treatment are a validated strategy to provide rapid and cost-efficient proof-of-concept for novel treatment approaches by assessing the direct effects of the study treatment on the tumour tissue. These studies provide access to tumour tissue before, under and after treatment for pharmacodynamic and correlative studies thus providing critical insight into the optimal patient population, differences in activity and mechanisms between agents, influence of the tumour biology on sensitivity, and molecular mechanisms of response or resistance.

Detailed studies in the neoadjuvant setting involving more than 860 patients in prospective randomised clinical trials have demonstrated the utility and validity of changes in Ki67 as a predictor of benefit from treatment and of long-term outcome [32-40]. In the neoadjuvant IMPACT study, suppression of Ki67 at 2 weeks was greater with anastrozole than with either tamoxifen or the combination of anastrozole plus tamoxifen [32, 33], mirroring the results of the much larger adjuvant ATAC trial without the requirement of a long follow-up [7, 8]. Similarly, a recent randomised trial demonstrated that the effects of combined therapy with letrozole and everolimus as measured by Ki67 down-regulation seem to be limited to patients with activating PI3K mutations, providing a rationale for selecting an optimal patient cohort for subsequent clinical trials [39].

Although Ki67 measurements in preoperative trials cannot replace the need for adjuvant trials with clinical endpoints, they can be highly instructive in selecting or rejecting candidate approaches for phase III studies and defining the most appropriate patient populations. Over recent years, the perioperative window setting of this study together with the incorporation of primary biological endpoints has been established in UK as a new approach for breast cancer research. The POETIC trial, a UK NCRN phase 3 randomised clinical trial with approximately 4000 patients, is currently testing prospectively whether short- term perioperative endocrine therapy with an AI followed by standard adjuvant therapy can improve outcome in postmenopausal women with ER-positive breast cancer, whether the proliferation marker

Ki67 as measured by immunohistochemistry (IHC) after 2 weeks of AI therapy will predict for relapse free survival (RFS) and whether molecular profiling 2 weeks after starting endocrine therapy predicts better for long-term outcome than at diagnosis. The implementation of this national trial followed wide consultation with consumers and clinical colleagues at the UK Breast Intergroup and the NCRI's Breast Clinical Studies Group to minimise changes to routine clinical practice and to ensure that the procedures and their timing are acceptable and practical (Adapted from the "POETIC Protocol v3, 22Dec2009, EudraCT: 2007-003877-21). This process highlighted the requirement for tissue taken from patients prior to their entry into a treatment study to be stored within an HTA licensed laboratory (Adapted from the "POETIC Protocol v3, 22Dec2009, EudraCT: 2007-003877-21). As a result, the POETIC Pathway B was introduced to accommodate patients diagnosed prior to having the opportunity to consider the trial, a strategy which has also been adopted for this trial.

2 OBJECTIVES

The main aims of this study were to:

- Determine whether adding a PI3K-inhibitor to pre-operative endocrine treatment of ER-positive breast cancer patients increases the effects on tumour cell proliferation or apoptosis,
- Identify predictors of sensitivity to PI3K-inhibition in order to characterize the patient population that benefits most from treatment with PI3K inhibitors, and
- Study the effects of combined endocrine and PI3K-inhibitor therapy on breast cancer biology

Primary objective

The primary objectives of this study were to evaluate:

- Changes in tumour-cell proliferation (as measured by the changes in Ki67 expression between pre- and post-treatment tumour samples) between anastrozole + GDC-0941 and anastrozole alone in all treated patients
- Changes in tumour-cell proliferation (as measured by the changes in Ki67 expression between pre- and post-treatment tumour samples) between anastrozole + GDC-0941 and anastrozole alone in patients with and without PI3K mutations and/or loss of PTEN

Secondary objectives

The secondary objectives of this study were to:

- Determine the effects of the study treatment on tumour-cell apoptosis (as measured by changes in the TUNEL assay between pre- and post-treatment tumour samples) in all treated patients and in patients with and without PI3K mutations and/or loss of PTEN
- Determine safety and tolerability of the study treatment in this population
- Make a preliminary assessment of the efficacy of the study treatment on clinical and pathological responses

Exploratory objectives

The exploratory objectives of this study were to:

- Identify predictors of sensitivity to GDC-0941 in order to characterize the population that benefits most from PI3K inhibition

- Explore the biologic effects of GDC-0941 on breast cancer and stromal cells and establish pharmacodynamic markers of GDC-0941 action
- Explore mechanisms of resistance

3 MATERIALS AND METHODS

3.1 Overall Study Design

OPPORTUNE was an open-label, randomized phase II trial performed in 10 academic medical centres in the United Kingdom. The study aimed to detect an increase in Ki-67 suppression with PIC in ER-positive patients and to assess the treatment effects in subgroups defined by PI3K mutations, luminal A/B subtypes, and baseline Ki-67 scores. The main analysis of the overall treatment effects was planned with 163 evaluable patients.

Patients were eligible if they were postmenopausal (aged ≥55 years with amenorrhoea for ≥1 year or aged <55 years with amenorrhea for ≥1 year with Oestradiol <20pg/mL, or prior bilateral oophorectomy) and had histologically diagnosed ER-positive, HER2-negative invasive breast cancer. ER positivity was defined as ≥ 1% of tumour cells positive on immunohistochemistry (IHC) or an Allred IHC score of ≥ 3. All patients had operable breast cancer ≥ 1 cm in diameter; adequate hematologic, hepatic, and renal function; and baseline fasting plasma glucose of < 7.8 mmol/L and a WHO performance status of 0 – 2. Prior treatment of breast cancer or use of hormone replacement therapy was not permitted. Patients with inflammatory cancer or distant metastases were excluded. In addition, patients with significant pulmonary dysfunction, cardiac disease, or diabetes mellitus were excluded.

Patients were randomly assigned (2:1, favouring the combination) to receive treatment with Anastrozole or Anastrozole plus GDC-0941. Computer-generated permuted blocks were used, and stratification was by centre and histologic grade, as assessed on the diagnostic core biopsy. Anastrozole was given at a dose of 1 mg once per day. GDC-0941 was initially administered at 340 mg once per day; from August 2012 onward, GDC-0941 was reduced to 260 mg once per day according to safety data from other studies that indicated a lower rate of mucosal and skin toxicity at 260 mg. Five evaluable patients received GDC-0941 at 340 mg; the remaining patients received GDC-0941 260 mg. Study treatment was given for 15 days, followed by surgical resection and adjuvant therapy as appropriate for each patient according to local practice guidelines.

Patients were monitored for adverse events (AEs) and changes in laboratory values, electrocardiogram, and physical examination findings.

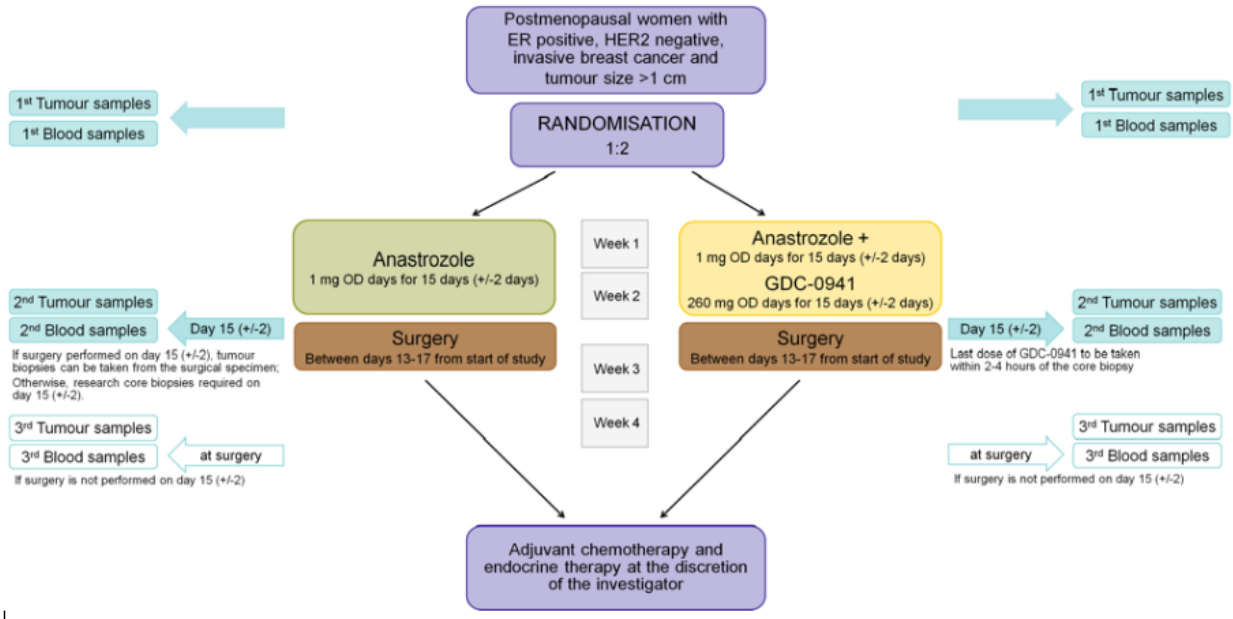


Figure 1: OPPORTUNE trial design. Eligible patients were randomised favourably to the combination arm. Patients participating in the trial consented for additional core biopsies at diagnosis and at 2 weeks post treatment (during surgical resection).

A minimum of two core-cut tumour biopsies (14-gauge) were taken at baseline and at the end of treatment. The last dose of study medication was required within 2-4 hours before the end-of-treatment biopsy.

Biopsies for histology were placed into 10% buffered formalin within 10 minutes of sampling and fixed for ≥6 hours before processing and embedding in paraffin wax. Snap frozen cores were placed in liquid nitrogen within 10 minutes.

All tumour core biopsies were reviewed centrally at Guys Hospital London and scanned in; histological sections were assessed by haematoxylin and eosin (H&E) staining to facilitate macro-dissection of carcinoma tissue for additional biomarker analyses.

3.1.1 Immunohistochemistry

IHC for Ki67, Caspase-3, PR and PTEN was performed and analysed centrally. IHC was performed on 3-4µm sections from the FFPE core biopsies after heat mediated antigen retrieval. Antibodies for Ki67 [Clone 30-9, Ventana], cleaved Caspase-3 [Clone Asp175, Cell Signalling], and PTEN (Clone 138G6, Cell Signalling) were used. Sections were only scored for Ki67, Caspase-3 and PTEN if the initial H&E stained section showed invasive cancer with clearly identifiable malignant epithelial cells and/or invasive tumour. For the trial, Ki67 and Caspase-3 IHC were recorded independently by two investigators, who were blinded as to treatment allocation and each other's assessment.

Ki67 analysis: A minimum of 1,000 invasive cancer cells were counted for Ki67 analysis; Ki67 was scored as the percentage of positively stained cells. A cut-off of 14% was selected to define high and low baseline Ki67 expression [41] [42]. Primary Ki67 analysis was based on estimating the mean Ki67 suppression in each group and the geometric mean ratio of proportional changes between groups. Secondary Ki67 analyses were geometric mean end-of-treatment Ki67 expression, individual end-of-treatment anti-proliferative response (RKi67-Day15) defined as Ln(Ki67Day15) ≤2, and individual anti-proliferative response (RΔKi67) defined as a ≥50% fall in Ki67 expression [43] [44].

Caspase-3 analysis: For Caspase-3, at least 3,000 invasive cancer cells were assessed, if available. Caspase-3 analyses included geometric mean change in Caspase-3 between day 15 and baseline and individual apoptotic response (RΔCasp3), defined as a ≥50% increase in Caspase-3 IHC.

Progesterone receptor analysis: PR was assessed centrally and regarded as positive if Allred score was ≥3.

3.1.2 PTEN analysis:

PTEN was classified as "positive" if any cytoplasmic and/or nuclear expression immunoreaction was observed in tumour cells and "negative" if no immune reactivity was observed, with the surrounding tissue microenvironment serving as a positive internal control.

3.1.3 DNA/RNA extraction

Following macro-dissection for tumour-enriched areas with >70% malignant tissue, DNA and RNA were simultaneously extracted from FFPE sections using Qiagen AllPrep DNA/RNA FFPE kit as per manufacturer's instructions. FFPE sections in tube were dewaxed and rehydrated using xylene-based protocol, and DNA/RNA extracted in a column-based approach. Nucleic acids were quantified and checked for purity using a UV spectrophotometer (NanoDrop).

3.1.4 Gene expression analysis

RNA expression analysis of approximately 800 breast cancer-related genes using the nCounter platform (NanoString Technologies, Seattle, US). RNA analysis was performed at Genentech, Inc., South San Francisco, CA, US. RNA (100ng) was hybridized overnight at 65°C according to the NanoString protocol. Samples were subsequently loaded onto the NanoString nCounter Prep Station and transcripts were counted using the NanoString nCounter Digital Analyzer at a FOV of 280. Samples were normalized to housekeeping genes. PAM50 analysis of Luminal A and Luminal B subtypes was carried out as previously reported [45]. Data were transferred back for integrated analysis.

3.1.5 Next Generation Sequencing

Analysis of mutations and copy number changes of PIK3CA and other key pathway components was assessed by targeted next generation sequencing using the Ampliseq Comprehensive Cancer panel assay with the Ampliseq Library Kit 2.0 according to the manufacturer's instructions (ion torrent, life technologies, US). NGS analysis was performed at the Centre for Personalized Nanomedicine at the Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Australia. NGS analysis was supported by a grant from the National Breast Cancer Foundation (NBCF) of Australia (CG-12-07).

Samples underwent 19 rounds of amplification and were barcoded using the Ion Xpress barcodes (Ion torrent, LifeTechnologies). Each pool was quantified post-adaptor ligation by qPCR. Samples were pooled to provide 300x coverage. The Ion PI Template OT2 200 v3 Kit, P1 chip and Ion PI Sequencing 200 v3 Kit were used as per the manufacturers protocol. Variant calling used the Torrent variant Caller (v4.0-r76860) set on Somatic PGM low stringency settings. Torrent Suite 4.0.2 was used for data processing, base-calling, and mapping. Data were transferred back for integrated analysis.

3.1.6 Reverse Phase Protein Arrays

Reverse Phase Protein Arrays (RPPA) analysis of 55 targets focused on PI3K pathway signalling, ER signalling, alternative intracellular signalling and cell cycle regulation. RPPA analysis was performed at Theranostics Health, Inc., Gaithersburg, MD, USA. 32 matched pairs of patient samples were selected for this study. Whole slide lysates were prepared and

approximately 6nl of protein were printed in 4 replicates of glass backed nitrocellulose slides. Protein was printed at approximately 2 concentrations, 0.5mg/ml or 0.25mg/ml. Slides were incubated with 55 different antibodies and target specific signal was captured at 635nm. The amount of protein printed at each spot was measured using a Sypro Ruby Protein Blot Stain (Invitrogen: S11791), captured at 532nm. The total protein yield is used as a denominator for primary antibody signal, giving us a total protein normalised signal. All results presented use the total protein normalised signal fit on a LOESS algorithm. Data were transferred back for integrated analysis.

3.2 Discussion of Study Design

Justification of selecting the target population: The trial was confined to postmenopausal women with newly diagnosed, ER-positive, HER2- negative, invasive primary breast cancer, as this was the subgroup considered for perioperative aromatase inhibitor therapy. Preclinical studies suggested that PI3K wild-type or mutant were equally affected by combined PI3K and endocrine therapy, suggesting that eligibility should not be restricted by PIK3CA mutation status. Pre-specified subset analyses were planned to characterise the relevance of PI3K pathway activation for the response to the study treatment. Patients with HER2 over-expressing tumours were excluded as these patients would be considered for HER2-directed therapy. Given the number of tumour biopsies required as part of this study, the trial was confined to patients with a tumour size of at least 1 cm.

Rationale for the use of short-term preoperative treatment: Short-term preoperative studies are a validated strategy to provide rapid and cost-efficient proof-of-concept for novel treatment approaches. Preoperative studies can be highly instructive in selecting or rejecting candidate approaches for phase 3 studies and defining the most appropriate patient populations. Short-term preoperative studies would not delay definitive surgery as the average time in the UK between the diagnostic biopsy and surgery was 2-4 weeks. The approach offers a direct assessment of the treatment effects in the tumour tissue which has been shown to correlate with long-term outcome. Access to tumour tissue before and after treatment enables pharmacodynamic and correlative studies thus providing critical insight into the optimal patient population, and mechanisms of resistance.

Selection of the primary endpoint: Endocrine treatment for breast cancer acts largely by inhibiting tumour cell proliferation. Ki67 expression measured by IHC (using the MIB-1 antibody) is a reliable, reproducible and validated biomarker of tumour cell proliferation. Prospective clinical trials have demonstrated that 2-week preoperative therapy with an AI or tamoxifen markedly reduces breast cancer cell proliferation as measured by Ki67. Change in 2-week Ki67 expression has been shown to be closely linked with 12-week Ki67 expression and clinical response to preoperative endocrine therapy. A highly significant relationship between 2-week Ki67 expression and relapse free survival has been confirmed on multivariate analysis [35]

Selection of GDC-0941: GDC-0941 is a potent, novel, selective, small-molecule inhibitor of Class I PI3K. Non-clinical anti-tumour activity has been seen in a number of single agent and combination therapy studies. In addition, early clinical anti-tumour activity has been seen in ER-positive breast cancer. GDC-0941 has demonstrated an acceptable toxicity profile in more than 223 cancer patients and 71 healthy volunteers treated to date.

