

2017 IMPAKT Breast Cancer Conference

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Summary

The IMPAKT Breast Cancer Conference was held from 4 to 6 May, 2017 in Brussels, Belgium and was organised in partnership with the Breast International Group (BIG) and the European Society for Medical Oncology (ESMO), in collaboration with a multidisciplinary alliance of European breast cancer organisations. The abstracts submitted to the conference were focused on biomarkers, systemic therapy in early and advanced breast cancer, detection and diagnosis, new drug development, genomics and imaging. Enthusiastic speakers with recognised clinical expertise presented best abstracts selected for oral presentation. One highlight of the Congress was the Pre-IMPAKT training course. The following meeting report is a summation of selected topics and the new research presented during the IMPAKT 2017 Conference.

Introduction

The IMPAKT Breast Cancer Conference was held from 4 to 6 May, 2017 in Brussels, Belgium and was organised in partnership with the Breast International Group (BIG) and the European Society for Medical Oncology (ESMO), in collaboration with a multidisciplinary alliance of European breast cancer organisations. The meeting was attended by 410 participants, of whom 33.3% were medical oncologists. Of the participants, 90.2% cited breast cancer as their primary field of interest, with clinical research and cancer biology being the primary topics of interest. The majority (71.2%) of attendees traveled from Europe; however, the international appeal of the conference was reflected by the 16.6% of participants coming from Asia, 9.0% from North America, 1.5% from Australia and the Pacific, 1.0% from South America, and 0.7% of the attendees came from Africa.

Of the 148 abstracts submitted, 51 were presented at the conference including 34 that focused on biomarkers, 14 with miscellaneous topics, and 13 abstracts that each addressed systemic therapy in advanced breast cancer, detection and diagnosis, and systemic therapy in early breast cancer. Other topics covered included new drug development, genomics and imaging. Enthusiastic speakers with recognised clinical expertise presented the 6 abstracts selected for oral presentation and the remaining 45 abstracts were well-covered in detailed poster presentations.

One highlight of the Congress was the Pre-IMPAKT training course, which was attended by 89 professionals from Belgium, Italy, the USA, France, the UK and Spain, in addition to other countries world-wide.

The following Meeting Report is a summation of selected topics and the new research presented during the IMPAKT 2017 Conference.

DETECTION AND DIAGNOSIS

Methods using fixed circulating tumour cells developed and optimised for the detection of biomarkers of treatment response in metastatic breast cancer

Kristina E. Aaltonen, Division of Oncology and Pathology, Department of Clinical Sciences, Lund University, Lund, Sweden and colleagues demonstrated the utility of circulating tumour cells (CTCs) in detecting biomarkers of response and monitoring treatment, and optimised methods to characterise protein biomarker expression and genomic changes in selected CTCs. The investigators spiked blood samples with cells from the SKBr3, MCF7, BT474, and Colo205 cell lines for method optimization and all applications were validated in samples from patients participating in the CTC-MBC study of metastatic breast cancer (Clinical Trials NCT01322893). CTCs were enriched with the FDA-approved CellSearch® system and mounted on glass slides using the CTC-DropMount method. They then characterised fixed CTCs using a multiplex fluorescence staining procedure developed in their lab for detection of outcome predictive markers, including human epidermal growth factor receptor 2 (HER2) and oestrogen receptor α (ER α). A protocol for HER2 gene amplification analysis was established that used fluorescence in situ hybridization (FISH). The team also optimised fluorescence staining for programmed death-ligand1 (PD-L1) expression on CTCs, which, at high expression levels, has been reported to associate with response to immunotherapy.

In addition, the investigators isolated individually selected CTCs using laser capture microdissection. After performing genome amplification of the selected cells, they found that the DNA quality allowed for a variety of genomic analyses to further characterise CTCs for mutations occurring after treatment that could be predictive of treatment outcome, and also enable increased understanding of tumour evolution. NCT01322893. Aaltonen *et al.* Abstract 2P

Practice point and future research opportunities

The presence of CTCs in the blood of patients with breast cancer is prognostic of poor prognosis; however, CTCs are accessible in patient blood samples and maybe used for monitoring treatment response and to improve treatment selection.

No relationship determined between the time to diagnosis of bone metastases in breast cancer and Nottingham Grade or specific histopathologic characteristics

Mark Wickre, Radiology Department, University of Minnesota, Minneapolis, USA, presented findings from a study that reviewed data of 287 patients diagnosed with breast cancer and bone metastases. Of these, 143 patients without secondary metastases that had given consent were included in this analysis. The investigators assessed the time to diagnosis of bone metastasis, pathological data including Nottingham Grade, receptor status, histological subtype, tumour size, and lymph node status.

At the initial presentation, 24% of patients with breast cancer in the study were diagnosed with bone metastases; the mean time to development of bone metastases if not diagnosed

at staging was 81 months. There was no significant difference in the time to diagnosis based on univariate analysis of Nottingham Grade ($p = 0.11$), receptor status ($p = 0.18$), histological subtype ($p = 0.62$), tumour size ($p = 0.92$), or lymph node status at diagnosis ($p = 0.24$). Wickre *et al.* Abstract 4P

Practice point and future research opportunities

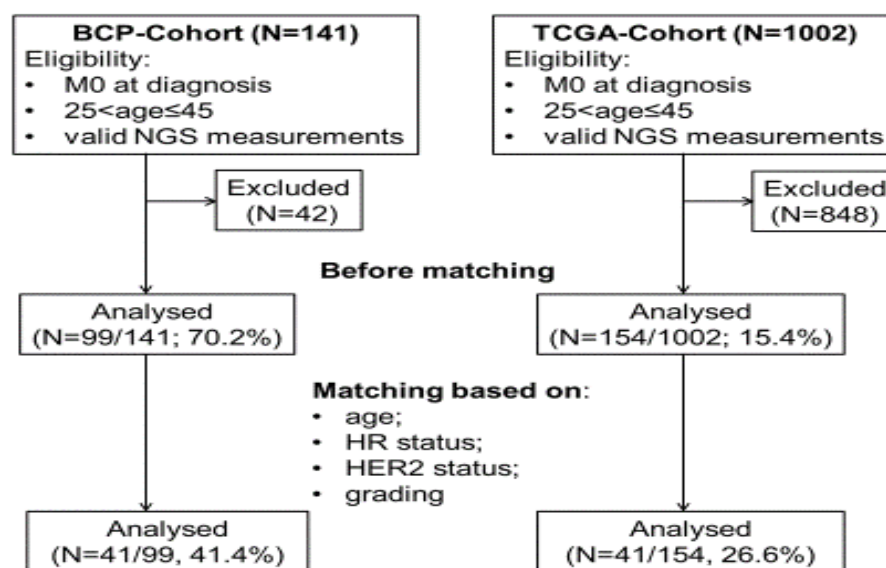
Better understanding of the metastatic distribution, incidence, and timing of bone metastases is needed to use the recent developments in the treatment for early bone metastases early in the course of treatment to improve outcomes. While there have been many attempts to characterise the prognostic factors for metastases from breast cancer, this study is one of the few studies that has focused on the time course to development of bone metastases. However, the analysis was unable to show a significant relationship between the Nottingham Grade or the histopathological factors studied and the time to diagnosis of bone metastases.

EARLY BREAST CANCER SYSTEMIC THERAPY

The tumours of pregnant and non-pregnant patients with breast cancer display similar somatic mutation patterns

Sibylle Loibl, Medicine and Research, German Breast Group, Neu-Isenburg, Germany and colleagues compared a dataset of pregnant patients enrolled in the BCP study (GBG 29; BIG 03-02), a multicentre, retrospective, observational study of women with simultaneous breast cancer and pregnancy to non-pregnant control patients with breast cancer obtained from the TCGA database. Pregnancy is estimated to be a factor in 1 to 3% of all breast cancers.

Figure 1. Consort statement

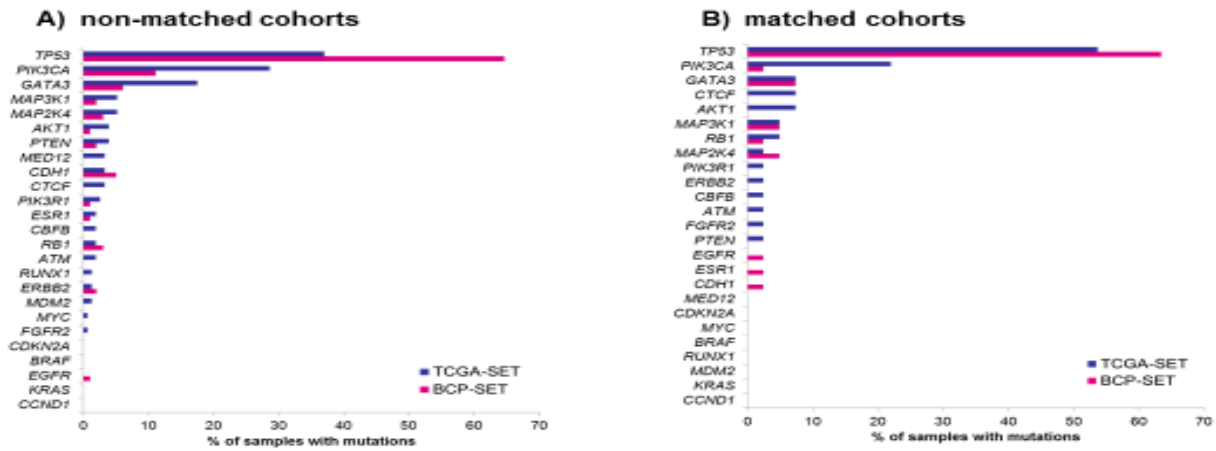


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In this study, investigators analysed FFPE core biopsies taken prior to therapy for somatic mutations using an Ion Torrent Proton/PGM sequencing platform. The samples were assayed on a custom designed breast cancer gene panel (BCPv2) comprising 236 amplicons split into two primer pools and covers 138 exons located in hotspot regions of 25 genes. Only non-synonymous mutations without germline origin were processed.

Overall, the investigation of the mutational patterns of BCP compared to TCGA data identified slightly fewer mutations in pregnant patients; an average of 1.03 mutations per patient was observed in the BCP cohort versus 1.27 in the TCGA cohort. The most frequent somatic mutations occurring in both cohorts were *TP53*, *PIK3CA*, and *GATA3*. *TP53* was seen more often in 65% of the BCP cohort compared to 37% in the TCGA cohort. *PIK3CA* was seen in 11% versus 29%, and *GATA3* in 6% versus 18% in the BCP versus TCGA cohorts, respectively.

