An analysis of ERBB2 alterations (amplifications and mutations) found by next-generation sequencing (NGS) in 2000+ consecutive solid tumor (ST) patients.

Gary A. Palmer, Kai Wang, Vincent A. Miller, Roman Yelensky, Phil Stephens, Jeffrey S. Ross, Doron Lipson; Foundation Medicine, Inc., Cambridge, MA; Foundation Medicine, Cambridge, MA; Albany Medical College, Albany, NY

Background: Testing for ERBB2 amplification by FISH and IHC is routine in breast and gastro-esophageal cancer. There are 3 approved and multiple targeted therapies in clinical trials that rely on the results of these tests. ERBB2 amplification and activation by mutation/fusion has also been described in a wide variety of other ST. As these alterations are not routinely tested for but may predict response to anti-ERBB2 agents we sought to determine their frequency in an unselected cohort of specimens from advanced ST patients.

Methods: We reviewed genomic profiles from the first 2,223 formalin-fixed, paraffin-embedded specimens received and analyzed by our CLIA-certified lab (Foundation Medicine) with our NGS platform. 3,230 exons in 182 cancer-related genes and 14 genes frequently rearranged were assayed for base pair substitutions, small insertions/deletions (indels), amplifications, and rearrangements.

Results: 110/2,223 (4.9%) specimens had 116 ERBB2 alterations: 67 (58%) amplifications, 29 (25%) substitutions, 16 (14%) indels, 2 (2%) splice site variants and 2 (2%) translocations, including a potential fusion. Six samples (5%) had multiple alterations, and two had both ERBB2 substitution and amplification. 14 ST types had evidence of ERBB2 alterations including 29% of esophageal, 20% of uterine, 14% of breast, and 12% of stomach carcinomas. 6% of all lung cancer samples had ERBB2 alterations. Amplifications predominated, but lung specimens had predominantly indels. Durable responses exist to anti-ERBB2 agents in STs with activating ERBB2 mutations.

Conclusions: Use of a broad NGS panel identifies an unprecedented number of actionable genomic changes including a significant rate of ERBB2 alterations across 14 different solid tumor types. The discovery of unanticipated ERBB2 amplifications and activating mutations in a wide variety of ST highlights the need to study a broad range of genes at a high level of sensitivity and specificity when searching for novel targets of therapy. Widespread use of this approach could provide more treatment options and enable more rapid accrual to ongoing and planned trials of agents targeting pathways under study.
Integrating molecular profiling into cancer treatment decision making: Experience with over 35,000 cases.

Zoran Gatalica, Sherri Millis, Sting Chen, Gargi Dan Basu, Wenhsiang Wen, Les Paul, Ryan P. Bender, Daniel D. Von Hoff; Caris Life Sciences, Phoenix, AZ; Caris Life Sciences, Irving, TX; Virginia G. Piper Cancer Center Clinical Trials at Scottsdale Healthcare/TGen, Scottsdale, AZ

Background: Molecular profiling of both common and rare cancer types provides for the identification of actionable targets for chemotherapy with many unexpected associations. Methods: Caris Life Sciences database of >35,000 profiled cancers was reviewed for well-established driver gene mutations and copy number alterations, and protein expression patterns that are relevant for selection of targeted therapy. Based on the published literature, these tumor characteristics were then associated with potential benefit or no benefit to the specific therapeutic agents. All relevant published studies were evaluated using the USPSTF grading scheme for study design and validity. Assay methodologies included sequencing (Sanger, pyrosequencing), PCR, FISH, CISH, and immunohistochemistry. Results: All common malignancies (10 most common cancer types in men and women) and 10 rare cancer types were well represented (minimum of 100 cases in each individual cancer type). Well established driver mutations and protein expression in common cancers were all identified with expected frequencies (e.g. HER2 amplification in breast, PIK3CA mutations in ER+ breast cancer, EGFR mutations in NSCLC, etc.). Importantly, unexpected new and potentially actionable targets were identified in common (e.g., 6.7% HER2 amplification in NSCLC, 1.6% KRAS mutation in prostatic adenocarcinoma) and rare cancers (e.g., 8.3% ALK alteration in soft tissue sarcomas, 10.5% c-MET and 26.4% EGFR gene amplification in melanomas, 16.3% KRAS mutation in cholangiocarcinomas, 10% AR expression in STS), as well in cancers of unknown primary site (approximately 4% of all tested cases). Conclusions: This review of the large referral cancer profiling database provided an unparalleled insight in the distribution of common and rare genetic and protein alterations with direct and potential treatment implications. Numerous targets were discovered that had a potential to be treated by the conventional chemotherapy as well as targeted therapy not usually considered for the cancer type. Comparison between an individual patient tumor profile and database for the matched cancer type provides additional level of support for targeted treatment choices.
Princess Margaret Cancer Centre (PMCC) Integrated Molecular Profiling in Advanced Cancers Trial (IMPACT) using genotyping and targeted next-generation sequencing (NGS).