Ethical considerations: Approximately 80% of invasive early breast tumours diagnosed in postmenopausal women are ER- and/or PR-positive. Postoperative endocrine therapy of ER or PR positive breast cancer results in significant survival benefits. Despite all progress, only a subgroup of patients will derive benefit from endocrine treatment, whereas other patients have refractory disease or will develop resistance. The PI3K pathway has been implicated as a major

contributor to de novo or acquired resistance to endocrine therapy, and multiple lines of preclinical, translational and clinical evidence demonstrate that inhibition of the PI3K pathway can overcome endocrine resistance. GDC-0941, a highly selective and effective inhibitor of PI3K, has been shown to effectively block PI3K signalling in preclinical models and early clinical studies.

In the UK, patients currently have to wait an average of 2-4 weeks between establishing the diagnosis of early breast cancer and definitive surgery. It is established practice in many centres to treat patients with endocrine therapy as soon as the diagnosis of breast cancer has been established, and several clinical trials have shown that two weeks preoperative therapy with an AI or tamoxifen markedly reduces proliferation as measured by Ki67 in human breast cancer. Experimental evidence furthermore suggests that short duration endocrine therapy shortly before and immediately after breast cancer surgery might improve long term outcome with no additional toxicity or resource implications [32, 33, 35, 46, 47]. This hypothesis is currently being tested clinically in a randomised study supported by the UK NCRN (Adapted from the "POETIC Protocol v3, 22Dec2009, EudraCT: 2007-003877-21).

The main potential risks associated with this trial are delay of surgery, adverse reactions from giving GDC-0941, and adverse effects of obtaining tumour tissue specimen.

Risk of delaying surgery and risks associated with surgery: Given the current average of 2-4 weeks between establishing the diagnosis of breast cancer and surgery, participation in this trial of 2-week preoperative treatment was not expected to result in relevant delays of surgery for participants. To ensure that current UK therapeutic standards were kept, the protocol required definitive surgery to be performed within 15 days from the start of the study treatment. There was no preclinical, clinical or mechanistic evidence to suggest that GDC-0941 had a relevant impact on operability or increases the risks associated with surgery.

Safety plan: The current experience with single agent GDC-0941 in cancer patients confirmed that GDC-0941 could be given safely and was associated with an acceptable toxicity profile. However, GDC-0941 remained an experimental agent and additional side effects might be described at later stages. Most studies to date included heavily pre-treated patients with advanced or metastatic cancers. The majority of adverse effects in these studies were grade 1 or 2 and were generally rapidly reversible. The incidence of moderate or severe toxicities was low, especially during the first 2 weeks of treatment. Consequently, the risks associated with 15 days of preoperative treatment with GDC-0941 as part of this trial were expected to be low.

All enrolled patients were evaluated clinically and with standard laboratory tests before and at regular intervals during their participation in this study. Safety evaluations consisted of medical interviews, recording of adverse events, physical examinations, ECG recordings, and laboratory measurements. Patients were evaluated for adverse events (all grades), serious adverse events, and any adverse events requiring drug interruption or discontinuation throughout the course of the study. Any outcomes of these pre-specified early safety reviews that affect study conduct were communicated in a timely manner to the investigators for notification to the appropriate ethics committees.

Tumour tissue specimen: As part of the implementation of the POETIC study, a UK NCRN randomised phase 3 clinical trial of short-term perioperative endocrine therapy in an almost identical setting involving approximately 4000 patients, wide consultation with consumers and clinical colleagues at the UK Breast Intergroup and the NCRI's Breast Clinical Studies Group took place to minimise changes to routine clinical practice and ensure that the procedures and their

timing are acceptable and practical. This process highlighted the requirement for tissue taken from patients prior to their entry into a treatment study to be stored within an HTA licensed laboratory and the need for obtaining additional biopsies at later stages. As a result, the POETIC Pathway B was introduced to accommodate patients diagnosed prior to having the opportunity to consider the trial (Adapted from the "POETIC Protocol v3, 22Dec2009, EudraCT: 2007-003877-21).

The study development group followed closely the discussions around the POETIC study group and decided to adopt comparable processes for this trial. To minimize the need for additional tumour biopsies participating study centres had agreed to adopt pathway A as the preferred pathway for obtaining tumour tissue specimen. Although diagnostic tissue must be taken as part of routine care in all patients, additional tissue would have to be obtained for the purposes of this trial, which might add to the duration of any discomfort experienced during this routine procedure. Patients may have also need to undergo additional core biopsies as a separate procedure, which would be the same as the procedure they had already undergone for diagnostic tissue to be taken. Biopsies were carried out under local anaesthetic. Tissue taken at surgery would not add to pain or discomfort, as the tissue sample was taken after the tumour tissue had been removed from the patient.

3.3 Administrative Structure

Patients were enrolled from 10 centres in the UK. OPPORTUNE was Sponsored by the Brighton and Sussex University NHS Trust. The Sponsor was responsible for the overall study management (monitoring), drug supply, data management, statistical analysis, biomarker analysis and pharmacovigilance. The Chief Investigator, Prof P. Schmid, was responsible for medical writing of this clinical study report.

Two committees were convened to evaluate the safety of this trial. The first committee the Trial Management Group (TMG), included the chief investigator, principal investigators from each site, the study statistician, the study co-ordinator and the trial pharmacist. The second committee was a scientific Trial Steering Committee (TSC), which included external advisors who advised the Sponsor and the TMG on data interpretation and appropriate modifications to the study, as appropriate.

This trial was registered under ISRCTN26131497.

3.4 Ethics and Study Conduct

This study was conducted in accordance with GCP, and investigators were trained according to applicable Sponsor SOPs.

The Sponsor and the investigators strictly adhered to the stated provisions in these guidelines. This was documented by the investigator's signature, which indicated the investigator's agreement to carry out all of its terms in accordance with the applicable regulations and law and to follow ICH GCP guidelines for good clinical practice.

Approval from the London City East Research Ethics Committee (11/LO/1559) and the United Kingdom Medicines and Healthcare Products Regulatory Agency was obtained before study start.

Protocol amendments were prepared by the Sponsor and were submitted to the Ethics Committee and to Regulatory Authorities in accordance with local regulatory requirements. Approval was obtained from the Ethics Committee and to Regulatory Authorities (as locally required) before implementation of any changes.

3.5 Selection of Study Population

The target population for this trial were postmenopausal women with newly diagnosed, ER-positive, HER2-negative, invasive primary breast cancer of at least 1cm size. Patients must not have had definitive surgery, prior radiotherapy

or any prior systemic treatment for breast cancer including any prior endocrine therapy. Specific inclusion and exclusion criteria are detailed below:

3.5.1 Inclusion Criteria

1. Histologically confirmed breast cancer
2. A palpable tumour of any size, or a tumour with an ultrasound size of at least 1.0 cm
3. No evidence of metastatic spread by standard assessment according to local guidelines
4. Oestrogen receptor (ER) positive tumours with ≥1% of tumour cells positive for ER on immunohistochemical staining or an immunohistochemistry score (Allred) of 3 or higher
5. No prior systemic treatment regimens for the new primary breast cancer currently under investigation; prior treatment for previous breast cancer is allowed as long as it was completed at least 1 year prior to inclusion into this trial.
6. Postmenopausal, defined as:
    a. Age $\geq$ 55 years and 1 year or more of amenorrhea
    b. Age $<$ 55 years and 1 year or more of amenorrhea, with an estradiol assay $<$ 20 pg/mL
    c. Age $<$ 55 with prior hysterectomy but intact ovaries with an estradiol assay $<$ 20 pg/mL
    d. Status after bilateral oophorectomy ($\geq$ 28 days prior to first study treatment)
7. Adequate hematologic function (ANC $\geq$ 1500 cells/µl, and platelet count $\geq$ 100000/µl).
8. Serum creatinine concentration < 1.5 x ULN
9. AST, ALT, bilirubin level < 1.5 x ULN
10. Fasting plasma glucose level < 7.8 mmol/L
11. ECOG performance status 0-2
12. Written informed consent prior to admission to this study

3.5.2 Exclusion Criteria

1. Men
2. Inflammatory breast cancer
3. HER2-positive tumours with 3+ intensity on IHC staining for HER2 or amplification of the HER2 gene on ISH
4. Evidence of distant metastases
5. Concurrent use of HRT (HRT users must stop HRT a minimum of four weeks before the
6. baseline diagnostic biopsy is taken)
7. Previous systemic or local treatment for the new primary breast cancer currently under investigation (including surgery, radiotherapy, cytotoxic and endocrine treatments); prior treatment for previous breast cancer is allowed as long as it was completed at least 1 year prior to inclusion into this trial.
8. Previous systemic treatment for other neoplasms within 1 year prior to inclusion into this trial.
9. Clinically significant pulmonary dysfunction
10. Significant cardiovascular disease, such as
    a. History of myocardial infarction, acute coronary syndromes (including unstable angina), or history of coronary angioplasty/stenting/bypass grafting.

b. History of symptomatic congestive heart failure (CHF) New York Heart Association (NYHA) Classes II-IV or LVEF <50% by either ECHO or MUGA
c. Severe cardiac arrhythmia requiring medication or severe conduction abnormalities
d. Poorly controlled hypertension (resting diastolic blood pressure >100 mmHg)
e. Clinically significant valvular disease, cardiomegaly, ventricular hypertrophy, or cardiomyopathy

10. QTc prolongation defined as a QTc interval >460 msecs or other significant ECG abnormalities including 2nd degree (type II) or 3rd degree AV block or bradycardia (ventricular rate <50 beats/min)
11. Uncontrolled Type 1 or 2 diabetes mellitus (diabetic patients must have been on a stable regimen of oral anti-hyperglycaemic therapy for at least 3 weeks duration and must have home monitoring levels without fasting blood glucose >8.9 mmol/L or hypoglycaemia for one week prior to study entry)
12. Any condition requiring anti-coagulants, such as warfarin, heparin, or thrombolytic drugs
13. History of documented haemorrhagic diathesis or coagulopathy
14. Malabsorption syndrome or other condition that would interfere with enteral absorption
15. Uncontrolled hypomagnesemia or hypokalaemia, defined as values below the lower limit of normal (LLN), or hypercalcemia above the ULN for the institution despite adequate electrolyte supplementation or management
16. Clinically significant history of liver disease, including cirrhosis, current alcohol abuse, or current known active infection with hepatitis B virus, or hepatitis C virus; Active infection is defined as requiring treatment with antiviral therapy or presence of positive test results for Hepatitis B (Hepatitis B surface antigen [HBsAg] and/or total Hb core antibody [anti-HBc]) or Hepatitis C (Hepatitis C virus [HCV] antibody); Patients who are positive for anti-HBc are eligible only if testing is also positive for Hepatitis B surface antibody [HbsAb] and polymerase chain reaction (PCR) is negative for HBV DNA; Patients who are positive for HCV serology are eligible only if testing for HCV RNA is negative.
17. Cerebrovascular disorders / vascular dementia
18. Serious intercurrent medical or psychiatric illness, including serious active infection
19. Concurrent treatment with other experimental drugs or participation in another clinical trial with any investigational drug within 30 days prior to study entry.
20. Any other disease, metabolic dysfunction, physical examination finding, or clinical laboratory finding that, in the investigator's opinion, gives reasonable suspicion of a disease or condition that contraindicates the use of an investigational drug or that may affect the interpretation of the results or render the patient at high risk from treatment complications

3.6 Study Treatments

3.6.1 Dosage and Administration

Patients were randomly assigned (2:1, favouring the combination) to receive treatment with Anastrozole or Anastrozole plus GDC-0941. Anastrozole was given at a dose of 1 mg once per day. GDC-0941 was initially administered at 340 mg once per day; from August 2012 onward, GDC-0941 was reduced to 260 mg once per day according to safety data from other studies that indicated a lower rate of mucosal and skin toxicity at 260 mg. Five evaluable patients received GDC-0941 at 340 mg; the remaining patients received GDC-0941 260 mg.

Treatment in both arms was continued until surgery on day 15 (± 2 days) unless there was evidence of unacceptable toxicity or the patient requested to be released. Patients in the anastrozole + GDC-0941 arm received the last dose of GDC-0941 within 2-4 hours prior to surgery. If patients experienced significant adverse events that could potentially impact on the operability, surgery was delayed until adverse events had improved to grade 1 or resolved. In those cases GDC-0941 should be discontinued permanently whereas anastrozole should be continued until surgery. If definitive surgery could be performed on day 15 (± 2 days) patients were required to undergo a core biopsy on day 15 to assess the effect of the study treatment.

Following surgery all patients will be treated in accordance with local policy based on national clinical guidelines that prevailed at the time. According to national guidelines in place at the time OPPORTUNE was designed, patients were expected to received adjuvant endocrine treatment with either tamoxifen or an aromatase inhibitor, or each sequentially, for a minimum of 5 years. It was recognised at the time that these practices could change during the course of the trial.

3.6.2 Formulation and Packaging

GDC-0941 was supplied by Genentech. Anastrozole was purchased by the sites and the Sponsor reimbursed this cost.

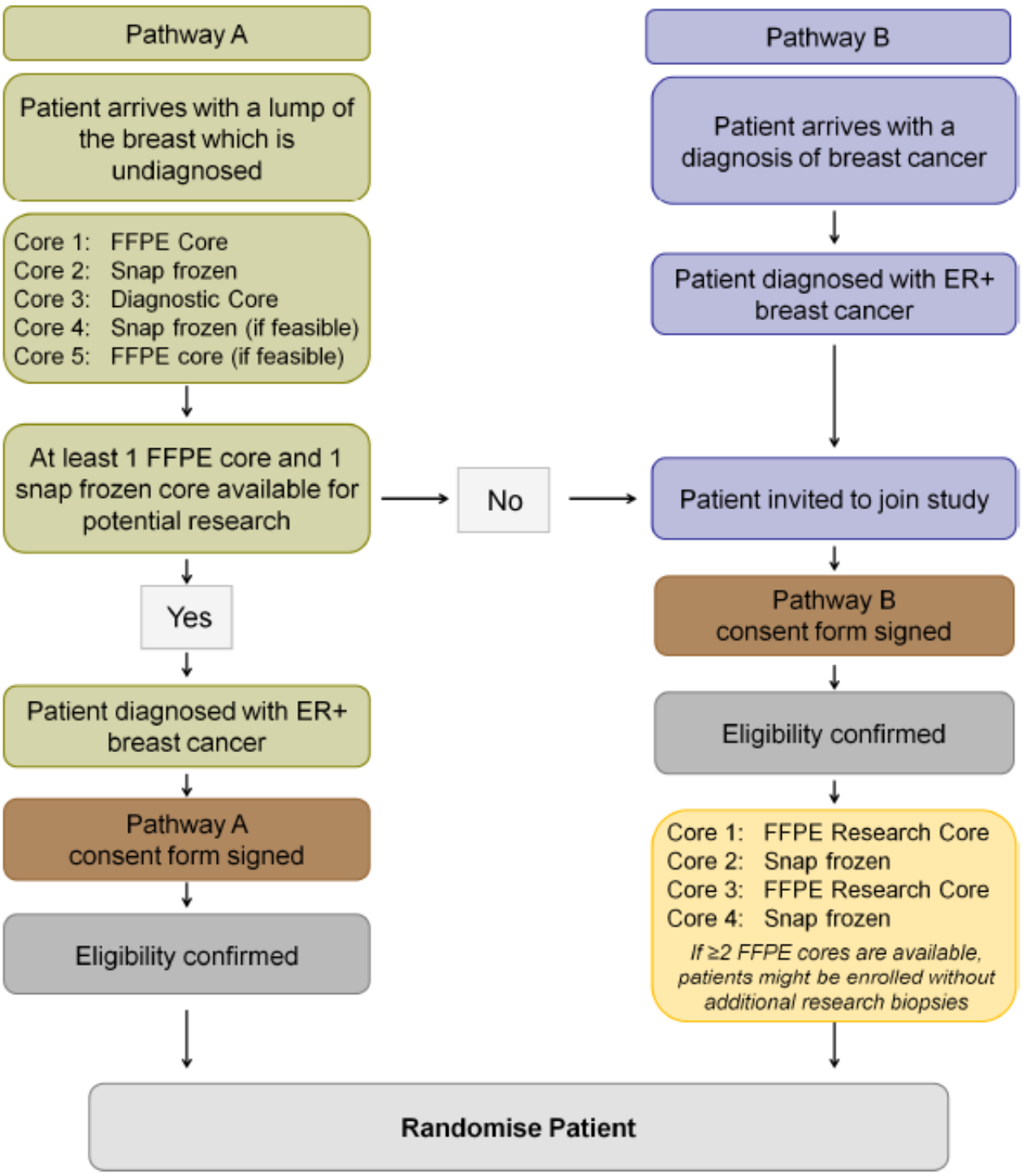
Anastrozole and GDC-0941 were supplied as open-label stock. All labels fulfilled all requirements specified by governing regulations. Anastrozole and GDC-0941 were stored in accordance with the relative governing regulations, the Summary of Product Characteristics (SmPC), the pharmacy manual, the Investigators Brochure (IB), and hospital procedure.