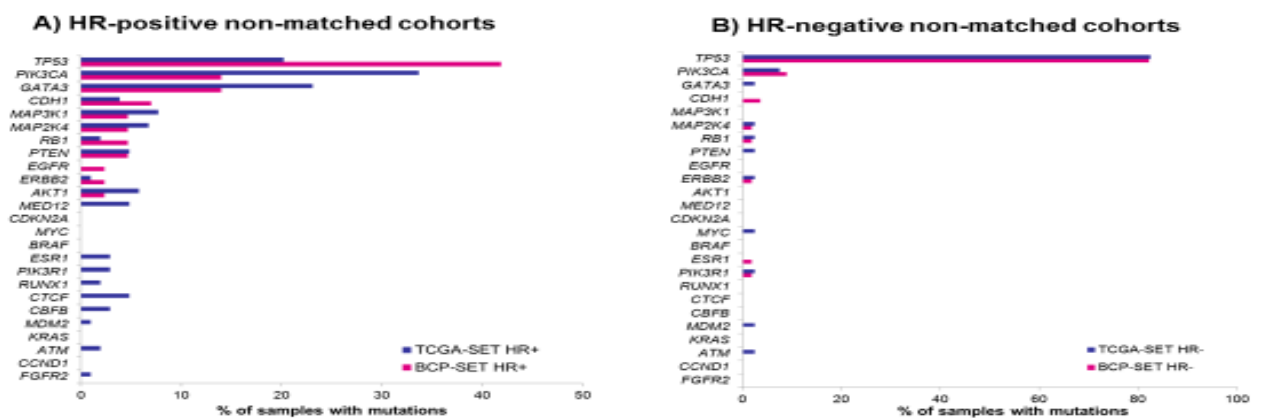
Figure 2. Mutation patterns overall in BCP vs. non-pregnant controls



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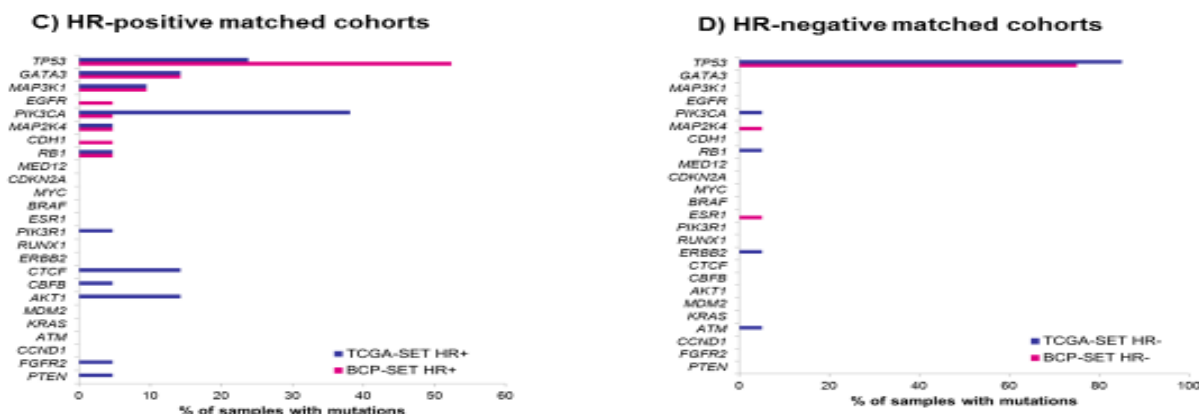
The investigators then performed exact matching of BCP and TCGA cohorts that identified 41 patients per cohort that were matched regarding age, grade, and hormone receptor (HR) and HER2 status. Within this comparison, lymph node positive tumours were less frequent in BCP compared to TCGA patients ($p = 0.046$). *PIK3CA* mutations occurred significantly less frequently in the BCP cohort; 2.4% of pregnant compared to 22.0% of non-pregnant patients harboured *PIK3CA* mutations ($p = 0.015$). However, no significant difference was observed for the frequency of *TP53* ($p = 0.502$) and *GATA3* ($p = 1.000$) mutations in these cohorts. Evaluation of the data by HR status revealed that *TP53* was the most frequently mutated gene overall with higher mutational rate in HR-negative compared to HR-positive patients; *TP53* mutations were observed in 52.4% versus 75% of HR-positive versus HR-negative patients in the BCP cohort and in 23.8% versus 85.0% of HR-positive versus HR-negative patients in the TCGA Cohort.

Figure 3. Mutation patterns by HR status in BCP vs. non-pregnant controls



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Figure 4. Mutation patterns by HR status in BCP vs. non-pregnant controls



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The investigators are continuing to collect tumour material for further translational research using other datasets and are currently looking into gene expression patterns. Loibl *et al.* Abstract 10P

Practice point and future research opportunities

This study compared the somatic mutation patterns of the tumours of pregnant versus non-pregnant women with breast cancer to determine whether there are biological differences in the disease. Currently, there is only protein expression data derived primarily by immunohistochemistry to illuminate the possible biological differences in breast cancer between pregnant and non-pregnant women that would afford the further personalisation of treatment. The study found that the frequencies of some specific mutations differ by hormone receptor status between pregnant versus non-pregnant patients, but on the whole, the mutational landscape does not seem to differ between the groups. More TP53 mutations were identified in pregnant patients with breast cancer but significantly fewer PIK3CA mutations were seen. The investigators addressed the imbalance in the PIK3CA mutational rate after matching and suggested that it reflected a possible remaining bias caused by differences in sensitivity or specificity of the methods used to detect mutations or by differences in variables not used for matching.

Statin treatment modulates 27-hydroxycholesterol and sterol 27-hydroxylase (CYP27A1) expression in breast cancer tumours

Siker Kimbung, Division of Oncology and Pathology, Department of Clinical Sciences, Lund University, Lund, Sweden investigated the role of systemic 27-hydroxycholesterol (27HC) and intra-tumoural CYP27A1 expression on the pathobiology and clinical response to statins in breast cancer. 27HC is an oxysterol produced from cholesterol by the monooxygenase CYP27A1, which regulates intracellular cholesterol homeostasis and also modulates the endogenous selective oestrogen receptor (ER). Therefore, 27HC may increase the rate of tumourigenesis and metastasis. In turn, 27HC levels are modulated to minimise these pro-

tumourigenic activities by statins or direct inhibition of CYP27A1. Professor Kimburg and colleagues investigated the effect of statins on serum 27HC and tumour-specific CYP27A1 expression in 42 breast cancer patients who received atorvastatin within a phase II clinical trial and in two independent patient cohorts.

The team found that statin treatment effectively decreased serum 27HC and deregulated CYP27A1 expression in the patients' tumours, but these changes were not associated with an anti-proliferative response to statin treatment. In primary tumours, CYP27A1 was heterogeneously expressed and high expression significantly associated with high tumour grade, ER negativity, and the basal-like subtype. High CYP27A1 expression also associated with longer recurrence-free and overall survival, but the beneficial effect of high CYP27A1 in ER-positive breast cancer seemed limited to women ≤ 50 years. Although these results establish a link between CYP27A1 and breast cancer pathobiology and prognosis, they also imply that the reduction in serum lipids observed with statin treatment does not directly translate to tumoural anti-proliferative effects, which suggests that other undetermined serum or tumour factors mediate anti-proliferative effects due to statins that are seen in breast cancer. Kimbung *et al.* Abstract 11P

Practice point and future research opportunities

A link between high CYP27A1 expression and better prognosis has been made in this study, which also demonstrated that reducing serum lipids with statin treatment does not directly associate with the observed anti-proliferative effects.

High dose vitamin D3 does not alter pre-surgical caspase 3 or Ki67 levels in patients with breast cancer

Angel Arnaout, Department of Surgery, Ottawa Hospital Cancer Centre, Ottawa, Canada headed a team of investigators located throughout Canada in evaluating the possible tumour suppressive effects of high dose vitamin D3. They conducted a prospective, randomised, double blind, placebo-controlled phase II trial to evaluate the effect of 40,000 IU of oral vitamin D3 on breast cancer biology in patients awaiting surgical management of their primary breast cancer. Blood and tumour samples were obtained from eligible patients who took high dose vitamin D3 for a minimum 2 weeks prior to the day of surgery. Pre- and post- 25-OH vitamin D blood levels, tumour Ki67 index, and tumour caspase 3 were analysed.

The study was completed by 80 patients, 38 in the control group and 42 patients in the vitamin D group. The mean duration on the study was 19 days. Sixteen (64%) patients had oestrogen receptor (ER)-positive tumours, 55 (55%) patients were progesterone receptor (PR)-positive, and 65 (61%) patients were negative for the human epidermal growth factor receptor 2 (HER2-negative). At baseline, the mean blood 25-OH Vitamin D level in the overall population was 76.4 nmol/L, and the mean Ki67 level at baseline was 35.4%.

The mean 25-OH levels increased to 241.9 nmol/L in the vitamin D treated cohort ($p = 0.0001$) by the date of surgery; however, no statistically significant difference in Ki67 expression was observed from the baseline to the surgical specimen in the vitamin D treatment group (mean Ki67 was 39.3%) compared to the control group (mean KI = 41.0%). The baseline caspase 3 level was 31.2% overall and no statistically significant difference was observed in caspase 3

levels in the surgical specimen between the vitamin D (mean = 13.1%) and control (mean =15.6%) cohorts. However, the overall caspase 3 level of 14% that was obtained from the surgical specimen from both study groups was significantly lower than that the mean caspase 3 level of 31.2% in the core biopsy taken at baseline (31.2%; $p = 0.04$). Arnaout *et al.* Abstract 13P

Practice point and future research opportunities

This is the first prospective randomised trial to evaluate the effect of short term, high dose vitamin D on the *in vivo* markers of proliferation and apoptosis. However, no statistically significant difference was seen in these markers as a result of vitamin D intake, despite significantly higher circulating levels of 25-OH vitamin D in the blood.