Philippe L. Bedard, Amit M. Oza, Ming-Sound Tsao, Natasha B. Leightl, Frances A. Shepherd, Eric Xueyu Chen, Ian Tannock, Monika K. Krzyzanowska, Neesha C. Dhani, Blaise Clarke, Hal K. Berman, Stefano Serra, Kenneth J. Craddock, Dianne Chadwick, Tong Zhang, Mahadeo A. Sukhai, Celeste Yu, Aaron Richard Hansen, Suzanne Kamel-Reid, Lillian L. Siu; Princess Margaret Cancer Center, University Health Network, Division of Medical Oncology & Hematology, Department of Medicine, University of Toronto, Toronto, ON, Canada; Department of Pathology, University Health Network, University of Toronto, Toronto, ON, Canada; Princess Margaret Cancer Center, Toronto, ON, Canada; Princess Margaret Hospital, Toronto, ON, Canada; University Health Network, Department of Pathology and Laboratory Medicine, Toronto, ON, Canada; Princess Margaret Cancer Center, University Health Network, Division of Medical Oncology & Hematology, Toronto, ON, Canada

Background: IMPACT is an institution-wide screening program to identify patients (pts) treated at PMCC with somatic alterations that can be matched to targeted therapies. Methods: Pts with advanced breast, colorectal (CRC), non-small cell lung (NSCLC), ovarian cancers and selected other solid tumors treated at PMCC were eligible. Tumor DNA was isolated from a FFPE archived sample and genotyped using a customized Sequenom panel (23 genes, 280 mutations) in a CLIA-certified laboratory. Verified mutations were reported in pts electronic health records. Selected FFPE samples were further characterized by NGS with the Illumina MiSeq TruSeq Amplicon Cancer Panel (48 genes, 212 amplicons, ≥500x coverage) for platform validation. Results: From Mar 1/12-Jan 10/13, 485 pts were enrolled with median 1 prior treatment for advanced disease (range 0-6). Of 33 (7%) screen failures, 5% were for insufficient tissue and 2% for clinical deterioration. Median DNA quantity from FFPE was 4250ng (range 15-32550ng). The median time from tissue receipt to reporting was 5 weeks (range 1-23). Mutations were identified by Sequenom in 137/349 (39%) pts, including 24/79 (30%) breast, 40/80 (50%) CRC, 54/88 (61%) NSCLC, 17/78 (22%) ovarian, and 2/24 (8%) other cancers. Mutations detected were: 76 KRAS, 35 PIK3CA, 22 EGFR, 5 NRAS, 5 ERBB2, 5 CTNNB1, 4 BRAF, and 1 AKT1. MiSeq was concordant with Sequenom in 112/113 (99%) pts, with mutations identified in 94/114 (82%). The average number of mutations detected by MiSeq was 1.72/pt (range 0-7) compared with 0.49/pt by Sequenom (range 0-2). After a median follow up of 5.0 months, 31/137 (23%) pts with mutations have been matched to targeted therapies, including 14 pts enrolled in clinical trials (15 trials) matched to their genotype. Of the 10 trial pts with at least one response assessment, 3 PR (1 confirmed) and 2 SD ≥ 24 weeks have been observed. Conclusions: Molecular profiling can be integrated into the routine care of advanced cancer pts. Genotyping and targeted NGS are feasible in a clinical laboratory using stored archival FFPE tumor samples. NGS identifies additional actionable mutations to inform clinical-decision making. Clinical trial information: NCT01505400.
Use of mutational profiling of metastatic ER+/HER2- breast cancers and the coexistence of KRAS, MET, BRAF, and FGFR3 with PIK3CA mutations.

Debora Fumagalli, Roberto Salgado, Carmen Criscitiello, Lina Pugliano, Ioanna Laios, Timothy Wilson, Denis Larsimont, Martine J. Piccart-Gebhart, Stefan Michiels, Mark Lackner, Christos Sotiriou, Sherene Loi; Jules Bordet Institute, Breast Cancer Translational Research Laboratory, Brussels, Belgium; Jules Bordet Institute, Department of Pathology, Brussels, Belgium; Istituto Europeo di Oncologia, Milan, Italy; Jules Bordet Institute, Breast International Group, Brussels, Belgium; Genentech Inc., San Francisco, CA; Institut Gustave Roussy, Villejuif, France; Jules Bordet Institute, Brussels, Belgium; Peter MacCallum Cancer Center, Melbourne, Australia

Background: ER+/HER2- breast cancers (BCs) constitute the most frequent BC subtype. Their response to endocrine therapy and degree of estrogen dependence are heterogeneous. There is little data available about the genetic changes associated with disease progression in this subtype. This information could facilitate drug development. Methods: A series of 132 ER+/HER2- BC patients diagnosed between 1982 and 2008, with known local-regional (n=10) or distant (n=98) relapse or both (n=24), and available FFPE blocks from their primary (P; n=132) and paired relapse (R; n=49) were identified at a single institution. ER and HER2 status were centrally confirmed. 120 mutations from 11 actionable genes and PTEN protein expression were determined using Fluidigm-based real-time PCR and IHC, respectively. Results: At primary diagnosis, median age was 57 years (27-90); median tumor size 2.5 cm (0.5-11); 75% had positive nodes, 26.5% were pre-menopausal; 80% received adjuvant hormonal treatment. Mutation frequency in P and R samples is presented in the Table. PIK3CA mutations were identified in 44% (58/132) P samples. HRAS, AKT1 and PIK3CA mutations were mutually exclusive. 62.5% (5/8) of KRAS-mutated, 75% (6/8) of MET-mutated, 100% (2/2) of BRAF-mutated and 33.3% (1/3) of FGFR3-mutated P had coexistent PIK3CA mutations. For the 49 evaluated pairs, high concordance for the mutations status was found between P and R. Conclusions: KRAS, BRAF, MET and FGFR3 mutations, found at relatively high frequency in this population of relapsed ER+/HER2- BCs, could represent clinically relevant targets and contribute to mechanisms of recurrence, particularly in PIK3CA-mutated BCs. Mutation profiling of additional paired samples is ongoing and clinical outcome data will be presented.
Concurrent driver mutations in non-small cell lung cancer (NSCLC) patients (p) on targeted therapy uncovered by comprehensive molecular profiling.