3.6.3 Method of Treatment Assignment

Participating centres entered patients into the study via Pathway A or Pathway B. All centres adhered to guidance from the Human Tissue Authority on taking and storing tissue for patients prior to their entry into this trial, details of which were available from the trials office.

Pathway A: Required tissue available for research to be taken from patients at the same time as the diagnostic core biopsy, and stored in accordance with any regulatory requirements. This may have included surplus tissue taken from the diagnostic core biopsy or additional tissue samples specifically taken for research. If additional core biopsy samples were taken for research, generic consent had to be gained prior to diagnostic core biopsy. The patient could be offered the trial as soon as ER positive breast cancer was confirmed.

Pathway B: Should be considered for patients whose diagnostic core biopsy was performed before the opportunity to consider possible entry to this trial. Patients consenting to the trial would be asked to have additional research biopsy cores taken after written informed consent to this study, and before randomisation. However, if two or more FFPE cores from the initial diagnostic core biopsy were available for analyses within this trial, patients might be enrolled onto the study without additional research biopsies.



Randomisation was stratified by histological grade. Separate randomisation lists were prepared for each participating centre. Treatment allocation was 2:1 (Anastrozole + GDC0941 : anastrozole alone) using computer generated random permuted blocks. Patients had to receive their first dose of study treatment no later than 7 days after randomisation. Surgery was booked as soon as possible after randomisation and scheduled for approximately two week after the initiation of study treatment.

3.6.4 Blinding

This was an open-label study.

3.6.5 Criteria for Dose Modification or Withdrawal from Treatment

No dose reductions were allowed for Anastrozole. Anastrozole could be held or discontinued at the discretion of the treating physician for endocrine-related toxicity. Patients could discontinue GDC-0941 and remain on study until surgery.

No dose reductions were allowed for GDC-0941. Treatment with GDC-0941 had to be interrupted and held until recovery to Grade ≤1 (or baseline value) in the event of

- Grade 2, 3 or 4 elevation of Liver function tests (LFTs)
- Symptomatic Grade 3 hyperglycaemia or Grade 4 hyperglycaemia (Changes in anti-diabetic medication are not allowed during the study; if changes in the anti-diabetic medication are required, the patient has to discontinue treatment with GDC-0941 and should be closely monitored after discontinuation because of the risk of hypoglycaemia).
- Grade 3 maculo-papular rash
- Grade 2, 3, or 4 pneumonitis

Treatment with GDC-0941 was discontinued in the event of any Grade 4 toxicities or Grade 3 toxicities other than elevation of LFTs or rash. Anastrozole could be held or discontinued at the discretion of the treating physician

3.6.6 Treatment Accountability and Compliance

Anastrozole and GDC-0941 were self-administered outpatient treatments. Patients were encouraged to take the required doses according to the treatment plan and asked to report and omissions to the investigator or study nurse and record these in the patient diaries and CRF as applicable. Any dispensed but unused study drug at the end of each treatment period was counted and recorded on the CRF to calculate the total dose received by the patient.

3.7 Concomitant Medications

Recommended concurrent treatment during study participation included:

- Standard anti-emetic therapy including a 5-HT3-agonist given as needed on a prophylactic and treatment basis in compliance with local centre standards.
- Loperamide for symptomatic treatment of diarrhoea ≥ grade 2

The following medications were prohibited:

- Any investigational agent other than anastrozole and GDC-0941
- Changes in anti-diabetic medication were not allowed during the study; if changes in the anti-diabetic medication were required, the patient had to discontinue treatment with GDC-0941 and should be closely monitored after discontinuation because of the risk of hypoglycaemia.
- Concomitant use of potent proton-pump inhibitors such as omeprazole, lansoprazole, pantoprazole, dexlansoprazole, esomeprazole, and rabeprazole (may decrease the plasma levels of GDC-0941).
- Antacids should not be taken within 4 hours of a GDC-0941 dose (may decrease the plasma levels of GDC-0941).
- H2-histamine receptor antagonists should not be taken within 10 hours before and 2 hours after a GDC-0941 dose (may decrease the plasma levels of GDC-0941).
- Concomitant use of CYP3A4/5 inhibitors: atazavanir, clarithromycin, indinavir, itraconazole, ketoconazole, nefazodone, nelfinavir, ritonavir, saquinavir, telithromycin (may increase the plasma levels of GDC-0941).

- Concomitant use of CYP3A4/5 and/or CYP2C8 inducers: rifampin, carbamazepine (may increase the plasma levels of GDC-0941).
- Anti-cancer treatments including cytotoxic, hormonal or specific immune therapy until surgery or discontinuation of the study treatment.

3.8 Assessments

Refer to section 8 of the protocol for full details of study assessments.

3.9 Statistical Hypothesis and Planned Sample Size

Patients were randomised 2:1 to anastrozole + GDC-0941 and anastrozole, respectively. Patients excluded from the Per-Protocol-Population were replaced. The planned study size was 94 evaluable patients in the anastrozole plus GDC-0941 group and 47 evaluable patients in the anastrozole group, respectively, to provide 80% power to detect an effect size of 0.58 between Anastrozole and Anastrozole + GDC-0941 inhibitor at the 5% significance level. The effect size (ES) is defined as the treatment difference divided by the standard deviation, i.e. ES = [M1 - M2]/σpooled where M1 and M2 were the mean values of the differences of proportional Ki67 changes and σpooled = $\sqrt{[(\sigma 1^2 + \sigma 2^2)/2]}$. Taking Cohen´s standard interpretation of effect sizes into account, 0.5 is the lower limit of medium effect. An effect size of 0.5 corresponds to 33% of non-overlap between the two treatment groups. The non-centrality parameter δ is 2.83. Critical t is 1.98.

Group sample sizes of 47 patients in the Anastrozole group and 94 in the Anastrozole + GDC-0941 group also achieve 80% power to detect a difference between the group response rates of 20%. Response is defined as a 50% or higher fall in Ki67 expression. The proportion of responders in the Anastrozole + GDC-0941 group is assumed to be 60% under the null hypothesis and 80% under the alternative hypothesis. The proportion in the Anastrozole group is assumed to be 60%. The test statistic used is the one-sided Z test with pooled variance. The significance level is 5.1%. If the difference between the group response rates is 25%, then the sample size will be needed 28+56=84. On the other hand, if the difference is 30%, then only 18+36=54 patients will be sufficient to detect this difference.

Individual end-of treatment anti-proliferative response ($Response_{Ki67\text{-}Post}$), defined as the natural logarithm of percentage Ki67 positive cells of less than 1 or 1-2 at the end of study treatment, is another endpoint under which required sample size might be slightly lower. Assuming that approximately 40% of patients have activating PI3KCA mutations or PTEN deletions, the study will provide 80% power at a 5% significance level to detect an ES of 0.8.

3.9.1 Analysis Populations

All Ki-67 and Caspase3 analyses were performed on a Per-Protocol Population, defined as all randomised patients who completed 15 days (±2 days) of study treatment and for whom tumour biopsy specimens at baseline and at 15 (±2) days were available for assessment of biological response. The per-protocol population also excluded patients who had a major violation of protocol inclusion or exclusion criteria. Patients excluded from the Per-Protocol-Population were replaced.

Safety analysis was conducted on all patients who received at least one dose of the study treatment, with patients analysed according to the treatment they actually received.

3.9.2 Efficacy Analysis

The main analysis of apoptosis and proliferation were from baseline to day 15 using non-parametric statistics to compare the log (surgical/Pre-treatment) scores. Additional analyses of apoptosis and proliferation were from day 15 to definitive surgery.

Treatment comparisons were tested with and without adjustment for baseline prognostic factors. In the absence of major confounding factors the latter would be considered secondary endpoints.

On the assumption of a log normal distribution, Ki67 values were log transformed before analysis of mean ΔKi67, mean Ki67post, and ResponseKi67-Post. Ln(Ki67post) and ln(Ki67pre) were used to calculate the geometric means. 0.1 was added to every untransformed Ki67 value to avoid the mathematical anomaly that arises because the log of zero is minus infinity. As a consequence of the assumption of a lognormal distribution, ln(Ki67post)–ln(Ki67pre) was also normally distributed. This formula gave the proportional change, and as a result mean log proportional changes and CI were calculated and displayed on their original scale by back transformation. Mean ΔKi67 and mean Ki67post was compared between groups by use of the t- test, and the proportional change within groups was analysed with the paired t- test. The proportional reduction was calculated as one minus the proportional change.

Anti-proliferative response ResponseΔKi67 and end-of treatment anti-proliferative response ResponseKi67-Post were calculated in all evaluable patients. An estimate of the anti-proliferative response rates RRΔKi67 and end-of treatment anti-proliferative response rates RRKi67-Post and 95% CIs (Clopper-Pearson 1934) was calculated for each treatment arm. CIs for the difference in response rates (Satner and Snell 1980; Berger and Boos 1994) was calculated. The relative risk (treatment : control) was reported along with the associated 95% confidence interval based on logistic regression model.

A similar analyses strategy was applied for Caspase3 endpoints.

The change in tumour size was expressed as the proportional change from baseline to post-treatment. A two-sided $\chi 2$ -test was used to compare clinical or pathologic tumour response for the treatments arms. Changes in secondary outcomes from baseline to post treatment was analysed between treatment groups with the Wilcoxon Mann-Whitney test and within treatment groups with the Wilcoxon signed rank test. Associations between outcomes was investigated by use of the Spearman's rank correlation coefficient.

3.9.3 Subgroup Analysis

The effects of the study treatment was assessed separately in patients with and without PI3K mutations and/or PTEN deletions, Luminal A and B subtypes and patients with high (>14%) or low (≤14% baseline Ki67) . Additional subgroups might be defined by the exploratory biomarker analysis.

3.9.4 Safety Reporting and Analysis

Safety data was reported for all patients who received at least one dose of the study treatment. Safety data were reported separately for each treatment group. The worst toxicity during each cycle and the worst toxicity during the entire treatment will be determined separately for each patient according to the criteria specified above.

3.9.5 Exploratory Analysis

The potential relationship of exploratory biomarkers with biological response (Ki67, Caspase3) and clinical response will be explored.

3.9.6 Changes in Conduct of Study or Planned Analyses

None

4 RESULTS: STUDY POPULATION

4.1 Disposition of Patients

Between January 2012, and September 2015, 167 patients underwent randomization (Figure 2). 54 patients were assigned to anastrozole alone and 113 patients to anastrozole plus pictilisib. Two patients were excluded because of violations of key eligibility criteria. Another two patients withdrew trial consent prior to the start of their study treatment. Assessment of the treatment effects was possible for 136 patients who successfully completed the protocol; 27 patients (8 in the anastrozole arm and 19 in the combination arm) had insufficient tissue for analysis.

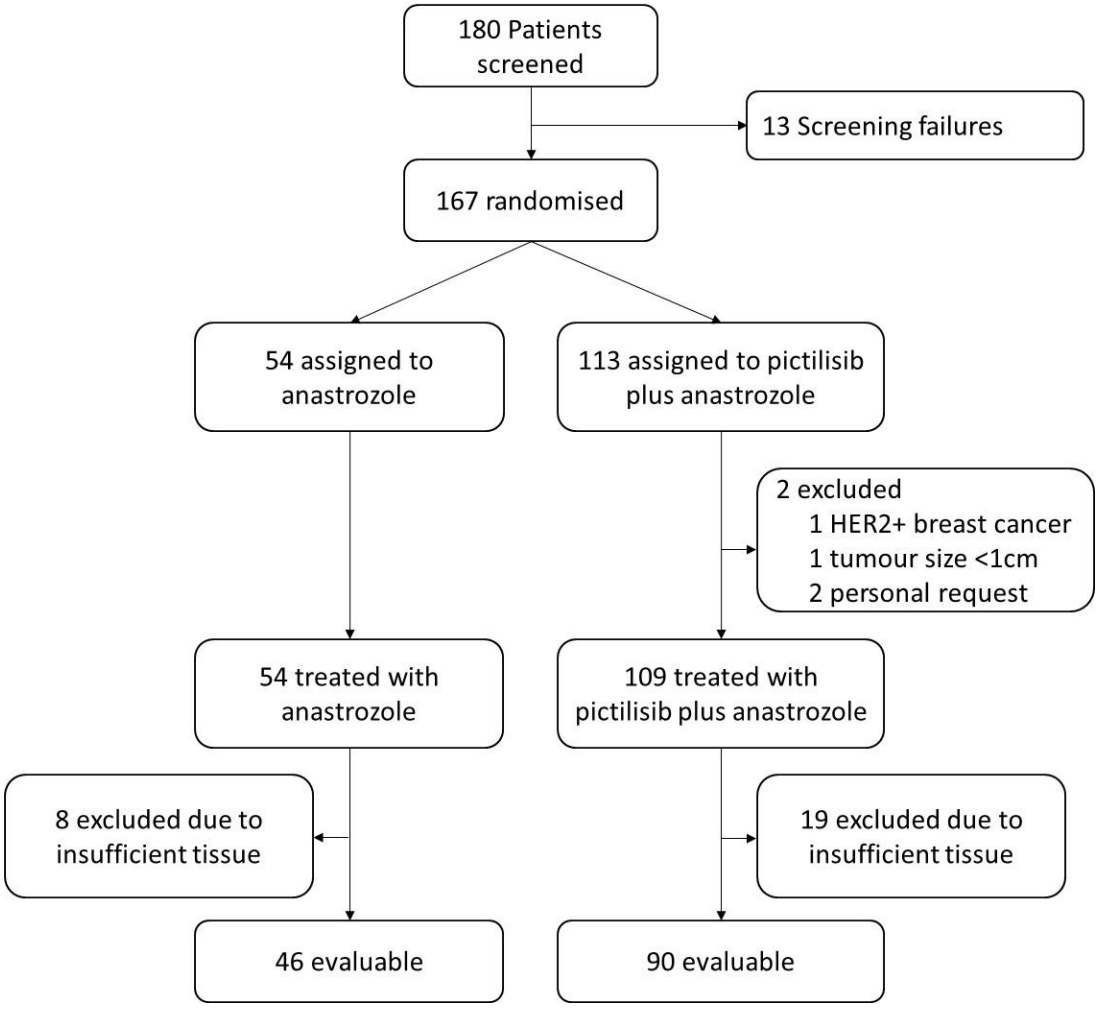


Figure 2: Trial Consort Diagram.

4.2 Demographic Data and Baseline Characteristics

Baseline distributions of patient and tumour characteristics were similar in the treatment arms (Table 1); 62% of tumours were classified as Luminal B according to PAM50 analysis and 63.2% according to baseline Ki67 analysis using a cut-off of 14%. 58.8% of tumours were PIK3CA wildtype. There was a slightly higher number of patients with PR positive tumours in the combination group.

	Anastrozole alone (n = 46)	Pictilisib plus Anastrozole (n = 90)
Age (years)		
Median (range)	66.9 (47.7-85.4)	64.1 (48.5-81.1)
Tumour status		
Grade 1	5 (10.9%)	13 (14.6%)
Grade 2	34 (73.9%)	62 (69.7%)
Grade 3	7 (15.2%)	14 (16.9%)
PR status		
Positive	33 (71.4%)	82 (91.1%)
Negative	11 (23.9%)	6 (6.7%)
Molecular Subtype (PAM50)		
Luminal A	6 (31.6%)	14 (41.2%)
Luminal B	13 (68.4%)	20 (58.8%)
Ki67 (% positive tumour cells)		
Mean (range)	23.0 (1.9-84.1)	22.7 (0.9-89.9)
0-14	15 (32.6%)	35 (38.9%)
>14	31 (67.4%)	55 (61.1%)
PIK3CA mutation status		
Wildtype	27 (58.7%)	53 (58.9%)
Mutation	19 (41.3%)	30 (33.3%)
Kinase-domain mutation	14 (30.4%)	15 (16.7%)
Helical-domain mutation	5 (10.9%)	14 (15.6%)

Table 1: Patient demographics and tumour characteristics a baseline. Kinase-domain mutations include H1047R/Y, H1048R, G1049D/R. helical domain mutations include E524K, E545K.

5.1.1 Effect of study treatment on cell proliferation

Tumour Ki67 expression decreased in 93.4% of patients over the course of the study treatment from baseline to day 15 (Figure 3 &4); in 9 patients Ki67 expression numerically increased, including 5 patients in the anastrozole group (10.9% of all patients treated with anastrozole) and 4 patients in the combination group (4.4%). More patients in the combination group (87.8%) had an EOT Ki67 expression of <10% compared to anastrozole alone (71.7%).