ADVANCED BREAST CANCER SYSTEMIC THERAPY

Hormone receptor expression remains the sole biomarker for response to ribociclib in HR-positive, HER2-negative advanced breast cancer

Mario Campone, Institut de Cancérologie de l'Ouest/Centre René Gauducheau, Saint-Herblain, France, used data from the phase III MONALEESA-2 trial to evaluate whether baseline levels of specific proteins or the expression of certain genes in the tumour could be predictive of response to CDK4/6 inhibitors. The MONALEESA-2 study randomised 668 postmenopausal women with hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative advanced breast cancer to ribociclib at 600 mg/day for 3 weeks on/1 week off plus 2.5 mg/day of continuous letrozole or to placebo plus letrozole. The trial demonstrated that ribociclib, an inhibitor of CDK4/6, plus letrozole significantly improved progression-free survival (PFS; primary endpoint) over placebo/letrozole.

Professor Campone and colleagues assessed ribociclib efficacy according to baseline tumour protein levels of retinoblastoma (Rb), p16, and Ki67 plus gene expression levels of the oestrogen receptor (ESR1), CDKN2A (p16) and the Cyclin D gene (CCND1), which both play a role in the phosphorylation of Rb; Rb phosphorylation may occur due to either amplification of CCND1 or by loss of p16, a negative regulator of CDK4/6, thereby increasing cyclin D–CDK4/6 activity. All patients were required to provide archival tissue or a fresh tumour biopsy at screening and these samples were evaluated for protein levels by immunohistochemistry and gene expression using NanoString nCounter® Human Cancer Reference panel.

Ribociclib plus letrozole uniformly improved PFS across all levels of protein and gene expression, where the median mRNA expression was used as a breakpoint between high and low gene expression. Rb levels could be evaluated in 479 patients, of whom 416 (87%) had high levels, defined as an H-score ≥ 100 . Of the 405 patients evaluable for p16 protein levels, 165 (41%) had low (H-score < 50), 182 (45%) had medium (H-score ≥ 50 –149), and 58 (14%) had high (H-score ≥ 150) levels. Ki67 was detected in $\leq 14\%$ of tumour cells in 216 (47%) patients and at $> 14\%$ of tumour cells of 247 (53%) patients. The hazard ratios (HR) for PFS according to expression ranged from HR 0.40 in patients with high p16 (95% confidence interval [CI] 0.16, 1.04; $p = 0.06$) to HR 0.68 in patients with low ESR1 (95% CI 0.43, 1.05; $p = 0.08$). NCT01958021. Campone *et al.* Abstract 160

Practice point and future research opportunities

An evaluation of the response to the CDK4/6 inhibitor, ribociclib uncovered no new prognostic markers and benefit was consistent with ribociclib/letrozole over placebo/letrozole irrespective of baseline Rb, p16, or Ki67 levels, or the CDKN2A, CCND1, or ESR1 gene expression levels. Hormone receptor expression remains the only established biomarker for benefit from CDK4/6 inhibitors.

Clinical benefit demonstrated with palbociclib combined with T-DM1 in metastatic HER2-positive breast cancer

Lead author Eric S. Knudsen, Medicine, University of Arizona, Tucson, USA, noted that agents targeting the amplified human epidermal growth factor receptor 2 (HER2) in HER2-positive breast cancer are effective; however, recurrence and progression ultimately occur despite the use of multiple HER2-targeted therapies, leading to investigation of therapies directed to different targets in conjunction with those targeting HER2. This study evaluated the safety and potential efficacy of combined TDM-1, a trastuzumab-cytotoxic drug conjugate targeting HER2, plus the CDK4/6 inhibitor, palbociclib, in previously treated metastatic HER2-positive breast cancer.

Palbociclib elicits a cytotoxic response that may prevent the outgrowth of tumours surviving HER2 targeting by T-DM1. Based on favourable preclinical data, this phase I study of standard 3X3 design enrolled patients with centrally confirmed HER2-positive and retinoblastoma (RB) intact advanced breast cancer, including those having prior TDM-1 exposure. TDM-1 was administered at 3.6 mg/kg i.v. every 21 days and palbociclib was given orally on days 5-18 of each cycle. Dose escalation occurred in 100, 150 and 200 mg cohorts and 9 patients entered the dose escalation, of which 4 (44%) had received prior TDM-1 therapy. Patients received a median of 8 treatment cycles and had an average time on study of 193 days.

The best observed responses included 3 partial responses (PR), 5 stable disease (SD), and one progressive disease. Two out of 4 patients relapsing on TDM-1 achieved stable disease and one patient showed PR. Study drug-related toxicities were mostly haematologic, with grade 3 thrombocytopenia occurring in 33% of patients and grade 3 palbociclib-related neutropenia occurring in 33% of patients at 100 mg, in 66% of patients at 150 mg, and in all (100%) of patients at the 200 mg dose level. Toxicity resolved with dose interruption. NCT01976169. Knudsen *et al.* Abstract 170

Practice point and future research opportunities

These findings provide evidence of clinical efficacy and support further evaluation of the T-DM1/palbociclib combination. The recommended dose for further study is T-DM1 at 3.6 mg/kg on day 1, with 150 mg of palbociclib on days 5 through 18 of a 21-day cycle. At this dose, study drug was well-tolerated and showed reversible haematologic toxicity.

GTx-024 proceeds to stage two of a phase II open label, international, randomised efficacy and safety study in patients with advanced ER-positive, AR-positive breast cancer

Beth Overmoyer, Medical Oncology, Dana Farber Cancer Institute, Boston, USA presented preliminary findings on behalf of a team located throughout the US and UK from a study of GTx-024. GTx-024 is a non-steroidal, tissue-selective, androgen receptor (AR) modulator (SARM), that targets the AR but without eliciting the virilization or oestrogenic effects that can limit the use of androgen therapy. The AR is the most highly expressed steroid receptor in breast cancer and is present in >95% of oestrogen receptor (ER)-positive and 10-50% of ER-negative disease.

This ongoing trial randomises post-menopausal patients to receive GTx-024 at 9 mg or 18 mg orally per day until disease progression. Patients are required to have demonstrated a prior response to endocrine therapy of either ≥ 3 years as adjuvant or ≥ 6 months for metastatic breast cancer. Patients with metastatic breast cancer are allowed one chemotherapy regimen and patients with measurable or bone-only disease or stable CNS disease may be included. The primary endpoint is clinical benefit (CB) at 24 weeks in each arm per modified RECIST v1.1 criteria. Secondary endpoints include objective response rate (ORR), progression-free survival (PFS), time to progression (TTP), duration of response (DoR), and overall survival (OS). The trial will test for a low CBR of $\leq 10\%$ versus a CBR $\geq 30\%$ among 88 evaluable patients who are identified as AR-positive by central review. The 2 dose arms will not be statistically compared; the study aims to determine which dose achieves an acceptable CB. This Simon two-stage design trial plans to enroll 118 patients for the second stage if ≥ 3 patients achieve CB during the first treatment stage.

The first stage of each dose arm is fully enrolled and both doses have been well-tolerated, with 18 patients receiving 9 mg and 15 patients on 18 mg GTx-024 either completing a 24 week evaluation or discontinuing the trial due to other reasons unrelated to treatment or disease progression. In the 9 mg arm, 10 patients demonstrated CB at 24 weeks. Of these patients, 5 were in the evaluable subset (AR-positive) and demonstrated a 28% CB rate, including one partial response (PR). In the 18 mg arm, 6 patients demonstrated CB at 24 weeks, including 4 patients in the evaluable subset, thus demonstrating a 27% CBR, with one patient achieving PR. NCT02463032. Overmoyer *et al.* Abstract 18P

Practice point and future research opportunities

This study of the novel non-steroidal, tissue-selective, AR modulator GTx-024 will proceed to increased enrolment in stage two, since both dosing arms of the study have exceeded the pre-determined stage one threshold, with 9 patients in the evaluable subset of patients with AR expression demonstrating clinical benefit at 4 weeks.

High disease burden does not alter response to ribociclib/letrozole in postmenopausal women with HR-positive, HER2-negative advanced breast cancer

Lead author Sunil Verma, of the Tom Baker Cancer Centre, Calgary, Alberta, Canada, reported findings from a study of patients with hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative advanced breast cancer with multiple metastases. Multiple metastatic sites associate with increased disease symptoms and may complicate clinical management and drug tolerability, leading Professor Verma and colleagues to compare the efficacy of ribociclib in subgroups of patients with low and high disease burden participating in the MONALEESA-2 study. MONALEESA2 enrolled 668 postmenopausal women with HR-positive, HER2-negative advanced breast cancer who had received no prior systemic therapy for advanced breast cancer and who were randomized 1:1 to ribociclib at 600 mg/day for a 3 weeks on/1 week off schedule plus continuous letrozole at 2.5 mg/day, or to placebo plus letrozole. Patients were required to have metastases, predominantly lytic bone lesion, and an Eastern Cooperative Oncology Group performance

status ≤ 1 . In the cohort of patients with low disease burden, defined as < 3 metastatic sites, 220 patients received ribociclib and 221 received placebo, and in the high disease burden cohort, defined as ≥ 3 metastatic sites, 114 versus 113 patients received ribociclib versus placebo, respectively.

Progression-free survival (PFS) was improved with ribociclib/letrozole regardless of the magnitude of disease burden. PFS was increased with ribociclib/letrozole over placebo/letrozole in patients with low disease burden, hazard ratio [HR] 0.607; 95% confidence interval [CI] 0.437, 0.845 ($p = 0.001$) and also in patients with high disease burden, HR 0.456; 95% CI 0.298, 0.700 ($p = 0.0001$).

In the low disease burden subgroup the median duration of ribociclib treatment was 12.1 versus 12.6 months with placebo and the median treatment duration in the high disease burden patients was 12.4 months with ribociclib versus 11.7 months with placebo. In the low and high subgroups, treatment was discontinued in 40% versus 51%, and in 45% versus 60% of patients receiving ribociclib versus placebo, respectively. Discontinuation due to disease progression occurred in 25% of patients on ribociclib versus 40% on placebo in patients with low disease burden and in 29% of patients on ribociclib versus 50% of patients on placebo in the high disease burden arm. The most commonly reported, all-cause grade 3/4 adverse events in the ribociclib plus letrozole arm were neutropenia and reduced white blood cells in patients with low and high disease burden overall. NCT01958021. Verma *et al.* Abstract 19P

Practice point and future research opportunities

Ribociclib plus letrozole significantly improved PFS over placebo plus letrozole in postmenopausal women with HR-positive, HER2-negative advanced breast cancer. This improvement was unaffected by the presence of multiple metastases and was comparable between cohorts of patients having low and high disease burden.