Collin M. Blakely, Luping Lin, Saurabh Asthana, Nikoletta Sidiropoulos, Tyrrell Nelson, Boris C. Bastian, Matthew A. Gubens, Barry S. Taylor, Trever Grant Bivona; University of California, San Francisco, San Francisco, CA

**Background:** NSCLC p with EGFR mutations respond initially to EGFR tyrosine kinase inhibitors (TKIs) but invariably develop acquired EGFR TKI resistance. Prior studies identified the EGFR T790M mutation and activation of MET, PI3K, AXL, HER2 and the MAPK pathway as drivers of acquired EGFR TKI resistance. To date, comprehensive molecular profiling to identify actionable modifiers of EGFR TKI response has not been conducted in NSCLC p on therapy. **Methods:** We performed next generation sequencing (NGS) using a 263-gene Nimblegen custom cancer panel on DNA isolated from primary patient lung adenocarcinoma FFPE specimens prior to initiating standard erlotinib treatment and upon the development of acquired erlotinib resistance after only 3 months of therapy. **Results:** In the pretreatment sample, we confirmed the presence of the \textit{EGFR}L858R mutation in 95% of the sequencing reads and discovered a concurrent BRAF V600E mutation with a frequency of ~ 6%. NGS performed on the acquired erlotinib resistance sample revealed acquisition of the EGFR T790M mutation with a frequency of ~ 14%. Notably, the frequency of the BRAF V600E mutation increased 10-fold upon acquired erlotinib resistance from ~ 6% in the pretreatment tumor to ~ 60% in the recurrent tumor. We found that overexpression of BRAF V600E in H3255 human NSCLC, which harbor EGFR L858R (but not BRAF V600E) and are erlotinib sensitive, caused resistance to erlotinib treatment (10-fold increase in erlotinib IC50). BRAF V600E-mediated erlotinib resistance was reversed by treatment with the BRAF inhibitor vemurafenib. Additional functional studies are ongoing and the complete dataset will be presented. **Conclusions:** These results indicate that EGFR-mutant NSCLC can harbor additional oncogenic driver mutations in BRAF at low frequencies prior to therapy. EGFR TKI treatment can lead to expansion of BRAF V600E expressing tumor cells, resulting in acquired EGFR TKI resistance that can be reversed by BRAF inhibitor treatment. The data demonstrate the utility of routine molecular profiling of NSCLC p on targeted therapy and offer unprecedented insight into the genetic basis of therapeutic resistance.
Amplification of the MET receptor to drive resistance to anti-EGFR therapies in colorectal cancer.

Alberto Bardelli, Simona Corso, Andrea Bertotti, Sebastijan Hobor, Giulia Siravegna, Andrea Sartore-Bianchi, Giorgia Migliardi, Francesco Galimi, Calogero Lauricella, Carlo Zanon, Alessio Amatu, Marcello Gambacorta, Luis A. Diaz, Victor E. Velculescu, Mark Sausen, Paolo M. Comoglio, Livio Trusolino, Federica Di Nicolantonio, Silvia Giordano, Salvatore Siena; Laboratory of Molecular Genetics - IRCC Institute for Cancer Research and Treatment at Candiolo, Candiolo, Italy; Institute for Cancer Research and Treatment at Candiolo, Candiolo (TO), Italy; Laboratory of Molecular Pharmacology - IRCC Institute for Cancer Research and Treatment at Candiolo, Candiolo, Italy; Ospedale Niguarda Ca’ Granda, Milan, Italy; Stuttura Complessa di Anatomia Patologica, Azienda Ospedaliera Niguarda-Cà Granda, Milan, Italy; Dipartimento Oncologico, Ospedale Niguarda Ca’ Granda, Milano, Italy; S.C. Anatomia Istologia Patologica e Citogenetica - Ospedale Niguarda Ca’ Granda, Milan, Italy; Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, MD; Ludwig Center for Cancer Genetics and Therapeutics, Johns Hopkins Kimmel Cancer Center, Baltimore, MD; Scientific Direction - IRCC Institute for Cancer Research and Treatment at Candiolo, Candiolo, Italy; Falck Division of Medical Oncology, Ospedale Niguarda Ca’ Granda, Milano, Italy