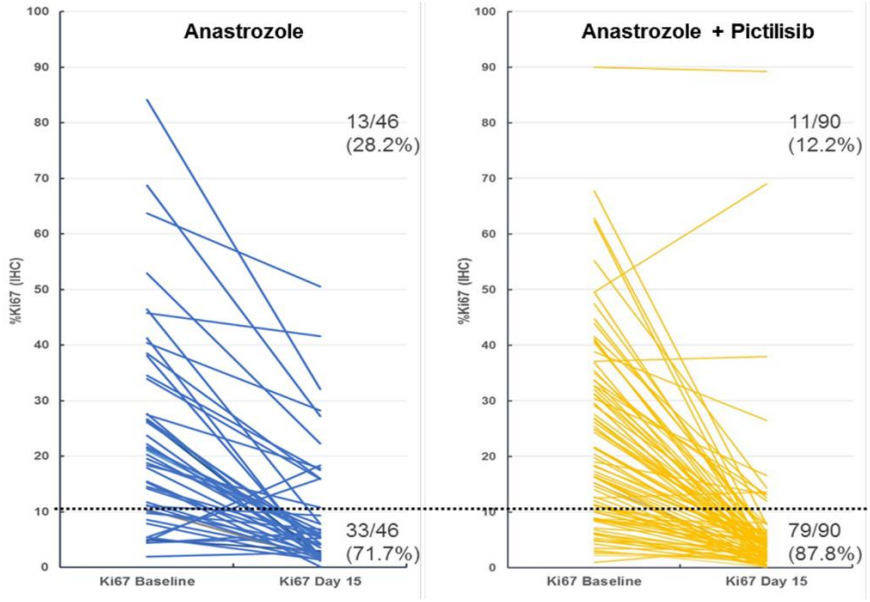


Figure 3: Individual changes in percentage Ki67 expression from baseline to Day 15; the number and percentage of patients achieving an end of treatment (EOT) Ki67 score of >10% or ≤10% are provided for each group.

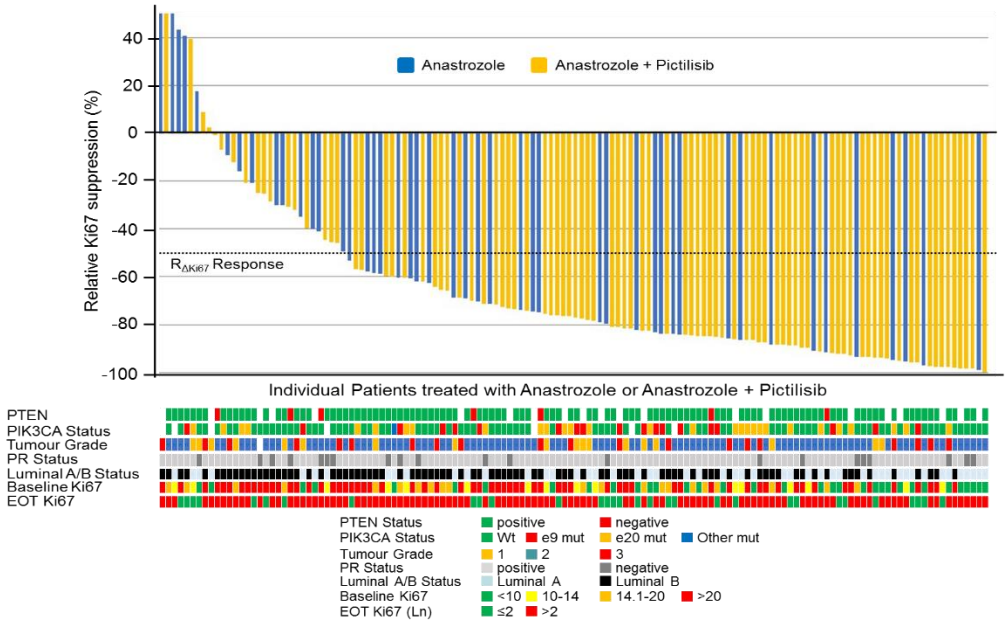


Figure 4: Individual relative Ki67 suppression sorted from low to high; relative Ki67 Suppression is defined as Ln(Ki67Day15) – Ln(Ki67baseline); results are displayed on their original scale by back transformation. Individual PTEN status, PIK3CA status, PR status, Luminal A/B status, tumour grade, Baseline and end of treatment (EOT) Ki67 expression are indicated as a coloured box under each patient.

Mean percentage suppression of Ki67 was 82.5% (95% CI, 78.3%-85.8%) for anastrozole plus pictilisib treated patients and 70.7% (61.0%-78.0%) for anastrozole treated patients (Table 2; Figure 5). The ratio (combination/anastrozole) of mean Ki67 suppression was 0.60 (0.58-0.85; p=0·01). The geometric mean end of treatment Ki67 expression was 6.3% (3.7%-8.8%) for anastrozole plus pictilisib and 9.5% (6.3%-12.8%) for anastrozole alone (p=0.02). The EOT response rate RKi67-Day15 was higher with the combination 83.3% (76.8%-90.9%) compared to anastrozole alone 65.2% (53.3%-77.1%; p=0.02).

	Anastrozole (n = 46)	Pictilisib plus Anastrozole (n = 90)	Relative Risk (combination/ anastrozole)	p-Value
Geometric mean Ki67 suppression [% (95% CI)]	70.7% (61.0%-78.0%)	82.5% (78.3%-85.8%)	0.60 [1] (0.58-0.85)	p=0.01
The geometric mean EOT Ki67 expression [% (95% CI)]	9.5% (6.3%-12.8%)	6.3% (3.7%-8.8%)	0.66 [1] (0.58-0.69)	p=0.02
$R_{\Delta KI67}$ response rate [% (95% CI)]	69.6% (58.0%-81.1%)	81.1% (74.2%-88.0%)	1.17 (0.97-1.40)	p=0.10
$R_{KI67\text{-}Day15}$ response rate [% (95% CI)]	65.2% (53.3%-77.1%)	83.3% (76.8%-90.9%)	1.36 (1.05-1.55)	p=0·02

Table 2: Anti-proliferative response to ANA or ANA+PIC. Geometric mean Ki67 suppression defined as Ln(Ki67Day15) - Ln(Ki67baseline); the ratio (combination/ anastrozole) of geometric mean Ki67 suppression provided with 95% CI. Geometric mean end-of-treatment (EOT) Ki67 expression defined as Ln(Ki67Day15)]; individual EOT anti-proliferative response RKi67-Day15 defined as Ln(Ki67Day15) ≤2; individual anti-proliferative response RΔKi67 defined as a ≥50% fall in Ki67 expression between baseline and Day 15.

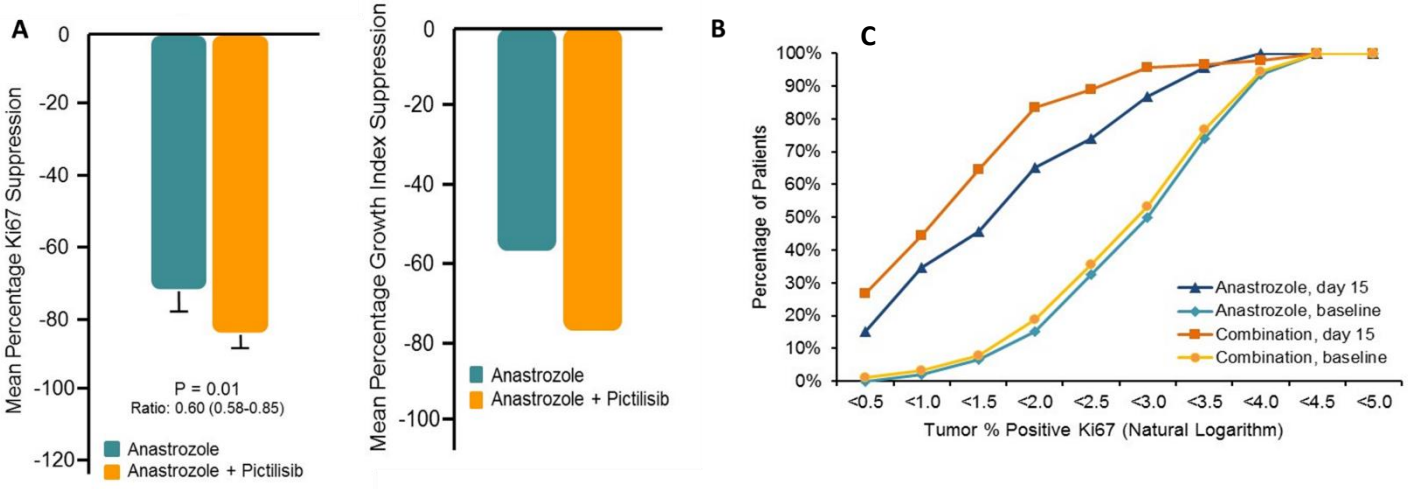


Figure 5: A) Anti-proliferative response expressed as the geometric mean Ki67 suppression in from baseline to day 15. B) Mean percentage Growth Index suppression. C) Figure 6: Cumulative proportion (by percentage) of patients who had tumours with %-positive Ki67 (expressed as natural logarithm) less than the value on the X axis is illustrated at BL and at D15

5.1.2 Effect of Study Treatment on Tumour Cell Apoptosis

Overall, the rate of apoptosis was low throughout the trial and there were no significant differences between baseline and end of treatment apoptosis levels within and between treatment groups (Table 3). Given the established positive correlation between Ki67 and apoptosis (Figure 7), the growth index defined as percent Ki67-expresion divided by percent Caspase-3 expression was analysed. There was a greater suppression in the growth index in the combination arm (75.2%) compared to anastrozole alone 55.9% (Table 4), (Figure 8).

	Anastrozole (n = 33)	Pictilisib plus Anastrozole (n = 56)	Relative Risk (combination/ anastrozole)	p-Value
The geometric mean baseline Casp-3 expression [% (95% CI)]	0.14% (0.10%-0.18%)	0.15% (0.11%-0.19%)	NS	NS
The geometric mean EOT Casp-3 expression [% (95% CI)]	0.14% (0.11%-0.18%)	0.14% (0.10%-0.19%)	NS	3NS

Table 3: Induction of apoptosis with anastrozole or anastrozole plus pictilisib. Geometric mean Ki67 suppression is defined as Ln(Ki67Day15) - Ln(Ki67baseline); the ratio (combination/ anastrozole) of geometric mean Ki67 suppression is provided with 95% CI. Geometric mean end-of-treatment (EOT) Ki67 expression is defined as Ln(Ki67Day15)]; individual EOT anti-proliferative response RKi67-Day15 is defined as Ln(Ki67Day15) ≤2; individual anti-proliferative response RΔKi67 is defined as a ≥50% fall in Ki67 expression between baseline and Day 15.

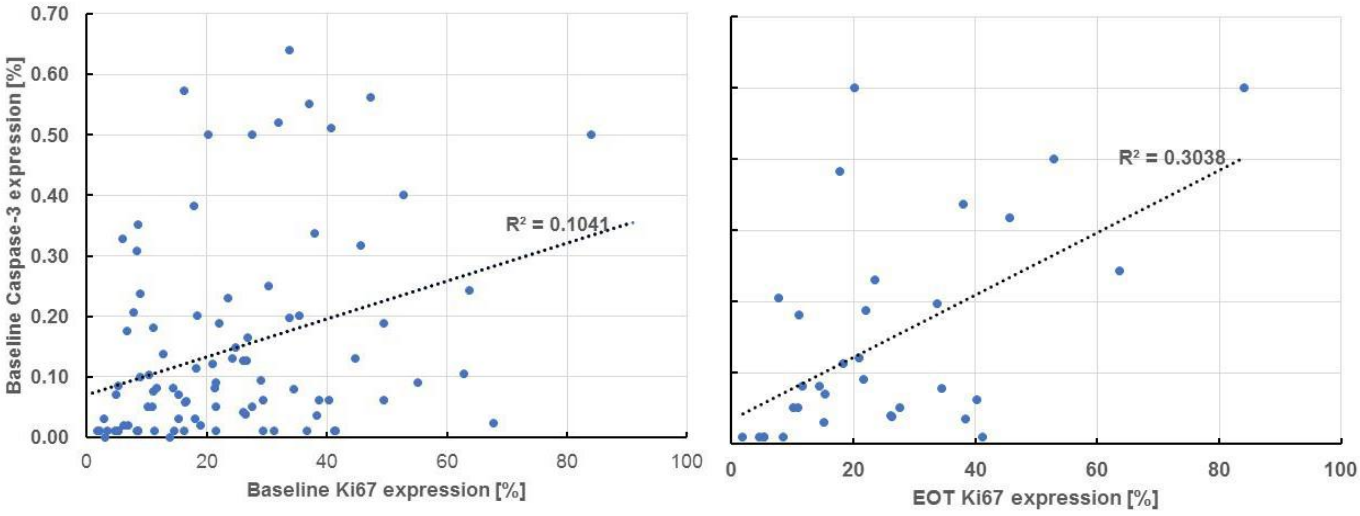


Figure 7: Relationship between Ki67(%) and apoptosis (%) before and after 2 weeks of treatment irrespective of treatment arm.

	Anastrozole (n = 33)	Pictilisib plus Anastrozole (n = 56)	Relative Risk (combination/ anastrozole)	p-Value
The geometric mean baseline growth index [% (95% CI)]	433.2 (190.0-676.4)	635.5 (381.2-889.8)	NS	NS
The geometric mean EOT growth index [% (95% CI)]	190.9 (71.5-310.3)	157.5 (93.0-222.1)	NS	NS
The mean relative growth index suppression [% (95% CI)]	55.9 (4.4-270.7)	75.2 (25.3-191.8)	NS	NS

Table 4: Treatment-associated change in growth index (GI), defined as Ki67[%]/Caspase-3[%], with anastrozole or anastrozole plus pictilisib

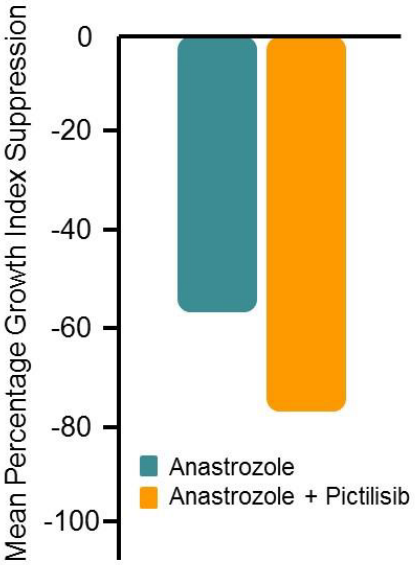


Figure 8: Mean percentage Growth Index suppression

5.2 Subgroup and Exploratory Analyses

5.2.1 PIK3CA mutation subtypes and response to study treatment:

Predefined subset analyses investigated potential interactions of PI3K mutations, luminal A/B subtypes, and baseline Ki-67 scores with Ki-67 response. Three major hotspots of mutations of the PIK3CA gene have been described; these are concentrated in the helical (E542K and E545K) and kinase (H1047R) domains, accounting to approximately 90% of all PIK3CA mutations. In the OPPORTUNE trial, PIK3CA mutations were tested using NGS. Given the limited power of these analyses, results must be considered exploratory and interpreted with caution.

At least one PIK3CA mutation was detected in 49 tumours (36.0%), including 19 helical domain and 29 kinase domain mutations. There was no significant correlation between PIK3CA mutation and added activity of pictilisib; the ratio (combination/anastrozole) of geometric mean Ki67 proportional change was 0.63 (0.39–1.0; p=0.05) for patients with PIK3CA-wildtype tumours and 0.72 (0.46–1.15; p=0.12) for patients with PIK3CA-mutated tumours.

A significant interaction was observed between PIK3CA mutation subtypes [helical domain mutations (HD), kinase domain mutations (KD), wildtype (WT)] and mean Ki67 suppression. The combination/anastrozole geometric mean ratio of Ki67 suppression was 0.48 (0.27-0.84; p=0.02) for patients with HD mutations and 0.63 (0.39–1.0; p=0.05) for patients with PIK3Ca WT, compared to 1.17 (0.57–2.41; p=0.64) for patients with KD mutations. This was largely due to patients with HD mutations showing a particularly poor response to anastrozole alone [mean Ki67 suppression 53.9% (9.5%-76.5%)], that was reversed by the addition of pictilisib [mean Ki-67 suppression 78.1% (71.0%-83.4%)]. On the other hand, patients with KD mutations responded well to anastrozole alone [mean Ki-67 suppression 77.7% (57.0%-88.4%)] and showed no benefit from the addition of pictilisib [mean Ki-67 suppression 73.9% (59.8%-83.0%)].