GENOMICS AND PROTEOMIC ANALYSIS OF BREAST CANCER

Next-generation sequencing in BRCA1/2-associated breast cancer

Mattias Van Heetvelde, Centre for Medical Genetics Ghent, Ghent University Hospital, Ghent, Belgium, and colleagues conducted a study evaluating somatic loss of the wild type BRCA1 or 2 allele, loss of heterozygosity (LOH), somatic point mutations, and hypermethylation of the promoter region in tumours from patients with a germline BRCA1/2 mutation. The investigators used multiplex PCR derived next-generation sequencing (NGS) libraries to analyse the complete coding region of BRCA1/2 on 84 formalin fixed and paraffin embedded (FFPE) breast tumours and matching blood samples obtained from patients with confirmed germline BRCA1/2 to evaluate loss of heterozygosity and the presence of somatic nucleotide variations. Also, exon-spanning deletions/amplification were investigated using MLPA in 72 samples and methylation-specific MLPA was used to determine the methylation status of both BRCA gene promoters in 38 samples.

The investigators found that the quality of the sequencing results showed a statistically significant association with the age of the FFPE sample, preventing LOH interpretation in 28 of the older samples. Nonetheless, 33 tumours were suggestive for loss of the wild type allele based on one or multiple markers across the germline mutated gene. They also observed somatic truncating point mutations and that none of the samples showed fully methylated promoters. The MLPA data suggested partial or complete deletion of BRCA1/2 alleles in several samples and the NGS data of 4 tumours suggested loss of the mutant allele. The investigators are validating these findings and correlating the results of the meta-data to the molecular data to gain more insight into the mechanisms underlying these results. Van Heetvelde *et al.* Abstract 21P

Practice point and future research opportunities

This study shows that determining the functionality of BRCA genes within breast tumours from BRCA1/2 germline mutation carriers remains challenging. Also, loss of the mutant allele implicates that use of LOH analyses in breast tumours is not informative to determine the impact of variants of unknown clinical significance. This cohort demonstrated a complex combination of somatic events that could potentially explain the less successful treatment effect of PARP inhibitors in breast cancer.

BIOMARKERS IN BREAST CANCER (PROGNOSTIC, PREDICTIVE AND PHARMACODYNAMIC)

High intra-tumour heterogeneity of the ER indicates higher long-term risk of death in patients with ER-positive, luminal A breast cancer

Linda Sofia Lindström, Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden, investigated the relationship between intra-tumour heterogeneity of the oestrogen receptor (ER) and an increased long-term risk up to 25 years of fatal breast cancer. The investigators used data from patients with postmenopausal lymph node-negative breast cancer participating in the Stockholm Tamoxifen (STO-3) trial, which randomised patients to receive tamoxifen or control. The patients had a complete long-term follow-up that included sample collection until 31 December, 2012. The fraction of cancer cells for each ER intensity level was scored by breast cancer pathologists and intra-tumour heterogeneity of the ER was calculated using Rao's quadratic entropy. Intra-tumour heterogeneity of the ER in ER-positive and Luminal A subtypes were determined by gene expression using the Agilent array.

Long-term breast cancer-specific survival analyses by Kaplan-Meier and multivariate Cox proportional hazard modeling that adjusted for patient and tumour characteristic revealed a significant difference in long-term survival according to intra-tumour heterogeneity of the ER. Long-term survival differed according to intra-tumour heterogeneity of ER in the tamoxifen treated arm for all ER-positive patients (Log rank, $p < 0.0001$), and in patients with luminal A subtype tumours (Log rank, $p = 0.012$). In addition, a two-fold increased long-term risk of fatal breast cancer was observed in patients with high intra-tumour ER heterogeneity as compared to patients with low intra-tumour ER heterogeneity (hazard ratio [HR] 1.98; 95% confidence interval [CI] 1.31, 3.00). This increased risk was also seen in patients with luminal A subtype that had high intra-tumour ER heterogeneity compared to patients with low ER heterogeneity (HR 2.43; 95% CI 1.18, 4.99). Lindstrom *et al.* Abstract 240

Practice point and future research opportunities

Intra-tumour heterogeneity is known to be a major factor in treatment resistance; this study demonstrates that patients with ER-positive or luminal A subtype tumours plus high intra-tumour heterogeneity of the ER are at increased long-term risk of fatal breast cancer as compared to patients with similar cancer subtypes and low intra-tumour heterogeneity. These findings suggest that routine clinical assessment of the intra-tumour heterogeneity of the ER may identify patients at high long-term risk of mortality. This study has a potential to affect the clinical management of patients with ER-positive disease and luminal A subtype tumours.

pSTAT3 signature associates with better outcome in luminal breast cancer

In order to determine whether the STAT3 signaling pathway plays a prognostic role in oestrogen receptor (ER)-positive breast cancer, Amir Sonnenblick, Sharett Institute of Oncology, Hadassah - Hebrew University Medical Centre in Jerusalem, Israel and an international team evaluated the proteomic and gene signature of phosphorylated-STAT3 (pSTAT3) status and compared its impact on outcome, both in a pooled data analysis and in a patient cohort from a large adjuvant phase III trial. First of all, gene signatures were

developed to correlate the level of expression of pSTAT3 with the corresponding level of pathway activation by analysing gene expression and matched proteomic data in ER-positive tumours from the TCGA repository. A pooled analysis of gene-expression data from over 7,000 breast cancer patients was then used to assess the association of these data with prognosis. The pSTAT3 prognostic effect was further validated using tissue from primary tumour tissue microarrays from the Breast International Group (BIG) 2-98, phase III, prospective randomised trial.

Analysis of pooled data revealed that the pSTAT3 signature score was elevated in luminal A tumours but not elevated in luminal B tumours (Kruskal–Wallis test $p < 10e^{-10}$ for all subtypes). The pSTAT3 signature was significantly associated with improved relapse-free survival (RFS; Log rank $p < 10e^{-10}$). This was confirmed by an analysis that was performed in luminal ER-positive breast cancer patients that were treated with endocrine therapy.

The pSTAT3 signature could also identify patients with improved RFS irrespective of the luminal molecular subtype (Log rank: luminal A $p = 0.026$; luminal B $p = 0.006$). pSTAT3 staining by immunohistochemistry in the tumour or stroma was positive in 174 (28.5%) of 610 ER-positive samples from the BIG 2-98 trial. At a median follow-up of 10.1 years, pSTAT3 associated with improved disease-free survival in ER-positive/HER2-negative breast cancer, Cox Univariate hazard ratio 0.66, 95% confidence interval 0.44, 0.98 ($p = 0.04$). Sonnenblick *et al.* Abstract 250

Practice point and future research opportunities

This analysis of pooled data found pSTAT3 to be associated with improved outcome in patients with luminal breast cancer that was confirmed by samples from a phase III trial wherein, at a median follow-up of 10.1 years, pSTAT was associated with improved disease-free survival.

Circulating levels of RANKL and OPG may be prognostic of ER-positive breast cancer survival

Studies, including one carried out by Danja Sarink, and colleagues at the Division of Clinical Epidemiology, German Cancer Research Centre (DKFZ), Heidelberg, Germany have shown that circulating Receptor Activator of Nuclear Factor Kappa-B ligand (RANKL) and osteoprotegerin (OPG) may be associated with increased breast cancer risk in humans. OPG is the decoy receptor for RANKL and together they may play a role in mammary tumour development and progression. In this current study, they aimed to determine whether circulating concentrations of these biomarkers are associated with breast cancer survival. Using the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, they evaluated overall survival (OS) and breast-cancer specific survival (BCSS) in 2006 women with breast cancer. Circulating levels of RANKL and OPG were quantitated by ELISA and electrochemiluminescent assay, respectively, in serum samples that had been collected a median 4.7 (range: 0.2 to 11.9) years prior to diagnosis.

During 21,253 person-years of follow-up, 421 deaths occurred in this cohort, of which 250 were breast-cancer specific. Oestrogen receptor (ER)-positive disease was diagnosed in

1,620 (81%) women. Previously, within-person reproducibility of OPG had been shown to be high (1 year, Spearman $r = 0.85$; 14 years, $r = 0.75$) and within-person reproducibility of sRANKL was lower (1 year, $r = 0.60$; 14 years, $r = 0.38$). Hazard ratios (HRs) and 95% confidence intervals (CIs) for overall and breast-cancer specific mortality were calculated for quintiles (Qs) of the biomarkers using multivariable Cox Proportional Hazards regression models.

In patients with ER-positive breast cancer, higher OPG was associated with risk of death of any cause: Q5 versus Q1, HR 1.47; 95% CI 1.01, 2.14 ($p_{\text{trend}} = 0.01$) and also with breast cancer-specific death (Q5 versus Q1, HR 1.89; 95% CI 1.11, 3.19 ($p_{\text{trend}} = 0.02$)). Among individuals with ER-negative disease, no associations between circulating RANKL or circulating RANKL and OPG and either overall survival or breast cancer-specific mortality were demonstrated. Sarink *et al.* Abstract 260.

Practice point and future research opportunities

The investigators provided new data showing an association between the RANK-axis and ER-positive breast cancer survival. Relatively high concentrations of OPG may represent a risk marker for mortality after an ER-positive breast cancer diagnosis. These observations warrant further study in patient cohorts.