Background: The anti EGFR monoclonal antibodies cetuximab and panitumumab are used to treat metastatic colorectal cancer patients but their clinical efficacy is limited by the development of acquired resistance. We recently reported that secondary KRAS mutations are responsible for acquired resistance in approximately 50% of the patients who initially respond to cetuximab or panitumumab (Misale et al., Nature 2012; Diaz et al., Nature 2012). Here we studied the molecular bases of relapse in CRC patients who do not develop KRAS mutations during the course of anti-EGFR therapy. Methods: Next generation sequencing was applied to tumor biopsies to identify genetic alterations associated with relapse to cetuximab and panitumumab in mCRC patients. Detection and quantitation of genetic alterations in circulating tumor DNA was used to monitor the occurrence of KRAS mutations and MET amplification in blood samples. Results: Molecular analyses of tumor biopsies from patients who did not develop KRAS mutations during anti-EGFR therapy revealed high level of amplification of the MET proto-oncogene in 3/5 cases. Quantitative PCR, FISH and IHC analysis confirmed high level of MET amplification in the post-therapy samples but not in the matched pre-treatment tissues. We developed a PCR based assay to detect the presence of the MET amplicon in circulating, cell-free, DNA. We found that MET amplification could be detected in the blood as early as 3 months after initiation of anti EGFR therapy. To functionally evaluate the role of MET amplification on resistance to anti EGFR antibody therapies we exploited patient-derived CRC xenografts (‘xenopatients). We found that (2/2) xenopatients established from MET amplified tumors were completely refractory to cetuximab but showed sensitivity to the Met inhibitor crizotinib. Conclusions: Amplification of the MET proto-oncogene is responsible for acquired acquired resistance to anti-EGFR antibody therapy in a subset of CRCs. The emergence of MET amplification in circulating, cell-free, DNA may be used to select patients most likely to benefit from anti MET therapies.
Development and validation of an immuno-PET tracer for patient stratification and therapy monitoring of antibody-drug conjugate therapy.

Ohad Ilovich, Arutselvan Natarajan, Ataya Sathirachinda, Richard Kimura, Ananth Srinivasan, Mathias Gebauer, Jochen Krüp, Chantal Carrez, Ingrid Sassoon, Veronique Blanc, Susanta K. Sarkar, Sanjiv Sam Gambhir; Stanford University, Palo Alto, CA; Sanofi-Aventis, Frankfurt, Germany; Sanofi R&D Vitry Research Center, Vitry-sur-Seine, France; Sanofi Oncology, Vitry-sur-Seine, France; Sanofi-Aventis, Cambridge, MA; Stanford University, Stanford, CA

Background: SAR566658 is an antibody-drug immunoconjugate consisting of a humanized monoclonal antibody (huDS6) against the tumor-associated MUC1-sialoglycope, CA6, conjugated to a cytotoxic maytansinoid (DM4). SAR566658 is currently undergoing phase I clinical trials in patients with CA6-positive solid tumors. A companion diagnostic based on huDS6 may facilitate patient stratification and early evaluation of therapeutic efficacy. The present study describes the development and preclinical evaluation of three novel Copper-64 (t1/2 = 12.7h) labeled antibody fragments (two Fabs and a diabody) derived from the huDS6 antibody. One fragment was chosen based on imaging figures of merit for further specificity evaluations. Methods: The affinity of all fragments and their DOTA conjugates to CA6 was validated using flow cytometry. The DOTA conjugates were labeled with Copper-64 and evaluated in human serum stability studies, in vivo small animal PET imaging and 24-hour biodistribution studies in nude mice bearing either CA6 positive (WISH) or CA6 negative (A2780) subcutaneous tumors. The specificity of the lead tracer was evaluated in vivo via blocking studies and isotype controls. Results: All fragments and their DOTA conjugates had high affinity (Kd = 4-20 nM) for WISH cells. 64Cu-DOTA-B-Fab gave superior results in radio-synthesis (RCY - 60%, SA - 55 GBq/µmole, >99% purity), serum stability (94±5%, n=3) and biodistribution profile. Its two isotype controls gave statistically significant (P<0.05) uptake values in WISH but not A2780 tumors (see table). Blocking with B-Fab or huDS6 prior to tracer administration afforded a 23% (p<0.05, n=8) and 26% (p<0.05, n=7) decrease in WISH tumor uptake, respectively. Conclusions: These preclinical studies suggest that 64Cu-DOTA-B-Fab may be a suitable companion diagnostic for SAR566658 in cancer patients and requires further investigation.

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<th>WISH tumor (%ID/g)</th>
<th>A2780 tumor (%ID/g)</th>
<th>WISH/A2780 ratio</th>
<th>Liver (%ID/g)</th>
<th>Kidneys (%ID/g)</th>
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<td>64Cu-DOTA-B-Fab</td>
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<td>64Cu-DOTA-anti-DM4-B-Fab</td>
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<td>3.03±0.24</td>
<td>1.33</td>
<td>10.4±1.6</td>
<td>52±8.3</td>
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Prognostic relevance at 5 years of the early monitoring of neoadjuvant chemotherapy using FDG PET in luminal HER2-negative breast cancer.

Olivier Humbert, Alina Berriolo-Riedinger, Alexandre Cochet, Mélanie Gauthier, Celine Charon-Barra, Isabelle Desmoulins, Séverine Guiu, Michel Toubeau, Inna Dygai-Cochet, Charles Coutant, Pierre Fumoleau, Francois Brunotte; Centre Georges François Leclerc, Dijon, France; Department of Nuclear Medicine, Centre Georges-Francois Leclerc, Dijon, France; Biostatistics and Epidemiology Unit, Centre Georges François Leclerc, Dijon, France and EA4184, College of Medicine, Dijon, France; Georges-François Leclerc Cancer Center, Dijon, France; Department of Surgery/Georges François Leclerc Comprehensive Cancer Care Centre, Dijon, France; Department of Nuclear Medicine, Centre Georges-François Leclerc, Dijon, France