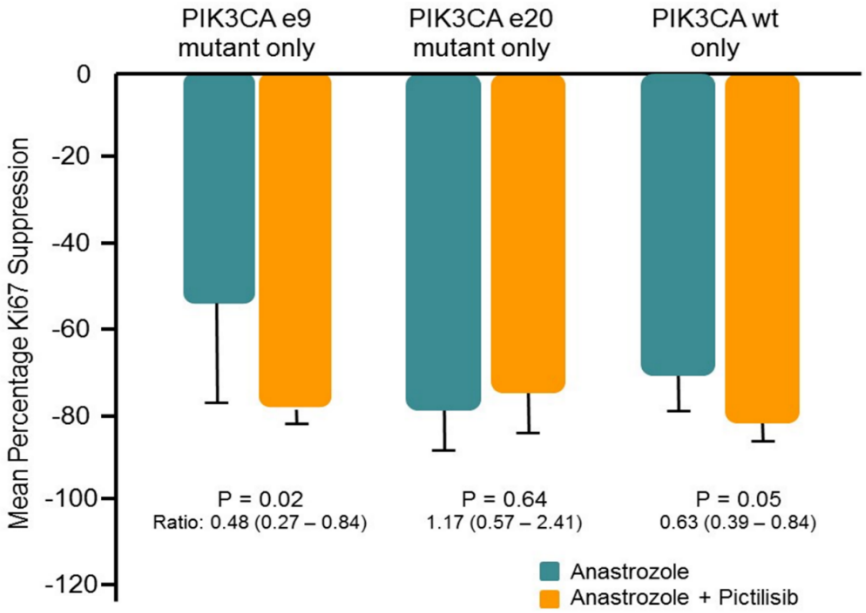


Figure 9: Anti-proliferative response to study treatment by PIK3CA mutation status; e9: exon 9 domain mutations (helical domain); e20: exon 20 domain mutations (kinase domain).

	Anastrozole (n = 46)	Pictilisib plus Anastrozole (n = 90)	Relative Risk (combination/ anastrozole)	p-Value
Geometric mean Ki67 suppression [% (95% CI)]				
PIK3Ca WT	69.9 (45.7 – 80.2)	81.1 (75.2 – 85.6)	0.63 (0.39–1.0)	0.05
HD mutations	53.9 (9.5 - 76.5)	78.0 (71.0 – 83.3)	0.48 (0.27-0.84)	0.02
KD mutations	77.7 (57.0-88.4)	73.9 (59.8 – 83.0)	1.17 (0.57–2.41	0.64
$R_{Ki67\text{-}Day15}$ response rate [% (95% CI)]				
PIK3Ca WT	59.3 (42.8-75.7)	84.9 (76.6-93.2)	1.43 (1.08-1.89)	0.01
HD mutations	75.0 (16.2-133.8)	84.6 (66.1-103.3)	1.13 (0.68 -1.88)	0.58
KD mutations	81.8 (59.7-103.9)	78.6 (58.4-98.7)	0.96 (0.69-1.33)	0.62

Table 5: PIK3CA status and anti-proliferative response to anastrozole or anastrozole plus pictilisib. Geometric mean Ki67 suppression is defined as Ln($Ki67_{Day15}$) - Ln($Ki67_{baseline}$); the ratio (combination/ anastrozole) of geometric mean Ki67 suppression is provided with 95% CI; individual EOT anti-proliferative response $R_{Ki67\text{-}Day15}$ is defined as Ln($Ki67_{Day15}$) ≤2.

Further NGS analysis demonstrated a range of somatic mutations in keeping with the expected mutational landscape of ER-positive early breast cancer. Figure 10 shows an overview of the somatic variants for each treatment group, divided by response to treatment. There was no specific mutational pattern associated with response to anastrozole or anastrozole plus pictilisib.

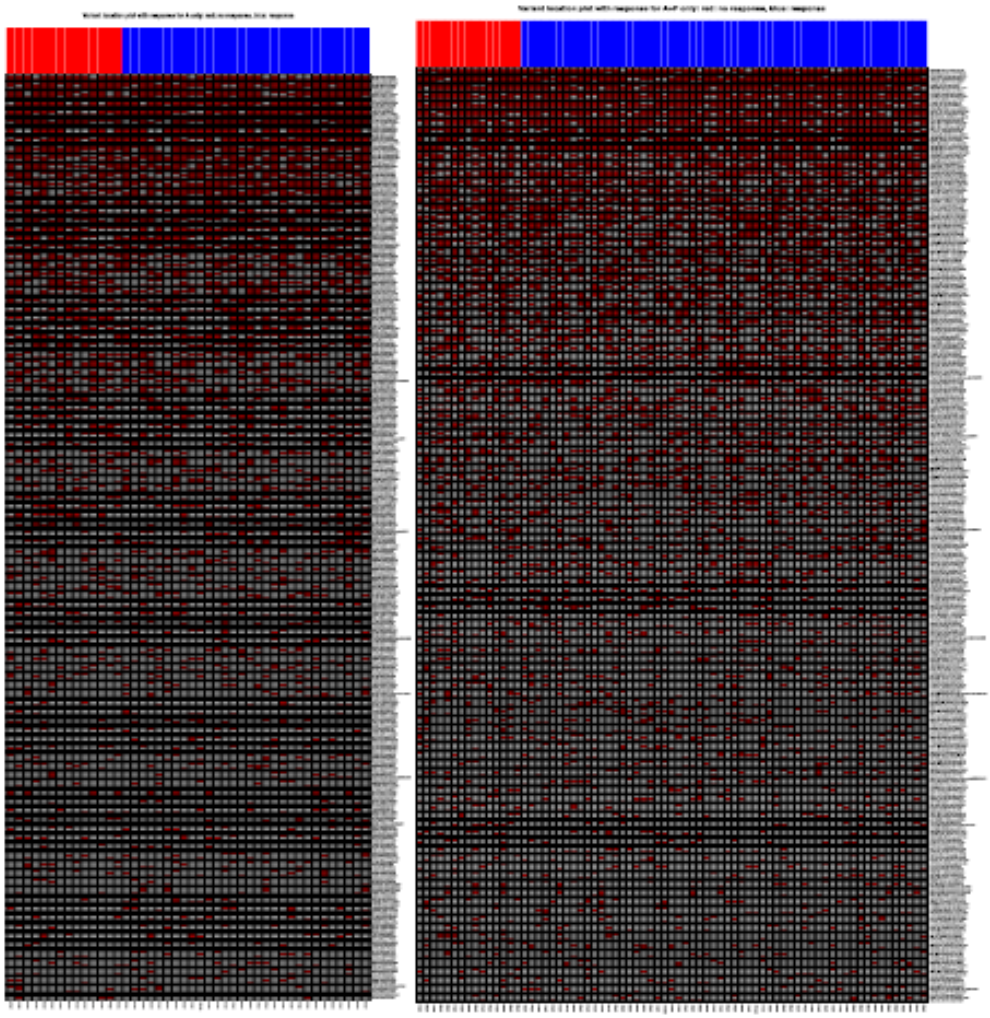


Figure 10: Somatic variant analysis and response to anastrozole (a) or anastrozole and pictilisib (b). Red demonstrates the presence of a somatic mutation; grey, no mutation detected.

5.2.2 PAM50 Luminal Status and treatment response:

NanoString PAM50 analysis was performed in a subset of patients (n=53) to assess luminal status. PAM50 results analysis showed that patients with Luminal B tumours had a significantly higher anti-proliferative response with the combination of anastrozole plus pictilisib compared to anastrozole alone [geometric mean Ki67 suppression, 86.5% versus 63.6%; ratio (combination/anastrozole) 0.37 (0.18-0.76; p=0.008)], whereas adding pictilisib to anastrozole had no apparent benefit for Luminal A tumours (ratio, 1.01; p=0.98).

5.2.3 Baseline Ki67 expression, PR, tumour grade and treatment response:

As it had been hypothesised that luminal B biology could be a determinant of suboptimal response to endocrine therapy alone and potentially therefore define a subgroup that might derive an increased benefit from combination therapy with pictilisib and anastrozole, the impact of several baseline characteristics that have been linked with luminal B phenotype were explored. These include baseline Ki67 expression, PR expression and tumour grade.

In an analysis involving all evaluable patients (n=136), luminal status was defined by baseline Ki67 expression in accordance to the St Gallen criteria using a Ki67 expression of 14% as the cut-off between luminal A and luminal B. In contrast to the PAM50 analysis, patients with Luminal A status (n=50) defined as baseline Ki67 of <14% had a significant benefit of the combination of anastrozole plus pictilisib compared to anastrozole alone [geometric mean Ki67

suppression, 74.1% versus 43.4%; ratio (combination/anastrozole) 0.46 (0.25 – 0.85); p=0.02)]. In patients with Luminal B tumours (n=86), defined as Ki67 >14%, geometric mean Ki67 suppression was 78.7% in the anastrozole alone group and 86.3% for patients treated with anastrozole plus pictilisib [ratio, 0.64 (0.43 – 0.97); p=0.04].

Using a Ki67 cut-off of 20%, mean geometric Ki67 suppression for Luminal A tumours was 61.6% in the anastrozole alone group and 77.6% for patients treated with anastrozole plus pictilisib [ratio, 0.58 (0.25 – 0.97); p=0.04]. For Luminal B tumours, geometric mean Ki67 suppression was 77.6% for patients treated with anastrozole alone and 86.7% for patients treated with anastrozole plus pictilisib [ratio, 0.59 (0.36 - 0.96); p=0.04].

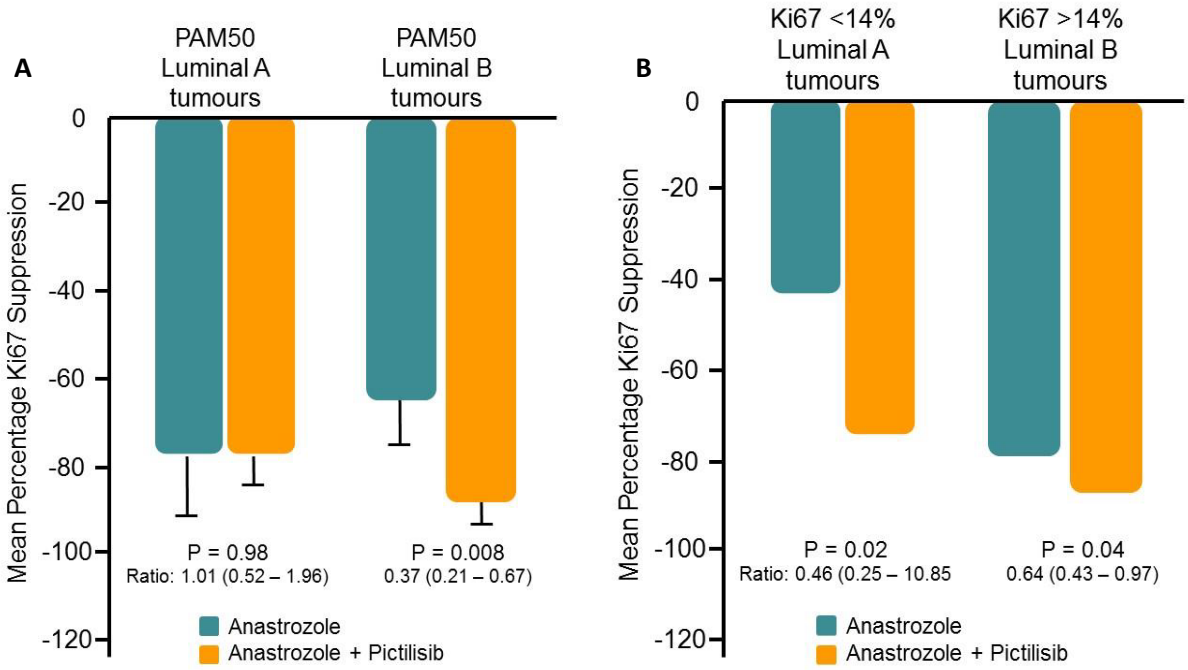


Figure 11: Anti-proliferative response to study treatment; a) anti-proliferative response by Luminal subtype defined by PAM50; c) anti-proliferative response by Luminal subtype defined by baseline Ki67 expression (cut-off 14%)

PR receptor status was available in 136 patients; the majority of tumours were classified as PR positive (84.6%), defined by an Allred score of 3 or higher. Only 21 tumours were PR negative (15.4%). The addition of pictilisib increased the anti-proliferative response in both subsets with a slightly more pronounced benefit in patients with PR negative tumours. In PR-positive tumours, the geometric mean Ki67 suppression was 72.1% with anastrozole compared to 81.7% with the combination [0.65 (0.43–0.98); p=0.04], whereas in PR-negative tumours the mean Ki67 suppression was 66.7% with anastrozole compared to 88.4% with the combination [0.35 (0.14–0.87); p=0.03].

Tumour grade was available for 135 patients; the majority of tumours were classified as Grade 1 or Grade 2 (n=115; 85.2%) with the remaining 21 tumours classified as Grade 3 (15.6%). Tumour grade was a strong predictor of response to anastrozole alone with a mean geometric Ki67 suppression of 73.2% (61.0%-81.6%) in patients with Grade 1 or Grade 2 tumours compared to 50% (19.4%-69.0%) in patients with G3 tumours. In contrast, patients responded to anastrozole plus pictilisib irrespective of the tumour grade with a mean geometric Ki67 suppression of 80.4% (74.8%-84.8%) for patients with Grade 1 or Grade 2 tumours and 90.3% (78.8%-95.5%) for patients with Grade 3 tumours.

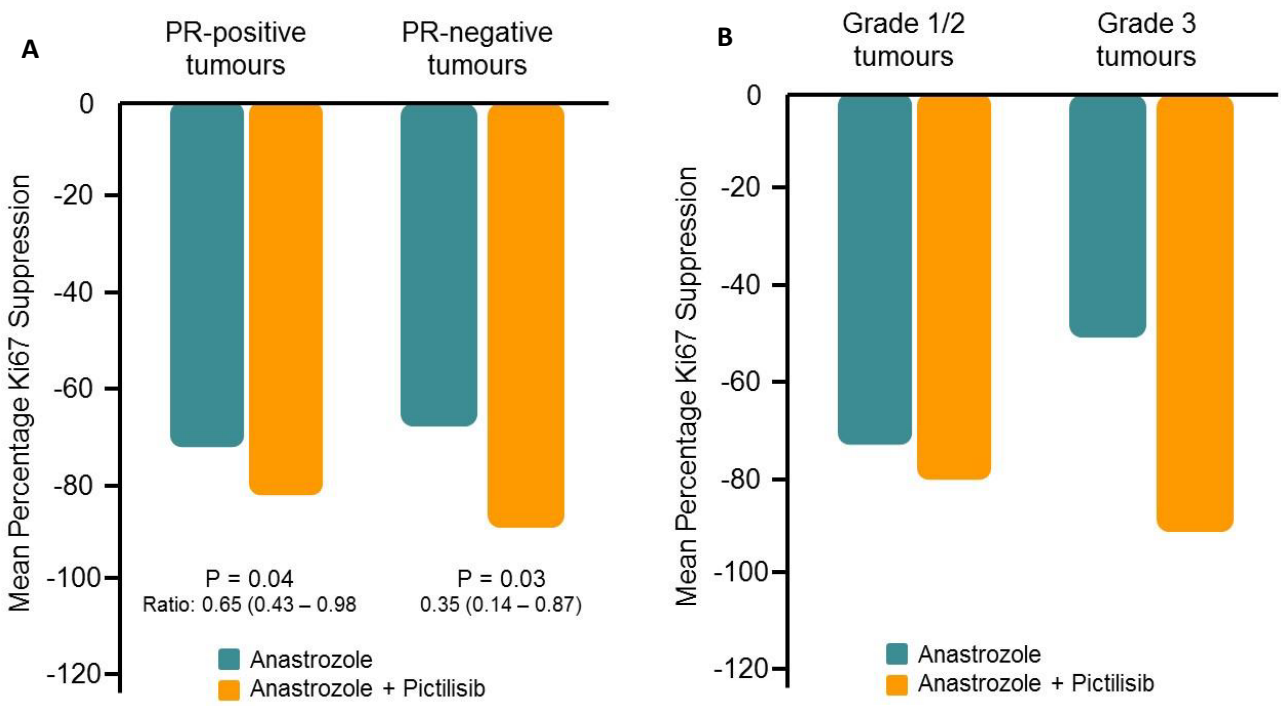


Figure 12: Anti-proliferative response to study treatment PR status (a) and tumour grade (b)

Multivariate linear regression analysis confirmed a significant interaction between treatment effect and molecular subtype by PAM50 (p=0.03), supporting the observation that the combination treatment is more effective than anastrozole alone for patients with Luminal B tumours irrespective of PR status or the baseline Ki67 expression. However, patients with PR-negative Luminal B cancers showed the greatest anti-proliferative effect from combination treatment (ratio=0.12). Furthermore, combined treatment also appeared to be more effective in PR-negative Luminal A cancers.