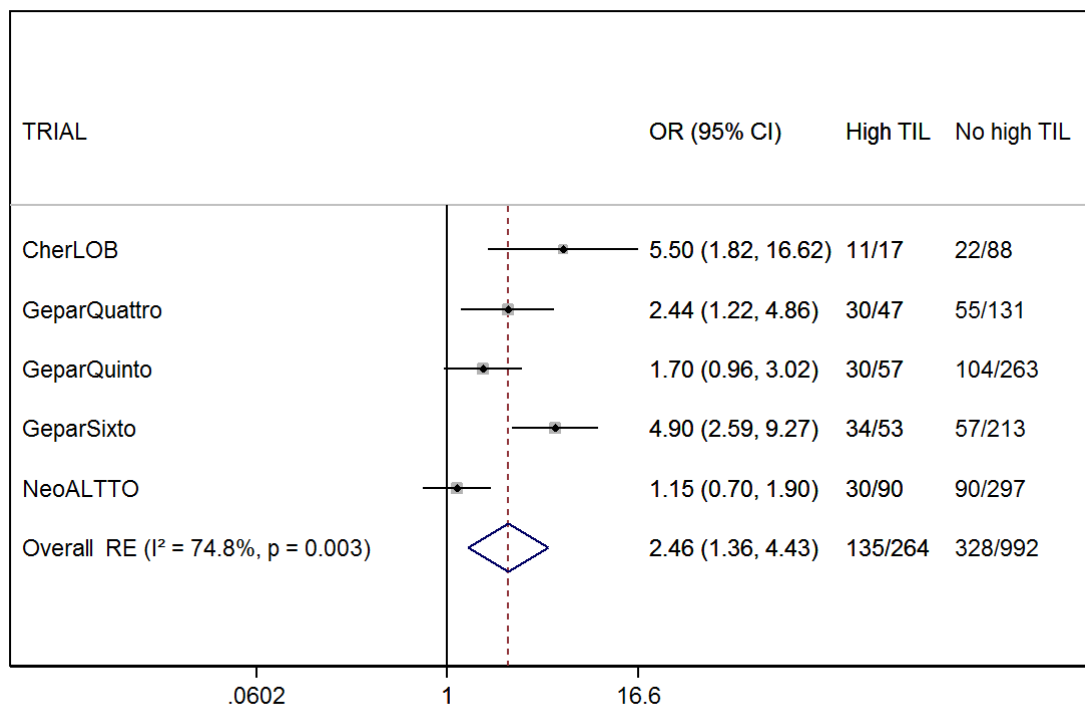
High baseline TIL levels are prognostic for pCR after neoadjuvant chemotherapy and anti-HER2 agents in HER2-positive breast cancer

Cinzia Solinas of the Institut Jules Bordet, Brussels, Belgium reported that high baseline levels of tumour infiltrating lymphocytes (TILs) in pre-treatment biopsies from patients with HER2-positive breast cancer significantly associated with pathological complete response (pCR) rates following neoadjuvant chemotherapy plus anti-HER2 agents. Professor Solinas and colleagues performed a systematic search of the PubMed, Embase and Cochrane library databases for randomised controlled trials investigating neoadjuvant chemotherapy plus trastuzumab, lapatinib, or their combination in HER2-positive breast cancer until 31 October, 2016. The investigators analysed the relationship between the frequency of pCR and pre-treatment levels of TIL by comparing subgroups of patients with high baseline TIL or 'other' levels of TIL (non-high TIL).

This meta-analysis comprised published data from 5 large trials and included 1,256 patients participating in the CherLOB, GeparQuattro, GeparQuinto, GeparSixto and NeoALTT0 neoadjuvant trials. Patients were stratified into TIL subgroups using the cut-off for high TIL defined in each study. The cut-off value for high TIL was 60% in the first 4 trials using trastuzumab, lapatinib or their combination plus anthracycline- and taxane-based neoadjuvant chemotherapy; whereas, in NeoALTT0 the cut-off was 30% and the treatment regime was trastuzumab, lapatinib or their combination plus taxane only-based neoadjuvant chemotherapy.

Evaluation of the data from all five trials using random and fixed effects models demonstrated a significant association between pCR rates and high pre-treatment levels of TIL with an odds ratio (OR) 2.46; 95% confidence interval (CI) 1.36, 4.43 ($p = 0.003$).

Association between TIL subgroups (high TIL versus non-high TIL) and pCR in the whole study population (any chemotherapy plus any anti-HER2 agent(s)).



← High TIL worse High TIL better →

Abbreviations: *: number of pathologic complete responses (pCR)/total number of patients in High TIL subgroup; †: number of pCR/total number of patients in non-high TIL subgroup; OR: odds ratio; CI: confidence interval; TIL: tumour-infiltrating lymphocytes; RE: random effect.

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No interaction was observed between subgroups of patients having high versus non-high TIL in terms of response to anti-HER2 agents, trastuzumab versus lapatinib versus trastuzumab/lapatinib ($p = 0.747$), or between regimes containing anthracyclines plus taxanes versus taxanes alone ($p = 0.201$). However, the association between pCR and high TIL was higher in the 869 patients participating in the CherLOB, GeparQuattro, GeparQuinto, GeparSixto trials of neoadjuvant anthracycline- and taxane-based chemotherapy, which employed a 60% cut-off to define high TIL, OR 2.88; 95% CI 2.03, 4.08 ($p < 0.001$). Solinas *et al.*

Practice point and future research opportunities

These findings indicate that high baseline TIL associate with increased probability of achieving pCR, irrespective of the anti-HER2 agent(s) and neoadjuvant chemotherapy regimes used in patients with HER2-positive breast cancer. The subgroup of patients with high (>60%) TIL prior to treatment demonstrated an increased benefit from chemotherapy combined with anti-HER2 therapy, suggesting that high TIL levels present in a baseline biopsy are prognostic of response to neoadjuvant anthracycline- and taxane-based chemotherapy plus anti-HER2 therapy, including trastuzumab, lapatinib, or their combination.

Analysis of samples from the BIG 02-98 adjuvant phase III clinical trial demonstrates that CD73 expression associates with poorer survival outcomes in patients with TNBC

Lead author Laurence Buisseret, Breast Cancer Translational Research Laboratory, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium presented findings from an analysis of CD73 and clinical outcome in patients with triple-negative breast cancer (TNBC). The investigators used multiplex immunofluorescence in combination with digital image analysis on whole tumour tissue sections to quantitate the level of CD73 expression and CD45⁺ tumour-infiltrating leukocytes (TIL) in 122 TNBC samples obtained from the BIG 02-98 adjuvant phase III clinical trial. BIG 02-98 compared docetaxel plus doxorubicin to doxorubicin-based chemotherapy in node-positive breast cancer.

Using the median as a threshold between low and high levels of CD73 on epithelial cells, high levels of CD73 on epithelial tumour cells significantly associated with reduced disease-free survival (DFS), hazard ratio (HR) 2.21; 95% confidence interval (CI) 1.15, 4.25 (p = 0.02), and with decreased overall survival, HR 2.47; 95%CI 1.21, 5.07 (p = 0.01) in patients with TNBC. This association adjusted for grade, number of positive lymph nodes and tumour size. High levels of epithelial CD73 were also found to negatively associate with CD45⁺ immune infiltration in TNBC (spearman R²: -0.50, p < 0.0001). Furthermore, patients with high levels of CD73 and low levels of TIL had the poorest clinical outcome in terms of DFS, HR 4.24; 95% CI 1.90, 9.45 (p < 0.001) and for overall survival, HR 3.91; 95% CI 1.65, 9.31 (p = 0.002), as compared to patients with low CD73 and high TIL.

Higher CD73 expression was observed in tumour-infiltrating myeloid cells and NK cells compared to peripheral blood by flow cytometry analysis, whereas CD73 expression was lower in tumour-infiltrating CD8⁺ T cells. Buisseret *et al.* Abstract 28P

Practice point and future research opportunities

CD73 is an ecto-enzyme that participates in tumour immune escape through the production of extracellular adenosine in the tumour microenvironment. CD73 gene expression is associated with a worse clinical outcome in various malignancies, including TNBC. In this study, higher CD73 expression on epithelial cells was found to associate with decreased disease-free and overall survival in patients with TNBC, and to negatively associate with TIL

infiltration of the tumour. Taken together, these findings support CD73 expression levels as an indicator of poorer patient outcome in TNBC.

High circulating 27-hydroxycholesterol levels indicate lower breast cancer risk in postmenopausal women

D. Lu, and colleagues at the Department of Cancer Epidemiology, German Cancer Research Centre in Heidelberg, Germany investigated the relationship between circulating 27-hydroxycholesterol (27-HC) and the risk of breast cancer in a nested case-control study using the well-characterised Heidelberg, Germany cohort of the European Investigation into Cancer and Nutrition (EPIC) project. The investigators included 530 patients with incident invasive breast cancer participating in EPIC who were each planned to be matched to two controls ($n = 1036$). Liquid chromatography–mass spectrometry was used for detection of serum 27-HC in blood samples obtained at study recruitment.

The association between circulating 27-HC and breast cancer risk differed by the menopausal status of the women at blood collection ($p_{\text{het}} < 0.05$). On multivariable conditional logistic regression model analysis, higher circulating 27-HC levels were associated with lower breast cancer risk among women who were postmenopausal at blood collection (one unit increase in log₂-transformed 27-HC, log odds ratio (OR_{\log_2}) 0.57; 95% confidence interval (CI) 0.34, 0.95, ($p_{\text{trend}} = 0.03$). This association did not differ significantly by postmenopausal hormone use ($p_{\text{het}} = 0.19$).

No association between 27-HC and breast cancer risk was found in women who were premenopausal at blood collection OR_{\log_2} 1.58; 95%CI 0.83, 2.99 ($p_{\text{trend}} = 0.16$). No heterogeneity in associations was found by tumour hormone receptor status ($p \geq 0.10$), nor in analyses stratified by age at diagnosis (<50 versus ≥ 50) years ($p = 0.91$). No risk associations were determined by tumour hormone receptor status, including oestrogen and progesterone receptors. Lu *et al.* Abstract 29P

Practice point and future research opportunities

27-HC was the first endogenous selective oestrogen receptor modulator to be identified and it has been reported to promote growth and metastasis in ER-positive breast cancer.

This is the first prospective study to show an association between higher circulating 27-HC and significantly lower risk of breast cancer in postmenopausal women; however, no association was seen in premenopausal women. Identification of the association of 27-HC with reduced risk of invasive breast cancer may offer novel avenues for breast cancer treatment and monitoring strategies in post-menopausal women.