Background: To evaluate, in the luminal breast cancer subtype, the prognostic value of tumor glucose metabolism at baseline and of its changes after one cycle of neoadjuvant chemotherapy (NAC). Methods: This prospective study included 61 women with immunophenotypically defined luminal HER2-negative breast cancer treated with NAC. 18F-FDG PET was performed at baseline. Hepatic activity was used as a reference to distinguish between low-metabolic and hypermetabolic tumors. In hypermetabolic tumors, a PET exam was repeated after the first course of NAC. The relative change in the maximal Standardized Uptake Value of the tumor (ΔSUV), corresponding to the metabolic response, was calculated. Results: Forty-two women had hypermetabolic tumors at baseline, corresponding to more proliferative breast cancers with higher Ki-67 expression (p=0.017) and higher grade (p=0.04). Nineteen women had low-metabolic tumors with lower proliferation indexes. Worse overall survival was associated with larger tumor size (>5cm, HR=6.52, P=0.009) and with hypermetabolic tumors achieving a low metabolic response after one cycle of NAC (ΔSUV<16%, HR=10.63, P=0.004). Five-year overall survival in these poor-response patients was 49.22% (95% CI=[14.76%-76.90%]). In contrast, overall survival in women with low-metabolic tumors or hypermetabolic/good-response tumors (ΔSUV≥16%) was good, 100% and 96.15%, respectively (95% CI=[75.69%-99.45%]). Conclusions: In luminal HER2-negative breast tumors, tumor metabolism at baseline and changes after the first course of NAC are surrogate markers of patients’ survival. A subgroup of women with hypermetabolic/bad-responding tumors correlated with poor prognosis can be identified. These results may create the ability to tailor the NAC regimen to the metabolic response at an early stage.
Early PET/CT scan compared with RECIST to predict long-term outcome of patients with liver metastases from colorectal cancer treated with preoperative chemotherapy plus bevacizumab.

Maria Carmela Piccirillo, Secondo Lastoria, Guglielmo Nasti, Corradina Caraco, Luigi Aloj, Cecilia Arrichiello, Alessandro Ottiano, Francesco Izzo, Elisabetta De Lutio, Vittorio Albino, Carmen Romano, Raffaele Palaia, Gennaro Daniele, Massimo Di Maio, Pasqualina Giordano, Simona Signoriello, Paolo Delrio, Rosario Vincenz Iaffaioli, Giovanni Romano, Francesco Perrone; Clinical Trials Unit, National Cancer Institute, Napoli, Italy; Nuclear Medicine Unit, National Cancer Institute, Napoli, Italy; Abdominal Oncology Department, National Cancer Institute, Napoli, Italy; Radiology 2 Unit, National Cancer Institute, Napoli, Italy; Medical Statistics, Second University, Napoli, Italy

Background: Early changes in tumor metabolism measured with positron-emission-tomography/computed tomography (PET/CT) could predict the long-term efficacy of treatment better than dimensional RECIST response. Methods: We performed PET/CT before and after 1 cycle of treatment in patients with resectable liver metastases from colorectal cancer, within a phase II trial of preoperative FOLFIRI plus bevacizumab. For each lesion, the maximum SUV (SUVmax) and the total lesion glycolisis (TLG) were determined. For both, based on previous studies, a ≤-50% change from baseline was used as threshold for significant response. Metabolic response was categorized no/yes by using three different methods that enter into the calculation (i) the largest observed value (highest SUVmax/TLG), or (ii) the sum of all the observed values (Total SUVmax/TLG), or (iii) each observed values (SUVmax/TLG-by-lesion). Standard RECIST response was assessed after 3 months of treatment. The association between metabolic and RECIST response was tested with the Mc Nemar’s test and their agreement was expressed as Kappa statistics; the ability to predict progression-free (PFS) and overall (OS) survival was tested with Log-rank test and a multivariable Cox model. Results: 33 patients were analyzed. After treatment, there was a notable decrease of all PET/CT parameters, with a median change of -33.9% for the highest SUVmax, -61.5% for the highest TLG, -34.9% for the total SUVmax, and -65.5% for the total TLG. The association of SUV-based metabolic response (but not the TLG-based) with RECIST was statistically significant. However, the agreement between RECIST and PET/CT responses was consistently small. PFS and OS were significantly longer among PET/CT responding patients, whichever the measure used. On the contrary, no significant outcome difference was evident according to RECIST response. Conclusions: Early PET/CT response was significantly predictive of long-term outcomes during preoperative treatment of patients with liver metastases from colorectal cancer and its predictive ability was higher than that of RECIST response. Clinical trial information: 2006-006572-38.
The genetic landscape of clinical resistance to RAF inhibition in melanoma.