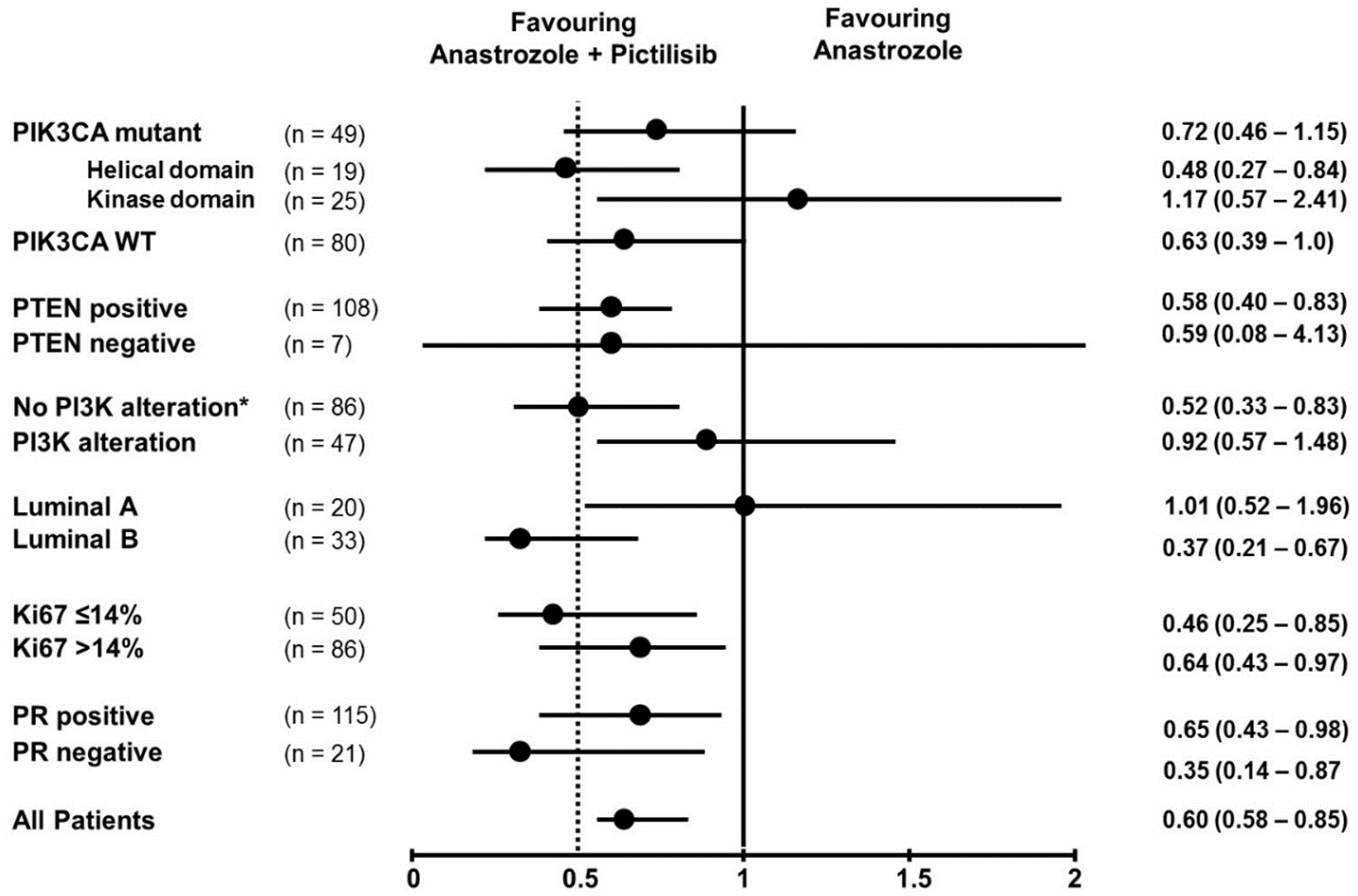


Figure 13: Ratio (combination/anastrozole) of geometric mean of Ki67 proportional changes in pre-specified subgroups

RPPA analysis focused on key genes involved in the activation of the PI3K pathway and cell cycle. There was substantial downregulation of cell cycle genes in both arms, associated with endocrine therapy. Phospho-AKT levels, pS6 levels or p4E-BP1 levels were comparable between both arms. Overall, the end-of-treatment profiles as well as the treatment-associated changes were largely comparable between both groups, suggesting a dominant anti-oestrogen effect. The effects on Cyclin D1 were more pronounced with the combination in keeping with the more substantial anti-proliferative effect as per Ki67 analysis.

5.2.4 PI3K kinase pathway activation and treatment benefit

To further assess the potential interaction of activation of the PI3K pathway and study treatment, an analysis of changes in gene/protein expression and phosphorylation of selected signalling markers was performed.

Two gene signatures (GS) were calculated as baseline and at the end of treatment and correlated with response to anastrozole and the combination therapy, respectively. The PIK3CA mutation associated GS [48] has previously been shown to negatively correlate with proliferation, AKT/mTOR activation and PTEN loss and strongly positively correlated with ESR1 and better outcome in ER-positive breast cancer. O'Brien et al identified a PIK3 inhibitor sensitivity GS, based on a number of genes that are differentially expressed between sensitive and resistant breast cancer cell lines; the PI3K inhibitor sensitivity GS [43] has been shown to correlate with activation of the PI3K pathway and can be used to characterise patients who are sensitive to PI3K inhibition.

The baseline PIK3 inhibitor sensitivity GS (O'Brien) score was associated with higher proliferation and Luminal B phenotype (Figure 14c). The baseline PIK3 inhibitor sensitivity (O'Brien) score was inversely associated with ΔKi67 in the anastrozole arm, characterising patients with partial endocrine resistance (Figure 14d). Post-treatment PIK3 inhibitor sensitivity GS (O'Brien) scores were significantly down-regulated in both arms, consistent with an attenuation of the flux through the PI3K pathway (Figure 14a).

In contrast, we observed no relevant modulation of the PIK3CA mutation-associated GS (Loi) with study treatment (Figure 14b). The PIK3CA mutation-associated GS (Loi) was not predictive of a treatment-induced change in Ki67 in either treatment arm.

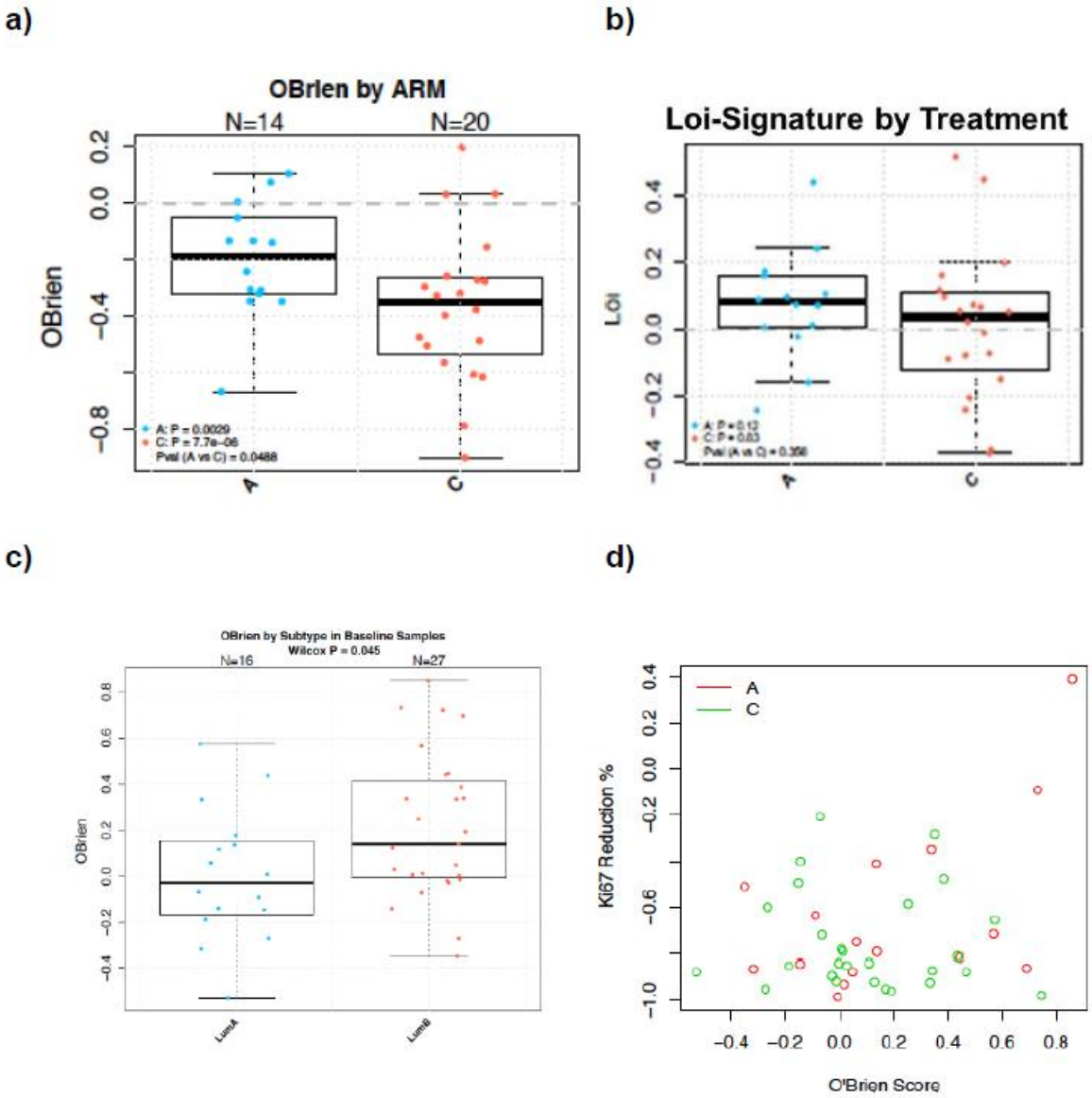


Figure 14: PI3K mutation and PI3K inhibitor sensitivity gene signatures: a) down-regulation of post-treatment PIK3 inhibitor sensitivity GS (O'Brien) scores in both treatment arms; b) post-treatment PIK3CA mutation-associated GS (Loi) in both treatment groups; c) association of baseline PIK3 inhibitor sensitivity GS (O'Brien) score and Luminal B phenotype; d) inverse association of baseline PIK3 inhibitor sensitivity GS (O'Brien) score with ΔKi67 in the anastrozole arm.

5.2.6 Treatment-induced changes in gene/protein expression and phosphorylation

Treatment-induced changes in protein expression and phosphorylation and gene expression were evaluated in subsets of patients, using RPPA (n=32) and Nanostring analysis (n=64). Figure 18a and 18b provide an overview of differentially expressed genes between pre- and post-treatment samples in the anastrozole and anastrozole and pictilisib groups, respectively. The top differentially expressed canonical pathways in the anastrozole group included cyclins and cell cycle regulation, oestrogen-dependent signalling and gene expression, ATM signalling, mitotic kinases, and aryl hydrocarbon receptor signalling. There was substantial upregulation of a number of genes associated with the immune system, whereas many of the most downregulated genes were involved in cell cycle control.

In the anastrozole plus pictilisib group, top upregulated canonical pathways included pancreatic adenocarcinoma signalling, aryl hydrocarbon receptor signalling, IL-8 signalling, bladder cancer signalling and GADD45 signalling. There was also substantial upregulation of genes associated with the immune system and downregulation of cell cycle genes.

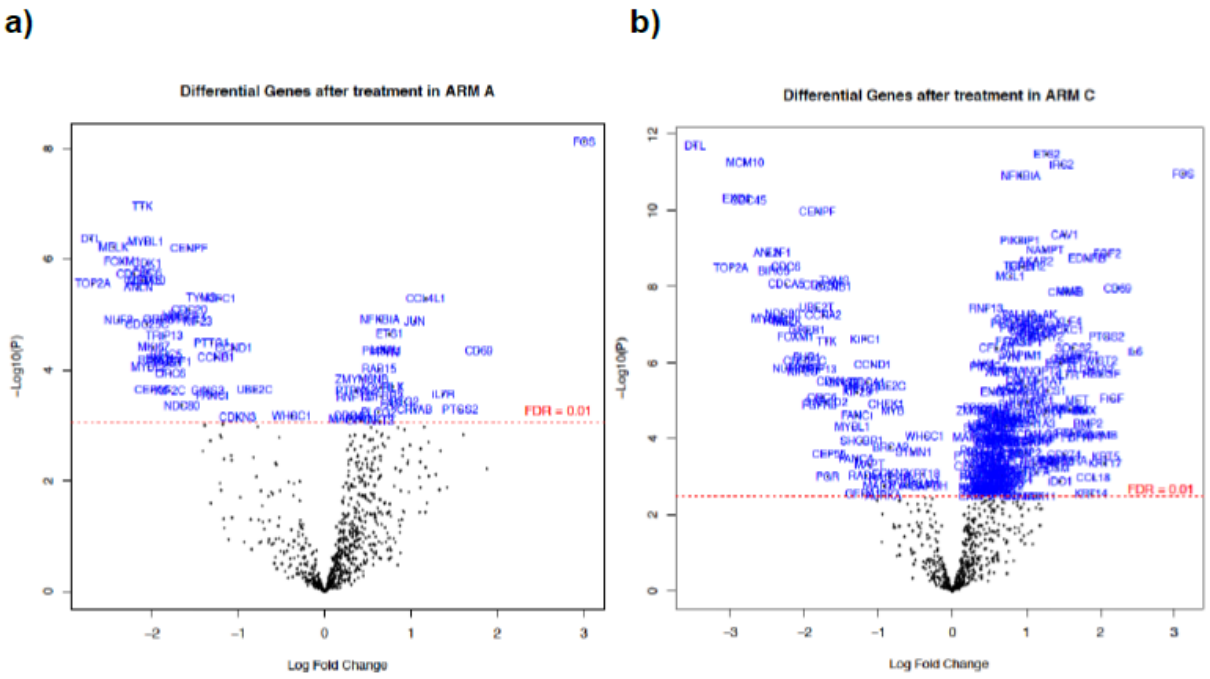


Figure 15: Differentially-expressed genes between pre- and post-treatment samples in the anastrozole arm (a) and anastrozole and pictilisib arm (b)

ER target genes: Previous data suggested that single agent PI3K inhibition up-regulates expression of ER target genes in vivo and in vitro [49]. In a preclinical study, treatment with the PI3K inhibitor BYL719 (p110a) was associated with substantially increased expression of ER-target genes. Furthermore, treatment with BYL719 upregulated ESR1 expression in tumour samples of treated patients.

The effect of treatment with pictilisib and anastrozole on ER target genes was therefore investigated, using Nanostring gene expression analysis. As illustrated in Figure 15 there was a significant treatment-associated reduction in the expression of ER target genes such as GREB1 or PR. No differences were observed between the 2 study arms, suggesting that induction of ER target genes by PI3K inhibition requires oestrogen.

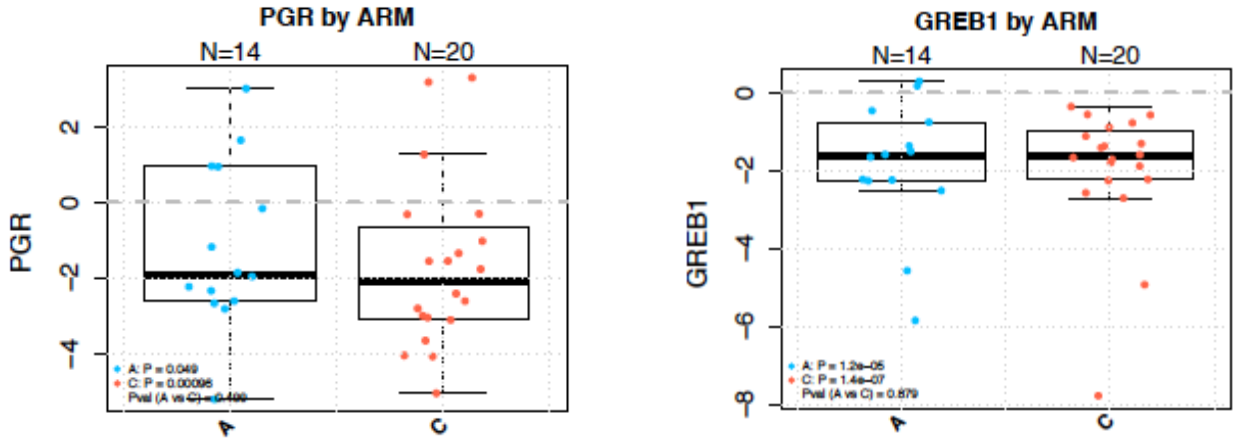


Figure 16: Treatment-induced changes in expression of ER target genes PR and GREB1; A, anastrozole alone; C, combination.

PI3K pathway and cell cycle. RPPA analysis focused on key genes involved in the activation of the PI3K pathway and cell cycle. Baseline protein expression and phosphorylation was comparable between both groups. There was substantial downregulation of cell cycle genes in both arms, associated with endocrine therapy. Phospho-AKT levels, pS6 levels or p4E-BP1 levels were comparable between both arms (Figure 18). Overall, the end-of-treatment profiles as well as the treatment-associated changes (Figure 17) were largely comparable between both groups, suggesting a dominant anti-oestrogen effect. The effects on Cyclin D1 were more pronounced with the combination in keeping with the more substantial anti-proliferative effect as per Ki67 analysis. Gene expression analysis in the anastrozole group demonstrated upregulation of genes involved in cell cycle arrest such as p21. In the combination group, gene expression analysis also demonstrated up-regulation of the PI3K-regulated genes IRS2 and PIK3IP1, confirming treatment-associated pathway inhibition.