Targeted mRNA sequencing for the quantification of immune and cancer-related genes can be successfully performed from small FFPE breast cancer samples

Bruno V. Sinn, Department of Pathology, Charité – Universitätsmedizin Berlin in Berlin, Germany presented results on the application of targeted RNA sequencing (RNA-seq) in 50 formalin-fixed and paraffin-embedded (FFPE) breast cancer core biopsies obtained from patients participating in the neoadjuvant GeparQuattro trial. The team investigated 13 genes for which matching quantitative PCR data was available, including ESR1, ERBB2, CCL5, CD80, CD8A, CTLA4, CXCL9, CXCL13, FOXP3, IDO1, ANXA1, ARID1A, and PDGFRB. One 5 µm tissue section per sample was processed with the HTG EdgeSeq library preparation system using the mRNA Oncology biomarker panel (2,560 genes) and sequenced on an Illumina NextSeq instrument. The PCR and RNA-seq data were compared for the 13 genes available on both platforms and a summarised 8-gene immune-score was calculated. The investigators performed additional analyses, including unsupervised clustering, and differential gene expression analysis to assess the overall biological signal.

The investigators found that the HTG EdgeSeq system can be successfully used in small FFPE tissue biopsies for mRNA sequencing. Library preparation was possible in 48 samples; of these, 45 were successfully sequenced and passed quality control. There was a good concordance between the sequencing data and quantitative PCR data: Pearson's correlation coefficients for the matched genes ranged from 0.959 to 0.243 with coefficients above 0.6 in 8 of 13 genes. The correlation of the summarised immune score was 0.889.

Levels of ESR1 and ERBB2 were highly concordant with the PCR-based classification ($p < 0.001$) and an exploratory analysis of global differential gene expression according to ESR1-status yielded known targets of ESR1. However, unsupervised clustering of the RNA-seq data resulted in incomplete separation according to oestrogen receptor status. Sinn *et al.* Abstract 30P

Practice point and future research opportunities

The HTG EdgeSeq system is a promising approach to targeted mRNA sequencing using small FFPE tissue samples from clinical trial cohorts. Overall, there was a good concordance between the sequencing data and quantitative PCR data. The strength of the biological signal, especially its correlation to clinical endpoints, warrants further investigation.

Pharmacogenomics of aromatase inhibitor associated arthralgia in patients with ER-positive breast cancer

Adrienne E. Borrie, Clinical Pharmacology, University of Western Ontario, London, Ontario, Canada and colleagues enrolled 135 patients with oestrogen receptor (ER)-positive breast cancer at the London Regional Cancer Program who were beginning aromatase inhibitor (AI) therapy. The investigators aimed to evaluate whether arthralgia was associated with this therapy, and reasoned that comprehensive assessment of pharmacogenetic and pharmacokinetic variables that affect AI disposition will improve AI selection and dosing for breast cancer patients. Patients were to complete questionnaires regarding arthralgia symptoms and to provide blood samples at baseline, 6 weeks, and 6 months after the initiation of treatment. Plasma AI drug concentration was measured by liquid chromatography

tandem mass-spectrometry (LC-MS/MS) and DNA was extracted and used for pharmacogenetic analysis of CYP3A4 and CYP2A6.

Of the 135 patients completing the baseline requirements, 95 patients also completed follow-up questionnaires and submitted blood at 6 weeks. In this cohort, 55% of patients reported measurable increases in pain in their hands, shoulders, and arms, and 45% of patients have had measurable increases in pain in their hips and knees. A preliminary LC-MS/MS analysis done on samples from 55 patients receiving letrozole revealed that plasma concentrations of letrozole were significantly associated with variation in the CYP2A6 gene ($p = 0.0061$). Borrie *et al.* Abstract 32P

Practice point and future research opportunities

Aromatase inhibitors are a standard first-line endocrine treatment for postmenopausal women with breast cancer, but their use is frequently associated with arthralgia, which was confirmed by this study. This study also determined that the plasma concentration of letrozole significantly associated with CYP2A6 variation. This finding contributes to the understanding of the impact of genes and drug levels on AI-induced adverse events and may allow better management of AI therapy.

Investigators define a DNA methylation signature in HER2-positive breast cancer cells to indicate trastuzumab response

Sònia Palomerias, Department of Medical Science, University of Girona, Girona, Spain and colleagues used HER2 breast cancer cells to develop a DNA methylation signature to predict response to trastuzumab in HER2 breast cancer patients. The investigators first determined the DNA methylation status to define the epigenetic changes that may be associated with trastuzumab resistance using the Infinium Human Methylation 450K array in SK trastuzumab-sensitive cells and in SKTR trastuzumab resistant cells. The threshold for differences between methylation groups was $\geq 60\%$.

Using the same cell lines, expression array data (RNAseq) was collected to determine the maximum number of biomarkers. Venn diagram comparison of the RNAseq and DNA methylation array identified candidate genes and their function was analysed by Gene Ontology. The DNA methylation and expression status was validated by expression techniques, including quantitative PCR and demethylation treatment with 5-aza-DC. Methylation-specific PCR was used to determine the DNA methylation promoter of candidate genes. Different gene status was identified by DNA methylation microarray and expression array data between SK and SKTR cells; of these, the investigators selected 5 genes for further study: TGF β 1, KILLIN, CXCL2, SLC38A1, and NR2F2. The expression and DNA methylation status of these candidate genes in SKTR were in agreement with their expression observed by qPCR before and after 5-aza-DC treatment and the CpG methylation profile of the candidate genes was confirmed by bisulfite pyrosequencing and methylation specific PCR. The authors have created a platform for additional study aimed at validating the hypermethylation status of the candidate genes as predictive biomarkers of trastuzumab resistance in patients with HER2 breast cancer. Palomerias *et al.* Abstract 33P

Practice point and future research opportunities

The major clinical problem surrounding treatment of HER2 breast cancer is that nearly 62% of patients develop resistance to trastuzumab within a year despite having a good initial response. The DNA methylation status of promoter gene regions has been described as a common epigenetic alteration for silencing or activation of transcriptional repression in various cancer types. The authors have defined candidate genes with differing hypermethylation status between trastuzumab sensitive and resistant cell lines and provided a basis for further studies to validate these genes as predictive biomarkers of trastuzumab resistance in patients with HER2 breast.

BCL2/p53 expression associates with improved breast cancer specific and overall survival in ER-positive breast cancer

Y.H. Eom, Department of Surgery, Seoul St. Mary's Hospital, The Catholic University of Korea, Seoul, Republic of Korea, conducted a retrospective analysis of data from 3,186 patients who were diagnosed with malignant breast cancer between August 2006 and December 2013 to evaluate the prognostic and predictive significance of BCL2 and p53 expression in oestrogen receptor (ER)-positive breast cancer. The relative expression of BCL2 and p53 was determined and the association of each with recurrence free survival (RFS), breast cancer-specific survival (BCSS), and overall survival (OS) was evaluated: BCL2-positive/p53-negative expression occurred in 511 (60.2%) cases of breast cancer, 97 (11.4%) had BCL2-positive/p53-positive expression, 169 (19.9%) had BCL2-negative/p53-negative expression, and 72 (8.5%) cases had BCL2-negative/p53-positive expression.

BCL2-positive/p53-negative expression associated with favourable prognostic factors, including a less advanced histological grade ($p < 0.001$), no lymphovascular invasion ($p = 0.004$), lower pathologic stage ($p = 0.033$), progesterone receptor positive status ($p < 0.001$), HER2-negative status ($p < 0.001$), lower Ki-67 level $< 14\%$ ($p < 0.001$), and EGFR negative status ($p = 0.001$).

In ER-positive breast cancer, BCL2/p53 double positive expression showed a significant association with BCSS ($p = 0.002$) and OS ($p = 0.026$). BCL2-positive/p53-negative and BCL2-positive/p53-positive breast cancer demonstrated a similar clinical prognostic pattern regarding recurrence and survival. Multivariate analysis revealed that lymph node metastasis and BCL2-positive expression were independent prognostic factors for BCSS and OS; however, no association between BCL2/p53 expression and RFS was seen. Eom *et al.* Abstract 35P

Practice point and future research opportunities

It has been reported that the B-cell lymphoma/leukemia 2 (BCL2) and p53 proteins are involved in growth control and the apoptosis pathways, which have a key role in tumour progression and outcome. This study suggests that the co-expression of BCL2/p53 in ER-positive breast cancer may be a predictor of favourable BCSS and OS.

BREAST CANCER HOST IMMUNE AND STROMAL BIOLOGY

Association of tumour genetics and tumour immune cell infiltration to prognosis in breast cancer

Jan Budczies, Charité Hospital, Berlin, Germany and colleagues analysed H&E sections together with specific mutations, mRNA expression, and the clinical data of patients in The Cancer Genome Atlas (TCGA) breast cancer cohort to identify patients that could profit from immunotherapy, and to overcome mechanisms of resistance. The levels of tumour infiltrating lymphocytes (TILs) were also assessed in 879 tumours as well as other cells, including including T cells, B cells, NK cells, monocytic cells, myeloid dendritic cells, and neutrophils were estimated for each of the tumours using specific mRNA makers (MCPcounter).

This investigation revealed that TILs were most abundant in triple negative breast cancer (TNBC), where the levels of T cells, B cells, NK cells, and myeloid dendritic cells were also highest. These 4 cell types were positive prognostic markers of overall survival in TNBC. Neutrophils were most abundant in luminal tumours, and were a positive prognostic marker of overall survival in hormone receptor (HR)-positive/human epidermal growth factor receptor 2 (HER2)-negative tumours.

The presence of TILs correlated significantly with T cells (Spearman's $R = 0.37$), NK cells ($R = 0.34$), monocytic cells ($R = 0.30$), B cells ($R = 0.28$), and myeloid dendritic cells ($R = 0.23$). In all, 22 mRNAs correlated strongly with TILs ($R > 0.40$), including T cell markers CTLA4, ICOS, and SRIPG plus the chemokines CXCL9, CXCL10, and CXCL11. High mutational load correlated positively with monocytic cells ($R = 0.14$) and negatively with endothelial cells ($R = -0.25$), neutrophils ($R = -0.19$), and fibroblasts ($R = -0.13$). MutSig 1, which is age-related correlated negatively with 9 of the 10 cell types and most strongly with myeloid dendritic cells ($R = -0.27$), endothelial cells ($R = -0.25$), neutrophils ($R = -0.20$), T cells ($R = -0.18$), B cells ($R = -0.17$), and NK cells ($R = -0.17$). MutSig 3, indicating defective DNA repair, correlated negatively with endothelial cells and neutrophils (both $R = -0.14$). Of the two apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-related signatures, MutSig 2 did not correlate with any of the cell populations in contrast to MutSig13 that correlated positively with monocytic cells ($R = 0.19$), cytotoxic lymphocytes ($R = 0.17$), T cells ($R = 0.16$), NK cells ($R = 0.15$), and negatively with endothelial cells ($R = -0.13$). Budczies *et al.* Abstract 39P

Practice point and future research opportunities

The markers of immune cell microenvironment investigated here warrant further evaluation in tissue from patient cohorts participating in clinical trials evaluating immunotherapies.