Eliezer Mendel Van Allen, Nikhil Wagle, Scott L. Carter, Antje Sucker, Deborah Norman Farlow, Eran Hodis, Amaro Taylor-Weiner, Carola Berking, Friederike Egberts, Jessica Cecile Hassel, Helen Gogas, Ralf Gutzmer, Simone M. Goldinger, Carmen Loquat, Selma Ugurel, Lisa Zimmer, Stacey B. Gabriel, Gad Getz, Levi A. Garraway, Dirk Schadendorf, DeCOG; Dana-Farber Cancer Institute, Boston, MA; Broad Institute, Cambridge, MA; University Hospital Essen, Essen, Germany; Department of Dermatology, Ludwig Maximilian University, Munich, Germany; University of Kiel, Kiel, Germany; University Hospital Heidelberg, Universitaets-Hautklinik, Hauttumorzentrum, Heidelberg, Germany; Hellenic Cooperative Oncology Group (HeCOG), Athens, Greece; Medizinische Hochschule, Hannover, Germany; University Hospital Zurich, Dermatology, Zurich, Switzerland; Universitätsmedizin der Johannes Gutenberg-Universität Mainz, Mainz, Germany; University of Würzburg, Würzburg, Germany; Department of Dermatology, University Hospital, Essen, Germany; Universitätsklinikum Essen, Essen, Germany

Background: Although single-agent RAF inhibition has proved effective in metastatic BRAFV600-mutant melanoma, most patients relapse and some are intrinsically resistant. While several genetic resistance effectors have been identified, a comprehensive assessment of the genetic resistance spectrum in a large patient cohort may further inform resistance patterns and treatment strategies. Methods: Pre-treatment and post-relapse biopsies were obtained from BRAFV600 melanoma patients treated with vemurafenib or dabrafenib. Whole exome sequencing of tumor and normal samples was performed to identify exome-wide mutations, insertion/deletions, and chromosomal copy number alterations. Since somatic allelic fraction comparisons across treatment time points must account for variable stromal admixture between biopsies, novel algorithms were applied to impute purity and tumor cell ploidy, thus determining the “cancer cell fraction” (CCF) of each alteration. Those with enriched CCF in post-relapse samples were nominated as potential resistance effectors. Results: 30 patients (towards a goal of 77) were sequenced to a mean depth of 145-fold coverage. In acquired resistance patients (on drug ≥ 12 weeks, n=18), NRAS Q61R (28%) and BRAF amplifications (6%) were observed. The remaining patients had multiple clonally enriched alterations in the post-relapse tumors that may drive resistance, including MEK1 mutations at residues previously noted in an in vitro mutagenesis screen for vemurafenib resistance. In the intrinsic resistance cohort (on drug < 12 weeks, n=12), multiple putative resistance mutations not previously observed in the setting of acquired resistance were identified. Conclusions: In addition to reported genetic resistance mechanisms, we identified new MEK1 mutations in resistant samples. For the majority of patients (67%) without events in these genes, computational algorithms identified new candidate drivers of RAF inhibitor resistance. Alterations that predict intrinsic or acquired resistance may also inform new approaches to melanoma therapy. Future studies incorporating longitudinal biopsies and genetic profiling will underpin a robust assessment of clinical resistance in melanoma and other cancers.

Background: NSCLC p with EGFR mutations initially respond to EGFR tyrosine kinase inhibitors (TKIs) but ultimately relapse. Sub-genomic molecular studies indicate that the EGFR T790M mutation and the activation of MET, PI3K, AXL, HER2 and MAPK can lead to acquired resistance to EGFR TKIs. To date, no integrated comprehensive genomic investigation of EGFR TKI resistance has been performed. Methods: FFPE biopsies of erlotinib-sensitive and erlotinib-resistant tumors were obtained from 11 EGFR mutant NSCLC p. DNA was extracted from all tumor and corresponding normal tissue samples and underwent whole exome sequencing using the Illumina HiSeq2500. RNA was extracted from all tumor samples and analyzed by whole transcriptome sequencing, also using the Illumina HiSeq2500. Results: Erlotinib resistant NSCLC specimens harbored upregulation of known resistance drivers including MET and AXL and novel alterations including upregulation of genes that are: 1) recurrently mutated in NSCLC, including ALK, STK11; 2) components of established embryonic stem cell signatures, including targets of Nanog, Oct4, Sox2, c-Myc; 3) neuronal lineage specific regulators, including NTRK3, NRCAM, ALK, LRP4. The analysis also revealed downregulation of several genes that are: 1) components of innate and acquired immunity, including HLA-A, -B, DQ, CD40; 2) phosphatases regulating survival signaling pathways, including PTEN, PTPRD; 3) proapoptotic components, including BNIP3L, IKIP. Conclusions: This study demonstrated the feasibility and utility of comprehensive genomic analysis in the clinical management of NSCLC p receiving targeted therapy. We identified known and novel molecular biomarkers of erlotinib acquired resistance in NSCLC p, and uncovered a previously unappreciated role for genetic events governing stem cell and neuronal phenotypes as well as immune evasion in erlotinib acquired resistance in NSCLC p. Together, our data provide unprecedented insight into the molecular pathogenesis of escape from EGFR oncogene inhibition in NSCLC. We are now conducting a prospective observational study in additional NSCLC p.
Tumor markers of efficacy and resistance to cetuximab (C) treatment in metastatic colorectal cancer (mCRC): Results from CALGB 80203 (Alliance).