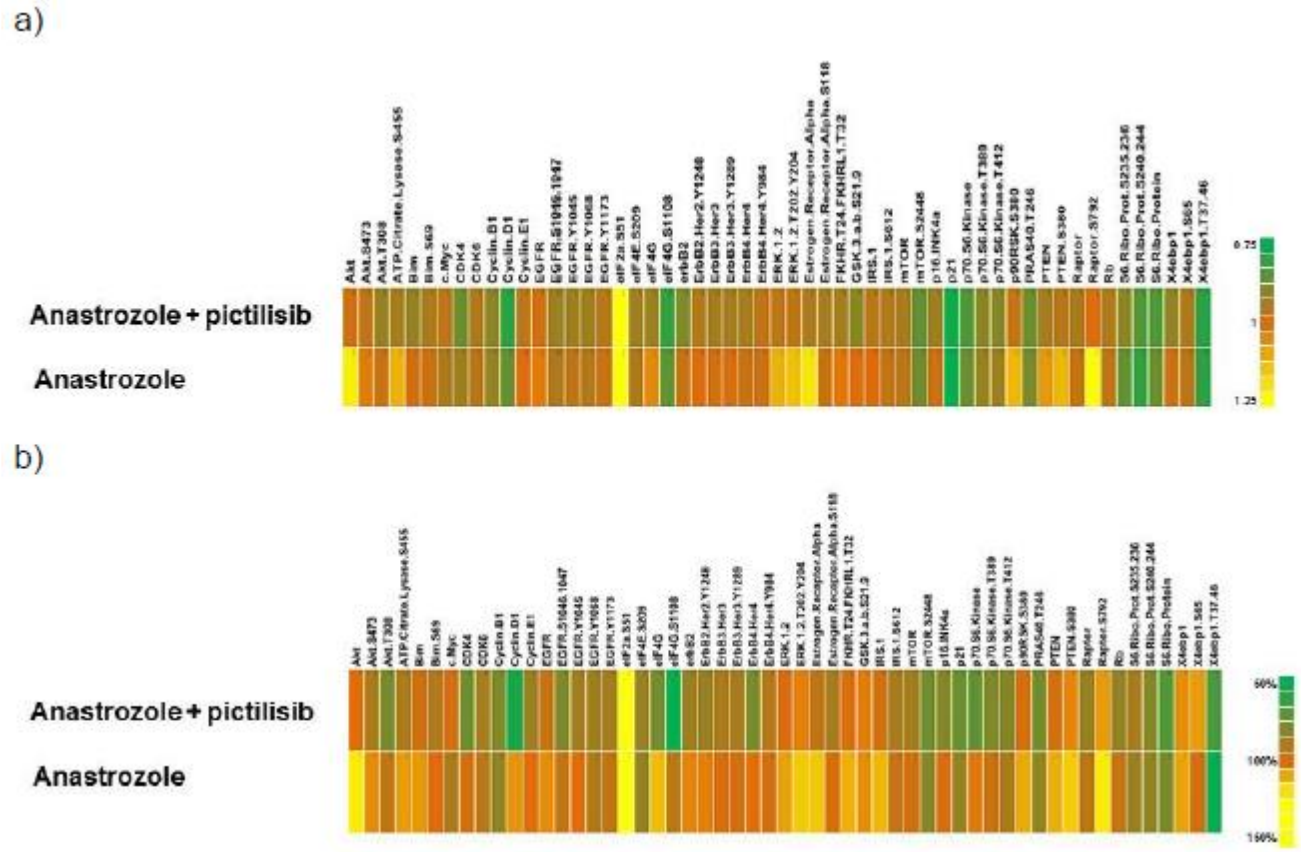


Figure 17: RPPA analysis focusing on key genes involved in the activation of the PI3K pathway and cell cycle. A) Mean end-of-treatment RPPA expression in the anastrozole and combination therapy groups; B) Mean treatment-associated changes in RPPA expression with anastrozole and anastrozole plus pictilisib.

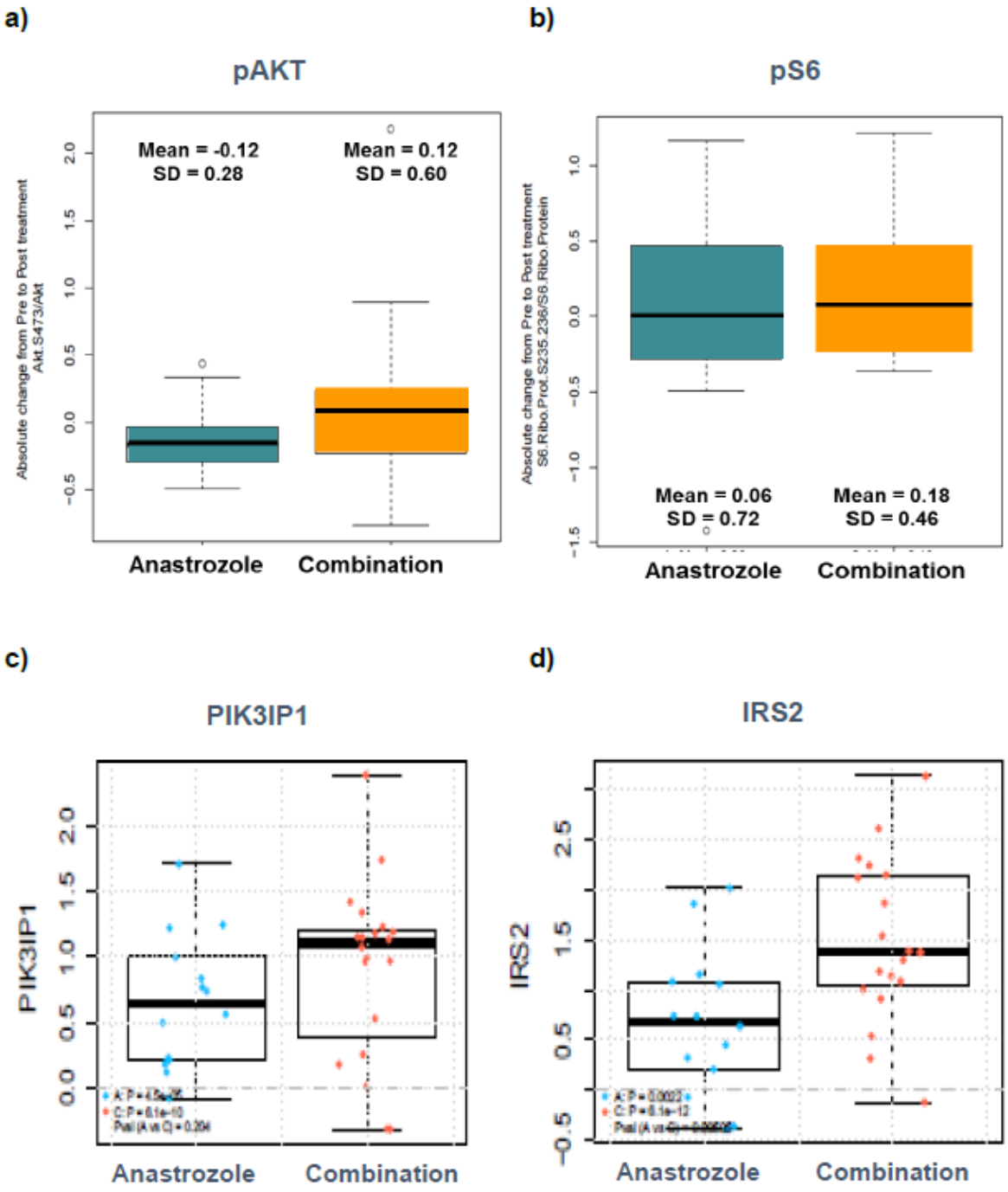


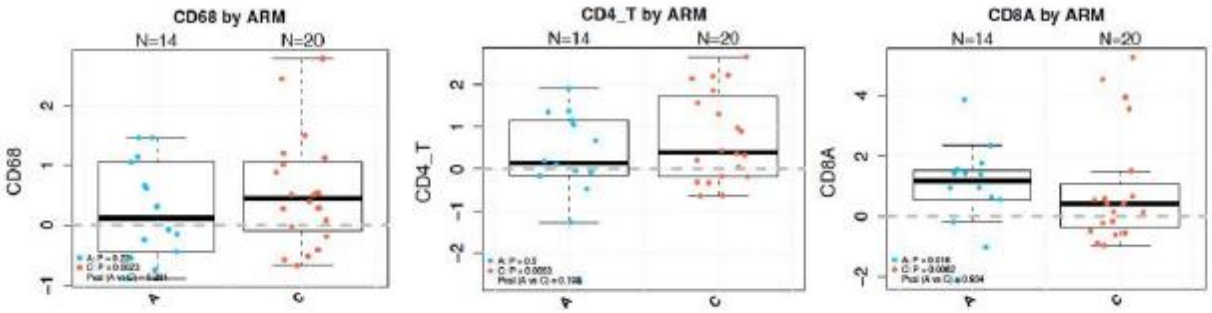
Figure 18: RPPA analysis of Phospho-AKT levels (a) and pS6 levels (b) after treatment with anastrozole or combination therapy; treatment-associated upregulation of PIK3CA-regulated genes PIK3IP1 and IRS2.

5.2.8 Effects of PI3K inhibition on the tumour microenvironment and immune system

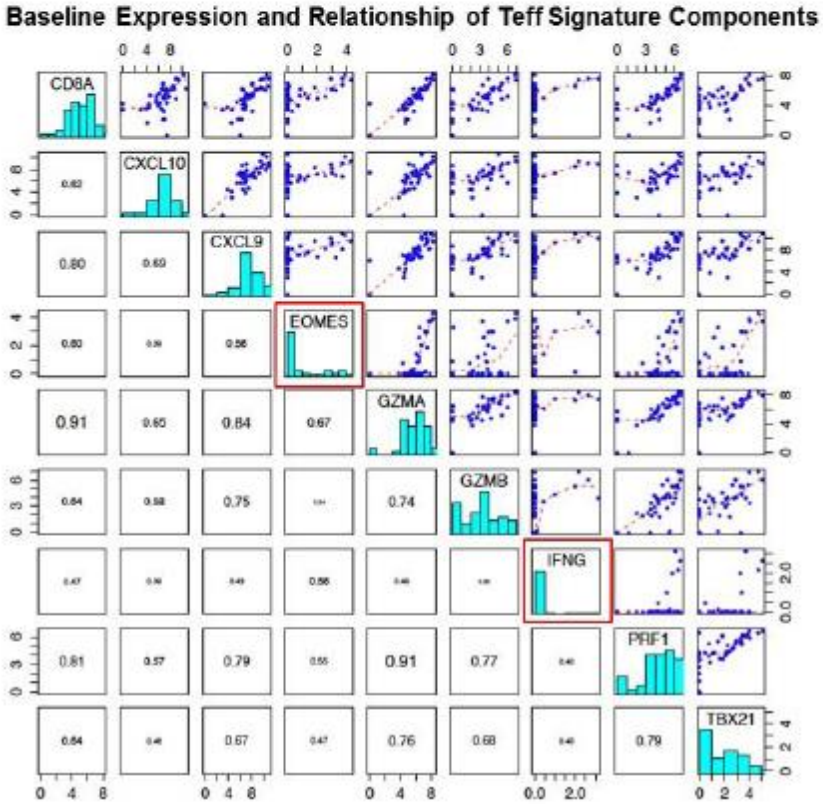
There is substantial evidence that oestradiol and/or ER signalling regulates the development and function of dendritic cells [50], B and T lymphocytes, NK cells, monocytes and macrophages [51, 52]. In addition, there is discussion around the role of PI3K signalling and the tumour microenvironment. We therefore performed gene expression analysis to assess the impact of anastrozole and the combination therapy on the tumour microenvironment and immune system.

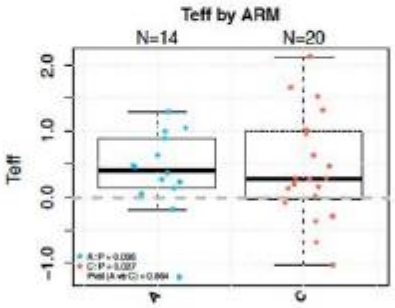
Analysis of pre- and post-treatment samples showed that a 2-week treatment of anastrozole and anastrozole plus pictilisib have a modest impact on the tumour immune microenvironment; the observed effects differed between the two treatment groups. Whilst in patients treated with anastrozole a modest increase in CD8A transcript was observed, combination therapy was associated with a modest increase in CD68, CD4 and CD8A transcripts. Study treatment had a minimal impact on Teff and Treg signatures and on Tcell immunosuppressive signature but a modest impact on APC immunosuppressive signature. In the tumour samples from patients that received combination therapy, there was an increase in the expression of markers indicative for macrophages, CD4 and CD8+ cell recruitment, as well as increase of immunosuppressive molecules such as PD-L1, PD-L2 and IDO. No significant increase in FOXP3+ effector cells was observed in either arm.

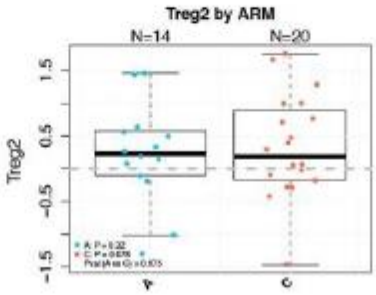
a)


CD68 by ARM
N=14
N=20
CD68
CD4_T by ARM
CD4_T
CD8A by ARM
CD8A
A
C

b)


Baseline Expression and Relationship of Teff Signature Components
CD8A
CXCL10
CXCL9
EOMES
GZMA
GZMB
IFNG
PRF1
TBX21


Teff by ARM
N=14
N=20
Teff
A
C


Treg2 by ARM
N=14
N=20
Treg2
A
C

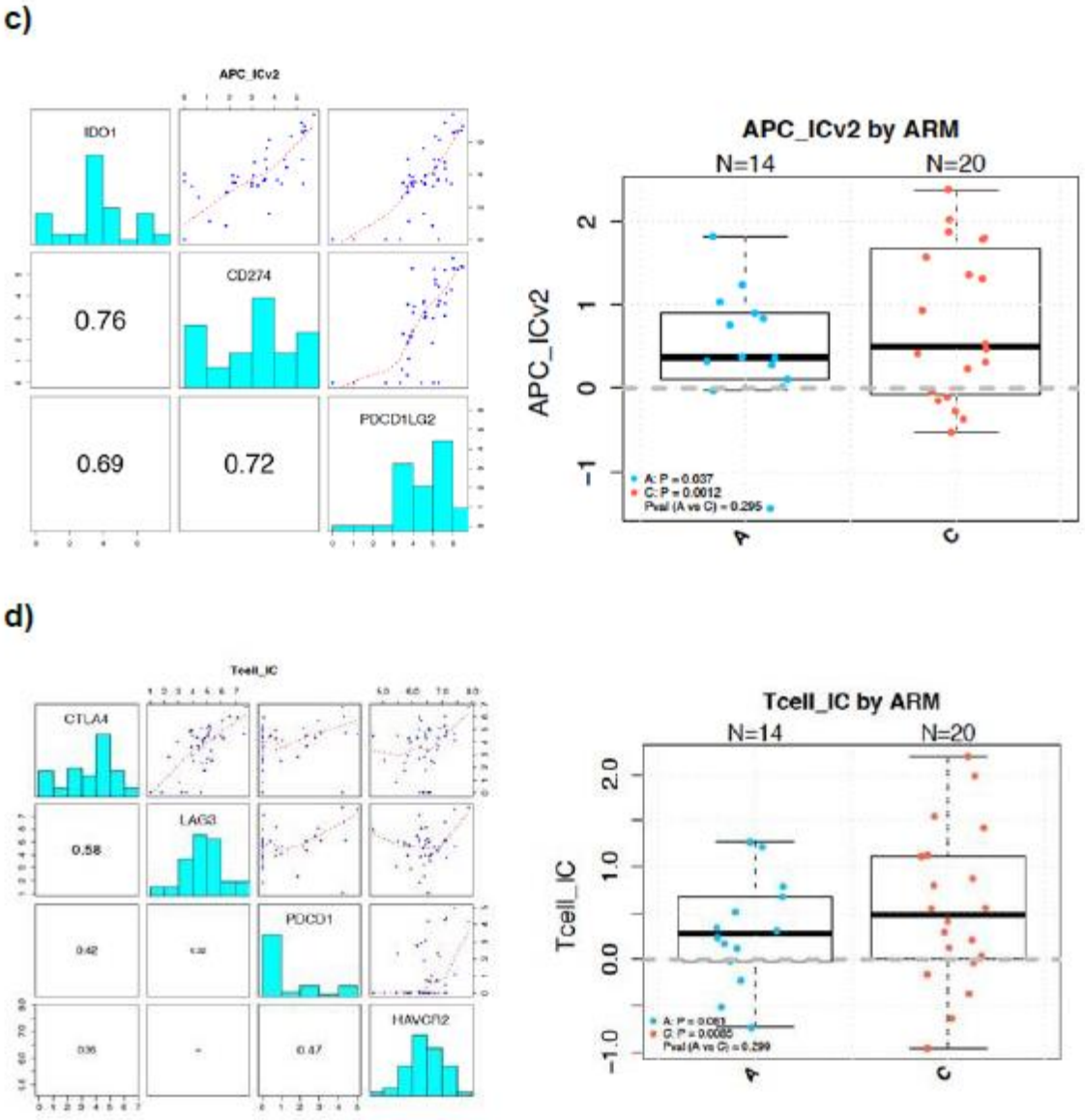


Figure 19: a) Impact on markers of immune cell populations (CD68, CD4, CD8A) in the post-treatment samples; b) Treatment effect on Teff and Treg signatures; c) APC immunosuppressive signature; d) Tcell immune-suppressive signature

6 RESULTS: SAFETY

Treatment-related AEs were consistent with those previously described for PIC and ANA with more AEs in the PIC-treated group (Table 3). No pulmonary toxic effects associated with PIC were identified. Reducing PIC dose from 340 mg to 260 mg reduced the skin toxicity significantly (grade 3, 38% v 3.3%; P =.013). At a PIC dose of 260 mg, grade 3 AEs were asymptomatic hyperglycemia and rash in one patient each. Treatment was discontinued in two patients receiving 340 mg PIC because of hypersensitivity reaction and rash. AEs were rapidly reversible, and all patients received subsequent standard therapy as planned.