Immune pruning of genomic heterogeneity in TNBC

Thomas Karn, Department of Gynaecology and Obstetrics, Johann-Wolfgang Goethe University, Frankfurt, Germany described the immunoediting hypothesis of cancer progression as the basis for this study, which suggests some cancers may be completely eliminated before diagnosis by an antitumour immune response, whereas most clinically apparent cancers represent states of partially controlled cancer or tumours that escaped

immune surveillance. It was hypothesised that extensive lymphocytic infiltration is caused by strong anti-tumour immune response that results in “pruning” of genomic heterogeneity of the cancer by eliminating many immunogenic cell clones. Therefore, cancers that have evaded immune surveillance may be expected to have low lymphocytic infiltration and to evolve towards greater clonal heterogeneity and genomic complexity. With colleagues, he tested this hypothesis by comparing clonal heterogeneity, somatic copy number alterations, mutations and neoantigen load, and the distribution of mutations in 119 canonical cancer genes among triple negative breast cancer (TNBC) samples obtained from the The Cancer Genome Atlas (TCGA) breast cancer cohort that were stratified by prognosis according to immune gene signatures or by histologically quantified lymphocyte infiltration.

The investigators found a strong inverse relationship between clonal heterogeneity and immune metagene expression ($\rho = -0.395$, $p = 2e-8$). They also observed a strong inverse relationship between immune metagene expression and somatic copy number alteration levels ($\rho = -0.484$, $p = 2e-10$). Moreover, patients with lymphocyte rich TNBC had a good prognosis and significantly lower mutation and neoantigen counts than patients with poor prognosis, lymphocyte poor TNBC, that also showed increased frequency of CASP8 mutation ($p = 0.007$). The results were validated using both different immune metagenes and histological quantification of infiltrating immune cells in an additional independent dataset. Karn *et al.* Abstract 40P

Practice point and future research opportunities

Mechanistic understanding of how immune surveillance shapes the cancer genome is important to aid in patient selection for immunotherapies and the development of more effective immunotherapy strategies. These findings suggest that immune cell rich TNBC is under a strong immune surveillance, which continuously eliminates many immunogenic clones, which results in lower clonal heterogeneity. These cancers may also represent the subset of TNBC that could derive benefit from immune checkpoint inhibitor therapy.

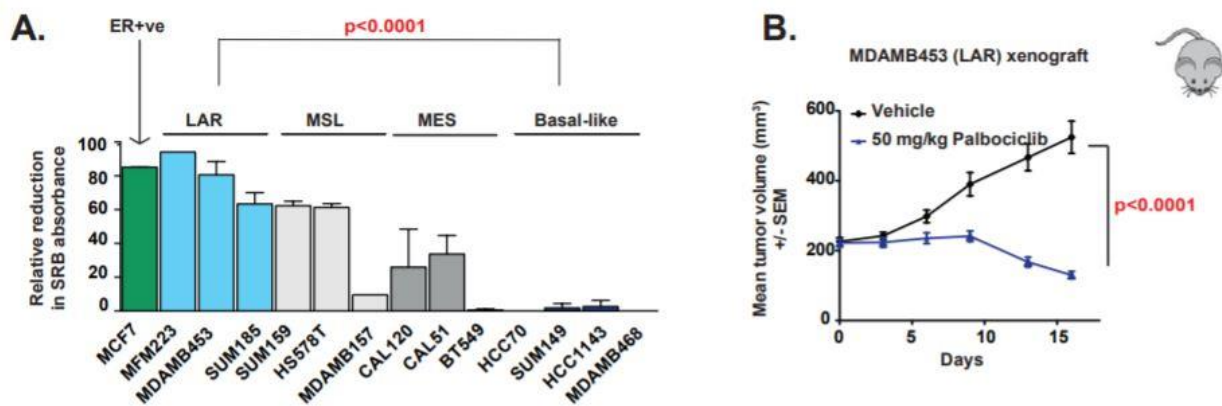
PRECLINICAL BREAST CANCER BIOLOGY

The LAR subtype of TNBC is highly sensitive to CDK4/6 inhibition *in vitro*

Uzma Asghar of Breast Cancer Now, Institute of Cancer Research, London, UK headed a research team in identifying a subtype of triple negative breast cancer (TNBC) that may be responsive to cyclin-dependent kinases 4 and 6 (CDK4/6) inhibition by agents such as palbociclib and ribociclib, which have been indicated in combination with an aromatase inhibitor for the treatment of hormone receptor (HR)-positive breast cancer. However, TNBC has demonstrated resistance to these agents, prompting the investigators to use several preclinical models to identify subtypes of TNBC that may be sensitive to CDK4/6 inhibition and to define the mechanisms of resistance seen in other subtypes of TNBC. *In vitro* assays on 18 TNBC cell lines were done to quantify sensitivity to palbociclib and ribociclib, which were also evaluated for *in vivo* drug toxicity, efficacy, and pharmacodynamics in mouse MDAMB453 xenograft models. The investigators performed single cell phenotypic analysis using a CDK2 live cell reporter to understand resistance mechanisms to palbociclib using time-lapse imaging technology. Additional experimental techniques employed included immunofluorescence, western blotting, and immunohistochemistry (IHC) to determine molecular determinants of sensitivity.

The luminal androgen receptor (LAR) subtype of TNBC was observed to be highly sensitive to CDK4/6 inhibition.

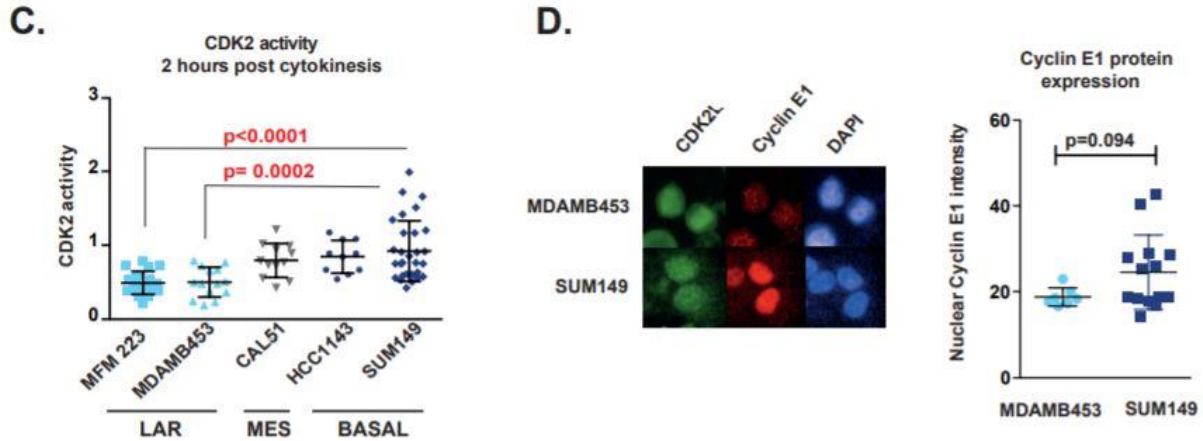
Luminal androgen receptor (LAR) subtype of triple negative breast cancer (TNBC) demonstrates *in vitro* and *in vivo* sensitivity to CDK4/6 inhibition.



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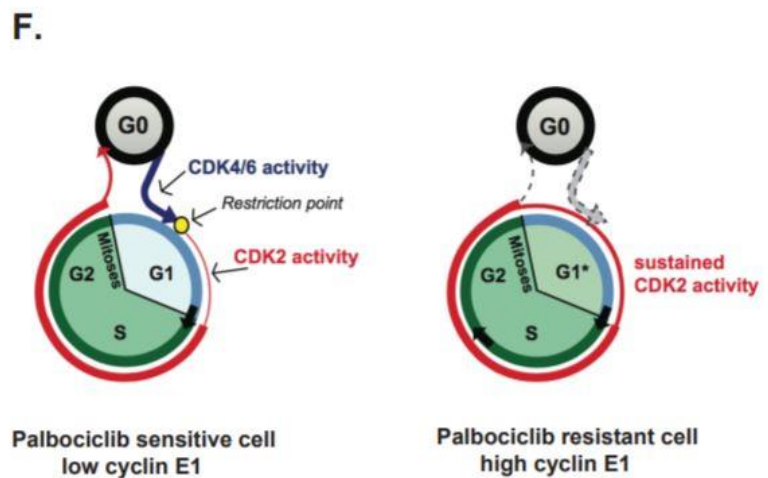
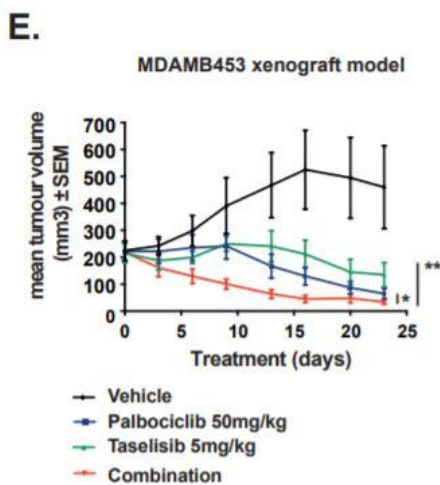
A. Sensitivity to 500 nmol palbociclib across 13 TNBC cell lines in clonogenic assays with LAR subtype highly sensitive to CDK4/6 inhibition [p < 0.0001 Student's T test LAR vs. basal-like]. ER-positive MCF7 cells are shown as positive control of sensitivity.

B. Mouse xenografts from LAR MDAMB453 cells, treated daily with vehicle (n = 10) and palbociclib (n = 10) demonstrating *in vivo* efficacy [p < 0.0001 Student's T test vehicle vs. palbociclib]. Error bars = mean tumour volume (mm³) and SD.



C. Post-mitotic (2 hours) CDK2 activity in individual cells from five TNBC cell lines. LAR MFM223 vs. Basal-like SUM149 cell lines (Student's T-test p < 0.0001). LAR MDAMB453 vs. basal-like SUM149 (p < 0.0002). Error bars = mean CDK2 ratio and SD. LAR=Luminal androgen receptor subgroup (light blue); MES=mesenchymal (grey) and basal-like (dark blue).