Herbert Hurwitz, Stephanie Cushman, Chen Jiang, Ivo Shterev, Michelle R. Mahoney, Donna Niedzwiecki, Robert J. Mayer, Alan Paul Venook, Kouros Owzar, Andrew B. Nixon, Alliance for Clinical Trials in Oncology; Duke University Medical Center, Durham, NC; Duke University, Durham, NC; Mayo Clinic, Rochester, MN; Dana-Farber Cancer Institute, Boston, MA; Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA

**Background:** Beyond KRAS status, there are no validated markers for anti-EGFR therapy in mCRC. While expression of genes within the EGF signaling axis has been reported to correlate with benefit, most reports used data from non-randomized trials which cannot distinguish between general prognostic markers and markers that predict for benefit from C. CALGB 80203 was a 238-patient (pt), 4-arm, randomized phase II study of FOLFOX or FOLFIRI +/- C in mCRC pts. Formalin-fixed, paraffin-embedded (FFPE) tumor samples from CALGB 80203 were analyzed for gene expression of HER family ligands, receptors and regulators. **Methods:** FFPE tumor samples from consenting pts were analyzed for AREG, BTC, CD73, DUSP4, EGF, EGFR, EPGN, EREG, HB-EGF, HER2, HER3, HER4, PHLDA1 and TGFa by RT-qPCR. Interaction between baseline gene expression levels and treatment (C) with respect to progression-free survival (PFS) and overall survival (OS) was modeled using a multiplicative Cox proportional hazards model. **Results:** Baseline tumor tissue was available from 103 pts; 55% of tumors were KRAS wild type (WT). Two prognostic markers were identified; higher tumor mRNA levels of HER2 (HR=0.64, p=0.002) and EREG (HR=0.89, p=0.016) were associated with longer PFS across all pts. Potential predictive markers of benefit for C were identified. In KRAS WT tumors, lower HER3 expression was associated with longer OS from C treatment while higher HER3 expression was associated with shorter OS from C treatment (chemo + C: HR=1.15; chemo only: HR=0.48, interaction p=0.029). No association was observed for HER3 and outcome in KRAS mutant (MT) tumors. Interestingly, higher CD73 expression was associated with longer PFS in C-treated pts in both KRASWT (chemo + C: HR=0.91; chemo only: HR=1.57, interaction p=0.026) and MT tumors (chemo + C: HR=0.80; chemo only: HR=1.29, p=0.025). **Conclusions:** In one of the first reports using data from a randomized study, gene expression of HER3 and CD73 were identified as potential predictive markers for C. These data implicate not only HER axis signaling but also immune modulation as potential mechanisms of C action and sensitivity, and warrant confirmation in other large randomized trials.
Prognostic impact of changes in circulating tumor cells (CTC) in metastatic breast cancer (MBC).

Markus Wallwiener, Andreas D. Hartkopf, Sabine Riethdorf, Martin Sprick, Christoph Wolfram Domschke, Sarah Schott, Irene Baccelli, Caroline Modugno, Birgitt Schoenfisch, Barbara Burwinkel, Frederik Marin, Joerg Heil, Christof Sohn, Klaus Pantel, Andreas Trumpp, Andreas Schneeweiss; Department of Obstetrics and Gynaecology, University of Heidelberg, Heidelberg, Germany; University of Tuebingen, Department of Gynecology and Obstetrics, Tuebingen, Germany; Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; DKFZ German Cancer Research Centre, Heidelberg Institute for Stem Cell Technology and Experimental Medicine, Heidelberg, Germany; University Hospital of Heidelberg, Heidelberg, Germany; Department of Gynecology and Obestics within the National Centre of Tumor Disease, University of Heidelberg, Heidelberg, Germany; HI-STEM, German Cancer Research Center (DKFZ), Heidelberg, Germany; NTC National Centre for Tumour Diseases, Heidelberg, Germany; Department of Obstetrics and Gynaecology, Tuebingen, Germany; Department of Molecular Epidemiology, German Cancer Research Center, and Department of Obstetrics and Gynecology, University Hospital of Heidelberg, Heidelberg, Germany; Department of Obstetrics and Gynecology, University Hospital Heidelberg, Heidelberg, Germany; Department of Obstetrics and Gynaecology, University of Heidelberg, Heidelberg, Germany; Institute of Tumor Biology, Campus Forschung, University Hospital Hamburg-Eppendorf, Hamburg, Germany; National Center for Tumor Diseases, University of Heidelberg, Heidelberg, Germany

Background: To prospectively assess the prognostic value of CTC counts at baseline and after one cycle of therapy and their kinetics for response, progression-free (PFS), and overall survival (OS) in MBC.

Methods: MBC patients underwent CTC enumeration (CellSearch, Veridex) at baseline (CTC\textsubscript{BL}), after one cycle of a new line of therapy (CTC\textsubscript{1C}), and at progression (CTC\textsubscript{PD}). CTC status was classified as negative (neg \((-\)) or positive (pos \((+)\)) for \(<5 \text{ and } \geq 5 \text{ CTC/7.5 ml peripheral blood, respectively.} \) CTC kinetics (CTC\textsubscript{KIN}) from CTC\textsubscript{BL} to CTC\textsubscript{1C} were classified as favorable if CTCs remained neg (neg\(\rightarrow\)neg) or became neg (pos\(\rightarrow\)neg), or as unfavorable (neg\(\rightarrow\)pos or pos\(\rightarrow\)pos). Tumor response was assessed every 2–3 months using RECIST criteria. CTC status and kinetics were associated with outcome using log-rank and Fisher’s exact tests.