	ANA Alone (n = 26)				ANA + PIC (340 mg) (n = 8)				ANA + PIC (260 mg) (n = 39)			
	G1/2		G3		G1/2		G3		G1/2		G3	
Most Common Adverse Events*	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Fatigue	6	23	1	4	7	88	0		6	26	0	
Rash	0		0		2	25	3	38†	3	8	1	3
Diarrhea	1	4	0		4	50	0		20	52	0	
Dysgeusia	1	4	0		2	25	0		4	10	0	
Dyspepsia	0		0		1	13	0		7	18	0	
Anorexia	1	4	0		2	25	0		5	13	0	
Nausea	3	12	0		7	88	0		16	41	0	
Vomiting	0		0		2	25	0		5	13	0	
Stomatitis	0		0		1	13	0		2	5	0	
Hyperglycemia	0		0		0		0		3	8	1	3
Creatinine	0		0		3	38	0		3	8	0	
Arthralgia	5	19	0		1	13	0		1	3	0	
Headache	4	16	0		1	13	0		3	8	0	
Hot flashes	6	23	0		0		0		2	5	0	

NOTE. The safety population includes all patients who received at least one dose of the study drug.
Abbreviations: ANA, anastrozole; G, grade; PIC, pictilisib.
*Included are all adverse events with an incidence of 10% or more in either group.
†Fisher's exact $P = .013$ between PIC 340 mg and 260 mg.

Figure 20: Adverse events in the safety population.

6.1 Deaths

None reported.

6.3 Serious Adverse Events

Subject	Category	Sub Category	Toxicity Category	SAE Details	Related to Study Product
OPP105	ALLERGY/ IMMUNOLOGY	Allergic reaction/hypersensitivity (including drug fever)	4	Anaphylactic reaction- treated at A&E	Yes
OPP107	HEMORRHAGE/ BLEEDING	Hematoma	3	AE caused prolongation of hospitalisation	No
OPP123	CARDIAC ARRHYTHMIA	Supraventricular and nodal arrhythmia Sinus bradycardia	2	Patient admitted to the ward post op in the absence of a carer at home. She experienced an episode of dizziness overnight, was assessed and found to have sinus bradycardie Heartrate 37-40 throughout the night. Hospital stay extended to allow assessment by a cardiologist. Cardiologist assessed that bradycardia was not related to the study drugs. The combination of anaesthetics and morphine overnight may have contributed to the event.	No
OPP123	CARDIAC ARRHYTHMIA	Supraventricular and nodal arrhythmia Sinus bradycardia	2	Prolonged hospitalisation	No
OPP840	DERMATOLOGY /SKIN	Ulceration	3	Wound breakdown and area of necrosis debrided in clinic but required further debridement of ulceration in theatre and removal of expander.	No
222	HEMORRHAGE/ BLEEDING	Hematoma	2	-	No

6.4 Pregnancies

None reported.

7 DISCUSSION

OPPORTUNE was the first trial of a PI3K inhibitor in ER-positive early-stage breast cancer. The study successfully met the primary end point, demonstrating that adding PIC to ANA significantly increased the anti-proliferative response. Both mean Ki-67 suppression and the percentage of tumours with significant Ki-67 reduction were substantially higher for ANA + PIC compared with ANA. Most importantly, the end-of-treatment Ki-67 suppression was also significantly higher for ANA + PIC. This is particularly relevant because only end-of-treatment Ki-67 expression but not baseline expression has been associated with improved recurrence-free survival (RFS) [32]. In the IMPACT (Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen) trial, 5-year RFS rates were 85%, 75%, and 60%,

respectively, for the lowest, middle, and highest tertiles of Ki-67 expression after 2 weeks of preoperative endocrine therapy [32]. End-of-treatment Ki-67 expression seemed to integrate the prognostic value of baseline proliferation and the predictive value of responding to endocrine therapy, thus making it an excellent predictor of outcome in this setting [32]. A significant interaction was observed between PIK3CA mutation subtypes, with patients with helical domain mutations showing a particularly poor response to anastrozole alone that was reversed by the addition of pictilisib.

In keeping with other studies in this field, the rate of apoptosis was low in this trial with the majority of tumour samples containing less than 1% apoptotic cells and a geometric mean expression of 0.15%. There was no clear evidence of a treatment-associated increase in Caspase-3 expression but the results have to be interpreted with caution, as the low rate of apoptosis together with the strong positive correlation between Ki-67 and apoptosis scores, found in this and other trials [35], could mask an effect of PI3K inhibition on apoptosis as observed in preclinical studies [53]. Other groups have therefore introduced growth index, defined as percent Ki67-expresion divided by percent Caspase-3 expression. Using this method, we were able to describe a greater suppression in the growth index in the combination arm (75.2%) compared to anastrozole alone 55.9%.

Pre-planned subset analyses suggest that the additional anti-proliferative effects of PIC may largely be limited to luminal B tumours, whereas luminal A tumours demonstrated no additional effect unless they were PgR negative. The latter result has to be seen in the context that negative PgR status and luminal B subtype are closely associated. Taking all types of PIK3CA mutations together, there was no association between overall PIK3CA mutation status and anti-proliferative response for anastrozole alone, in keeping with other studies suggesting that the presence of PIK3CA mutations has limited impact on the effect of preoperative anastrozole therapy in patients with primary, ER-positive breast cancer [54] [55] [56]. There was also no correlation between overall PIK3CA mutation status and added activity of pictilisib with a ratio of geometric mean Ki67 proportional change of 0.63 (0.39–1.0) for patients with PIK3CA-wildtype tumours and 0.72 (0.46–1.15) for patients with PIK3CA-mutated tumours. This is consistent with results from trials of pictilisib or the mTOR inhibitor everolimus in pre-treated, metastatic breast cancer, where patients derived benefit from everolimus or pictilisib regardless of their tumour PIK3CA genotype [57-59]. However, recent trials with more specific PI3K inhibitors (α-specific or β-sparing), have demonstrated an increased benefit in patients with PIK3CA mutated tumours (SOLAR1, SANDPIPER). This might be down to more profound target inhibition in the tumour which might be achievable due to the improved efficacy/tolerability ratio associated with the lower degree of β-inhibition which has been associated with driving toxicity.

Interestingly, we found a significant interaction between *PIK3CA* mutation subtypes and Ki-67 suppression. Whilst patients with helical domain mutations [ratio, 0.48 (0.27-0.84; p=0.02] or PIK3CA wildtype status [ratio, 0.63 (0.39–1.0; p=0.05] demonstrated a substantial relative benefit from the addition of pictilisib, there was not clear additional effect of pictilisib in tumours with kinase domain (KD) mutations[ratio, 1.17 (0.57–2.41; p=0.64]. This was largely due to patients with helical domain (HD) mutations showing a particularly poor response to anastrozole alone [mean Ki-67 suppression 53.9% (9.5%-76.5%)], that was reversed by the addition of pictilisib [mean Ki-67 suppression, 78.1% (71.0%-83.4%)], whereas patients with KD mutations responded well to anastrozole alone [mean Ki-67 suppression 77.7% (57.0%-88.4%)] and showed no benefit from the addition of pictilisib [mean Ki-67 suppression 73.9% (59.8%-83.0%)]. A similar observation was reported from a neoadjuvant trial of the mTOR inhibitor everolimus and letrozole in ER-positive breast cancer, in which exon 9 mutations seemed to be associated with an increased benefit of mTOR inhibition relative to exon 20 mutations [39] and this may merit testing in future studies of early breast cancer.

PTEN expression was not associated with benefit of the combination therapy and did not add significantly to PIK3CA mutations as determinant of PI3K inhibitor benefit. As expected being associated with PIK3CA mutations rather than PI3K pathway activation, the Loi GS did not change significantly with study treatment and was not predictive of a treatment-associated change in Ki67 expression in either treatment arm. On the other hand, the O'Brien GS, which was developed based on several genes that are differentially expressed between sensitive and resistant breast cancer cell lines, was significantly down-regulated with both study treatments, consistent with an attenuation of the flux through the PI3K pathway. It was also inversely associated with Ki67 suppression in the anastrozole arm, suggesting it might be useful for characterising patients with partial endocrine resistance.

As there is increasing evidence that luminal B biology is a determinant of suboptimal response to endocrine therapy alone, it was hypothesized that the intrinsic subtype could potentially define a subgroup that might derive an increased benefit from combination therapy with pictilisib and anastrozole. We therefore explored the possible interaction between intrinsic subtypes defined by NanoString PAM50 analysis and anti-proliferative response in a subgroup of tumours (n=53) with available pre- and post-treatment RNA. Additional analysis was performed on the entire study population using alternative markers that have been associated with the luminal B phenotype including baseline Ki67 expression, PR expression and tumour grade.

In keeping with our hypothesis, PAM50 analysis showed that patients with Luminal B tumours had a significantly higher anti-proliferative response with combination treatment compared to anastrozole alone [mean Ki67 suppression, 86.5% versus 63.6%; ratio 0.37 (0.18-0.76; p=0.008)], whereas adding pictilisib to anastrozole had no apparent benefit for Luminal A tumours (ratio, 1.01; p=0.98). It is unclear whether this result is more a reflection of the fact that luminal B tumours are partially endocrine resistant compared to the highly endocrine sensitive luminal A tumours, or a true differential effect of PI3K inhibitors in the respective subtypes.

Defining luminal A and B status through baseline Ki67 expression in accordance with the St Gallen criteria (using a Ki67 cut-off of 14%) provided somewhat contradictory results, demonstrating a significant benefit of combination therapy in patients with Luminal A and Luminal B tumours, using alternative cut-offs of 14% and 20%, respectively. However, much of the benefit in Luminal A tumours seems to be driven by an unexpectedly low Ki67 suppression with anastrozole alone. This contrasts with other studies. A possible explanation is that the Ki67 suppression results might be less reliable for patients with low baseline expression (<10%) considering the variability in the Ki67 assessment as illustrated in the mean difference of 2.6%-3.9% between the 2 analyses. Some trials therefore exclude patients with a Ki67 baseline expression of <10%. We explored the possibility of an additional analysis, excluding patients with baseline Ki67<10%, but the number were too low for achieving reliable results. Of note, mean baseline Ki67 expression was 15.4% for PAM50 Luminal A and 30.7% for PAM50 Luminal B tumours, with 60% of Luminal A tumours showing baseline Ki67 values of >10%, suggesting that this analysis might be less likely affected by a possible technical limitation in tumours with low baseline Ki67 levels.

Additional, pre-planned, subset analyses suggest that the effects of pictilisib added to anastrozole are predominantly seen in patients with PR negative and/or grade 3 tumours. Multivariate linear regression analysis demonstrated an increased treatment effect for the pictilisib-containing arm in patients with Luminal B cancers independent of baseline Ki67 expression, suggesting an impact of molecular subtype on the response to pictilisib independent of baseline proliferation. Overall, these findings are supportive of an association between luminal subtype, insensitivity to endocrine therapies and response to treatment with a pan-PI3K inhibitor, which have implications for future trial design and therapeutic strategies.

To further evaluate treatment-induced changes in gene expression and protein expression/phosphorylation, we used RPPAs and Nanostring gene expression analysis in subsets of patients. We were able to demonstrate profound down-regulation of ER-mediated transcription and cell cycle progression. Interestingly, we found no differences in the expression of ER target genes between both study arms, suggesting that the preclinically observed induction of ER target genes by PI3K inhibition requires oestrogen and is therefore not relevant in the context of combined endocrine and PI3K inhibitor therapy.

RPPA analysis focused on key genes involved in the activation of the PI3K pathway and cell cycle. There was substantial downregulation of cell cycle genes in both arms but Cyclin D1 levels were more suppressed in the combination arm consistent with the more substantial anti-proliferative effect demonstrated in the primary Ki67 analysis. Surprisingly, there was no discernible differences between both groups in the expression and/or phosphorylation of PI3K downstream targets Phospho-AKT, pS6 and p4E-BP1. On the other hand, gene expression analysis demonstrated up-regulation of the PI3K-regulated genes IRS2 and PIK3IP1, confirming treatment-associated pathway inhibition. It remains unclear whether the lack of a clear effect on PI3K downstream targets has technical reasons or might instead be reflective of the complex biology of the PI3K/AKT pathway. It is well recognised that Phospho-AKT levels can change rapidly during the processing of tissue samples. In the OPPORTUNE trial, we therefore defined strict criteria for rapid processing of tissue samples to minimise these effects, but it cannot be excluded that RPPA results have been affected by this.

We also demonstrated that short-term treatment with pictilisib and/or anastrozole has a modest impact on the tumour immune microenvironment. Effects differed between the two treatment groups suggesting that PI3K inhibition has additional effect to endocrine therapy. Most of these effects were modest and the potential clinical implications remain to be determined but the emerging role of immunotherapy in breast cancer makes underlines the importance of further studies in this context.

Overall, these findings were supportive of an association between luminal B subtype, insensitivity to endocrine treatment, and anti-proliferative response to treatment with PIC, which has implications for future trial design. As expected, the rate of apoptosis was low, with the majority of tumours containing , 1% apoptotic cells. No differences were observed between treatment groups, but the strong correlation between Ki-67 and apoptosis scores found in this and other trials [32] could mask an effect of PI3K inhibition on apoptosis as observed in preclinical studies [32].

Although the OPPORTUNE trial showed an increased response with ANA + PIC in early breast cancer, the FERGI (A Phase II, Double-Blind, Placebo-Controlled, Randomized Study of GDC-0941 or GDC-0980 With Fulvestrant Versus Fulvestrant in Advanced or Metastatic Breast Cancer in Patients Resistant to Aromatase Inhibitor Therapy) trial failed to demonstrate a significant benefit of adding PIC to fulvestrant in metastatic disease [59]. Because the OPPORTUNE trial did not allow dose modifications and excluded patients who discontinued treatment before surgery, results might reflect the potential of PI3K inhibitors if a sufficient dose can be maintained. Alternative strategies to specifically target the alpha subunit of PI3K, which may have a wider therapeutic index than pan-PI3K inhibitors, might overcome these limitations. There are a few caveats with respect to the data presented here. First, the study was not sufficiently powered for detailed subset analyses, and there is a risk of false-positive findings. Second, baseline PgR status was imbalanced between treatment arms with fewer PgR-negative tumours in the combination arm; this limits the study's ability to verify results from the recent FERGI study subset analysis, which suggested that only PgR-positive patients benefit from PI3K. Third, not all patients in the combination arm received the same dose of PIC. However, mean Ki-67

suppression was comparable for patients treated with 340 mg (68.8%) and 260 mg (76.7%). Finally, although previous studies have clearly established an association between Ki-67 response and RFS, it is unclear to what degree the same applies for combinations of endocrine treatment with other agents. Results of the OPPORTUNE trial therefore must be interpreted with caution in terms of potential long-term benefits.

8 CONCLUSIONS

Overall, the OPPORTUNE trial is, to the best of our knowledge, the first study to demonstrate that addition of the pan-PI3K inhibitor PIC significantly increases the anti-proliferative response to ANA in ER-positive early-stage breast cancers. It provided proof-of-concept by demonstrating that addition of the PI3K inhibitor pictilisib significantly increased the anti-proliferative response to anastrozole in ER-positive early breast cancers. The trial also provided important information on the subgroup of patients who might benefit most from combined therapy. We showed that PIK3CA mutations were not predictive of response to PI3K inhibitors (but highlighted potential differences between the mutations subtypes) and provided clinical evidence that Luminal B cancer, PR-negative cancers and/or high-grade tumours have an increased benefit of PI3K inhibition, which is in keeping with preclinical data. These data should guide optimal patient selection for future trials and could be critical for the successful clinical development of this group of agents in early breast cancer.

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