D. Immunofluorescent staining of nuclear Cyclin E1 (red) in CDK2L positive (GFP) MDAMB453 cells and SUM149 cells 1 hour after mitosis; DAPI (blue). Cyclin E1 nuclear intensity was assessed by immunofluorescence in individual cells transfected with the CDK2-L sensor, 1-2 hours post-mitosis in LAR MDAMB453 and basal-like SUM149 cell lines (p = 0.094 Student's T-test). Error bars = mean nuclear intensity and SD.



E. Mouse xenografts from MDAMB453 cells, treated daily with Vehicle (n = 10), palbociclib (n = 10), taselisib (n = 10) or combination (n = 10) (p = 0.02 palbociclib vs. combination p = 0.02; taselisib vs. combination p = 0.01). Error bars are mean tumour volume and SD.

F: Left: Schematic of cell cycle dynamics for palbociclib-sensitive model. CDK4/6 inhibition sensitive cell (CDK2^{low}) exits mitosis into a quiescent state (G0), requiring CDK4/6 activity (blue) to pass through the restriction point (yellow circle) after which CDK2 activity (red) promotes S phase entry.

Right: Schematic of cell cycle dynamics for palbociclib-resistant model. CDK4/6 inhibitor resistant cells (CDK2^{high}) exit mitosis in an active proliferating state with high CDK2 activity, bypassing the restriction point, making CDK4/6 activity redundant to enter S phase.

The LAR subtype of TNBC was highly sensitive to CDK4/6 inhibition in the *in vitro* assays compared to basal-like subtypes, which were resistant (p < 0.001). This sensitivity to palbociclib at 50 mg/kg was confirmed *in vivo* using MDAMB453 xenografts wherein 7 partial responses were observed out of a total 10 xenografts. Furthermore, IHC analysis revealed a reduction in tumour phosphorylated retinoblastoma (pRB) levels, a key factor in cell cycle progression, as early as 4 hours after treatment.

The mechanism of resistance was studied by phenotypic single cell analysis using 3 palbociclib sensitive and 3 palbociclib resistant TNBC cell lines, which revealed distinct cell cycle dynamics. Cells sensitive to palbociclib exited mitoses in a CDK2^{low} quiescent state that required CDK4/6 activity for cell cycle re-entry. However, cells resistant to palbociclib exited mitoses directly into a CDK2^{high} proliferative state with rapid transition to S phase with shorter cell cycles. Higher cyclin E1 levels were observed during the early G1 phase in resistant CDK2^{high} cells than measured in palbociclib sensitive CDK2^{low} cells. Interestingly, blocking cyclin E1 activity made resistant cells, sensitive to palbociclib, leading to the conclusion that dysregulation of cyclin E1 expression is a key determinant of sensitivity to CDK4/6 inhibition. Based on these pre-clinical findings, the authors concluded that the LAR subgroup of TNBC demonstrated high sensitivity to CDK4/6 inhibition, and noted that an ongoing clinical trial is currently assessing CDK4/6 inhibition in TNBC (phase Ib PIPA, NCT02389842). Asghar *et al.* Abstract 44P

Practice point and future research opportunities

In vitro cell line analysis demonstrated that the LAR subgroup of TNBC had high sensitivity to CDK4/6 inhibition. Distinct differences were noted between cell cycle exit from mitosis between palbociclib sensitive and resistant TNBC cell lines. Dysregulation of cyclin E1 expression has a key role in sensitivity to CDK4/6 inhibition. Higher levels of cyclin E1 were observed in resistant cells, which could be made sensitive to palbociclib by silencing cyclin E1. This study also demonstrated dual inhibition of CDK4/6 and PI3 kinase inhibitors in PIK3CA mutant TNBC cell lines was synergistic and could make other TNBC subtype cell lines sensitive to palbociclib. These pre-clinical findings suggest that CDK4/6 inhibition may be active in TNBC and support the ongoing clinical trial that is assessing CDK4/6 inhibition in TNBC.

Identification of chemotherapies that selectively target the vulnerability of RB tumour suppressor loss in TNBC

Eric S. Knudsen, University of Arizona, Tucson, USA investigated using a targeted approach to identify pharmaceutical vulnerabilities in triple negative breast cancer (TNBC) created by the loss of the retinoblastoma (RB) tumour suppressor, a key element of TNBC that deregulates cell cycle progression and is also associated with the aberrant expression of several genes that are drug targets. Genetic analysis done in 94 TNBC samples and histological analysis performed in 220 samples revealed that approximately 30% of cases exhibit loss of function of the RB tumour suppressor. The investigators used high-throughput drug screening to identify available agents that selectively target the loss of RB in TNBC. They screened cell lines having naturally occurring RB loss and clustered regularly interspaced short palindromic repeats/caspase9 (CRISP/CAS9) deletions to define selectivity. The results were validated by counter-screening with RB “super-activation” and competitive assays. Biochemical studies were performed to elucidate the mechanisms of action and xenograft studies were done to determine efficacy.

The investigators found that RB loss was associated with increased sensitivity to selective chemotherapies and drugs targeting DNA-damage checkpoints, such as CHK1, and chromosome segregation, for example PLK1, and also defined combination treatments that were particularly active in RB-deficient TNBC xenograft models that resulted in durable disease control in these models. They report that they have optimised immunohistochemical staining and molecular approaches that will enable the prospective identification tumours that have lost RB function. Knudsen *et al.* Abstract 45P

Practice point and future research opportunities

The genetic complexity of TNBC and the loss of the RB tumour suppressor function as the key driving event in TNBC have hampered the development of a precision approach in the treatment of this disease, and treatment remains predominantly cytotoxic chemotherapy-based. This study provides a basis for a targeted approach for treating the substantial number of TNBC cases that involve loss of RB tumour suppressor. These findings are in agreement with the function RB has in controlling cell cycle regulatory processes, and suggest a role for RB in the response to neoadjuvant chemotherapy.

BREAST CANCER TARGET IDENTIFICATION, VALIDATION AND PRECLINICAL MODELS

Novel therapeutic target identified by epigenomic analysis of primary breast cancer tumours

Citing the emergence of super-enhancers (large clusters of cis-acting enhancers) as regulatory features of oncogenes and other key tumour drivers in cancer cells, Matthew G. Guenther, Epigenomics and Gene Regulation, Syros Pharmaceuticals, Cambridge, USA and colleagues have shifted the focus to genomic non-coding regions to identify oncogenic cell state drivers and pinpointing novel druggable targets. They used H3K27ac ChIP-seq in 42 primary breast cancer patient samples to map enhancers and super-enhancers throughout the genome, and found that the super-enhancer maps also picked up known oncogenic drivers. In addition, the maps recapitulate established clinical subgroups; for example, most samples classified as HER2-positive contain a super-enhancer at the ERBB2 locus and most samples classified as ER-positive contain a super-enhancer at the ESR1 locus while neither tend to appear in TNBC samples.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) mediated gene ablation, as well as chemical validation was used in a panel of cell lines that either exhibit or do not exhibit the gene-associated super-enhancer to validate the new targets detected by mapping the patient samples. Applying the chemical approach in a panel of breast cancer cell lines, a super-enhancer was identified at the RARA locus that predicts sensitivity to a potent RAR α agonist, SY-1425. The investigators stated that the sensitivity of these cell lines to SY-1425 is associated with the enhancer size, thereby identifying RAR α as an enhancer-correlated vulnerability in breast cancer, and further state that this correlation extends to *in vivo* xenograft models. Guenther *et al.* Abstract 47P

Practice point and future research opportunities

Findings from this study support super-enhancer analysis as a tool to discover breast cancer dependencies *de novo*, independent of somatic mutations and to define new biomarker-linked breast cancer vulnerabilities for therapeutic intervention. By the CRISPR approach, new targets were identified by their association with super-enhancers in primary samples, which were validated in a panel of breast cancer cell lines.

NEW DRUG DEVELOPMENT

Novel xentuzumab targets IGF1R/IR signalling in ER-positive breast cancer cell lines

Agnese Losurdo, Department of Oncology, IRCCS Clinical and Research Institute Humanitas, Rozzano, Italy, and colleagues assayed xentuzumab (BI 836845) in a panel of oestrogen receptor (ER)-positive breast cancer cell lines, including MCF-7, T47D, CAMA-1, as a sole agent and in combination with hormonal therapeutics such as tamoxifen, fulvestrant, oestrogen deprivation, and/or RAD 001 mTOR inhibitor. Each proliferation assay was carried out in triplicate and involved cells seeded at 3×10^3 cells in 96-well plates, treated with the drug compounds and incubated for 5 days. Proliferation was compared to untreated controls using the acid phosphatase assay.

Growth was inhibited by nearly half (45%) in MCF-7 cell lines when treated with xentuzumab at 10 $\mu\text{g}/\text{mL}$ and T47D cell lines showed 35% growth inhibition at the same dose. Xentuzumab as a sole agent showed modest activity consisting of 5% growth inhibition in luminal-type human breast cancer CAMA-1 cells, which is consistent with the known biological heterogeneity in luminal breast cancer. Xentuzumab was synergistic with fulvestrant, tamoxifen, and RAD 001 in sensitive cell lines, and the strongest combination was xentuzumab plus tamoxifen together with RAD 001. Under oestrogen deprivation conditions, which mimic aromatase inhibition, both xentuzumab and RAD 001 were more active than in the presence of oestradiol. Losurdo *et al.* Abstract 49P

Practice point and future research opportunities

Although phase II trials of anti-IGF1R monoclonal antibodies in ER-positive breast cancer provided no significant increase in progression-free survival, xentuzumab (BI 836845), a humanised monoclonal antibody with high affinity to human IGF1 and IGF2, has shown effective inhibition of IGF-induced activation of both IGF1R and the insulin receptor *in vitro*. Insulin receptor activation has been implicated in resistance to anti-IGF1R therapy. In this study, sole agent xentuzumab showed promising activity in breast cancer cell lines and potentiated growth inhibition when used in combination with an mTOR inhibitor under oestrogen deprivation conditions. This study supports the rationale for the development of xentuzumab as a treatment strategy in ER-positive breast cancer.

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Affiliations and Disclosure

Affiliation

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Disclosure

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