Results: Of 326 patients enrolled (median age (range) at first diagnosis: 50 (23–81) years), 115/326 (35%) were CTC\textsubscript{BL}, 51/162 (31%) were CTC\textsubscript{1C}, and 39/108 (36%) were CTC\textsubscript{PD}. Median follow-up was 15.9 months (mos). Median PFS and OS were significantly reduced in CTC\textsubscript{BL} compared to CTC\textsubscript{1C} patients (PFS, 4.3 vs. 7.1 mos, \(p = .019\); OS, 15.0 mos vs. not reached (nr), \(p < .001\)) and for CTC\textsubscript{1C} compared to CTC\textsubscript{IC} patients (\(p < .001\) for PFS and OS). CTC\textsubscript{KIN} were predictive of progressive disease as best response (neg\(\rightarrow\)neg, 33%; pos\(\rightarrow\)neg, 38%; pos\(\rightarrow\)pos, 60%; neg\(\rightarrow\)pos, 75%; \(p = .040\)). PFS and OS for patients with unfavorable CTC\textsubscript{KIN} were significantly shorter than for favorable CTC\textsubscript{KIN} (PFS, 3.7 vs. 6.8 mos, \(p < .001\); OS, 7.8 mos vs. nr, \(p < .001\)).

Conclusions: CTC at baseline, CTC after one cycle and CTC kinetics are highly predictive of outcome in MBC. Serial CTC enumeration could serve as a useful adjunct to standard diagnostic tests in tailoring therapy. Further details of CTC\textsubscript{PD} will be presented at the meeting. Clinical trial information: S-295/2009.
Cell-free DNA copy number variations as a marker for breast cancer in a large study cohort.

Julia Beck, Ekkehard Schütz, Howard B. Urovnitz, Adel Tabchy, William M. Mitchell, Gordon B. Mills, Funda Meric-Bernstam; Chronix Biomedical, Göttingen, Germany; The University of Texas MD Anderson Cancer Center, Houston, TX; Department of Pathology, Vanderbilt University, Nashville, TN

**Background:** Massive parallel sequencing provides high numbers of cell-free nucleic acid serum DNA sequences (cfDNA) that can detect trace amounts of tumor derived chromosomal imbalances and copy number variations (CNVs) in patients with cancer. The aim of this study was to determine if there is a difference between the cfDNA CNVs from patients with breast cancer (BrCa) compared to healthy controls.

**Methods:** DNA extracted from serum samples of 225 BrCa (Stage 1 to 4) and 205 gender and age-matched healthy controls (HC) was amplified using random primers, tagged with a unique molecular identifier per sample, sequenced on an Illumina HiSeq system and aligned to the human genome (Build 37). Hits were counted in sliding 1Mbp interval regions and normalized. Using a Random-Resampling procedure, a model was established to distinguish BrCa from HC using the copy number variations (CNV) and cross validated.

**Results:** From 1,100 rounds of random resampling (50/50), a set of 31 regions was selected, based on the frequency of occurrence in the models. Using 20 random sets of a 10-fold cross validation, the selected regions were found to be highly significant discriminators between BrCa and HC ($p<10^{-5}$). When using a final linear model with 16 regions the AUC of a diagnostic ROC curve was found to be 0.895 for all samples, for Stage I and II the AUC was 0.86 compared to 0.93 for the higher stages. The final model included three regions from chromosome 8 and 1 and two regions from chromosome 15, the remaining regions were found as one per chromosome.

**Conclusions:** Using comparative massive parallel sequencing of cfDNA from cancer patients vs. controls, we were able to show that a 16-region model based on CNV, is useful to distinguish patients with breast cancer from matched controls. Genomic instabilities that are shed into the circulation from breast cancer may play a role in screening, monitoring or as companion diagnostic tests in breast cancer.
Massively parallel sequencing (MPS) of circulating DNA in patients with metastatic colorectal cancer (mCRC): Prognostic significance and early changes during chemotherapy (CT).

Jeanne Tie, Isaac Kinde, Hui-Li Wong, Joseph James McKendrick, Peter Gibbs, Madhu Sudan Singh, Christos Stelios Karapetis, Jayesh Desai, Ben Tran, Justin Kelvin Roebert, Kenneth W. Kinzler, Bert Vogelstein; The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia; Ludwig Center for Cancer Genetics and Therapeutics, Johns Hopkins University School of Medicine, Baltimore, MD; Royal Melbourne Hospital, Melbourne, Australia; Box Hill Hospital, Melbourne, Australia; Andrew Love Cancer Centre, Geelong, Australia; Flinders Medical Centre and Flinders Centre for Innovation in Cancer, Flinders University, Adelaide, Australia; The Royal Melbourne Hospital, Melbourne, Australia; Western Hospital, Melbourne, Australia; Medical Imaging Australia, Melbourne, Australia

Background: Prognostic and predictive biomarkers in mCRC are urgently needed. Circulating tumor cells are a promising blood biomarker, but are detectable in only a minority of pts. Recently, analysis of circulating tumor DNA (ctDNA) has shown promise as a liquid biopsy, reflecting the evolving mutational status of the tumor. Here we explored baseline ctDNA as a prognostic marker, and early changes in ctDNA as a marker of CT response. Methods: Serial plasma samples and CEA were collected at baseline (D1), day 3 (D3) and cycle 2 day 1 (C2D1) from 40 mCRC pts receiving standard combination CT. Restaging scans performed at 8 weeks were centrally assessed using RECIST criteria. Samples were analyzed at Johns Hopkins Kimmel Cancer Center. Initially tumor tissue was analyzed for hotspot mutations in TP53, APC, KRAS, BRAF, PIK3CA and FBXW7. The same mutation was queried and quantified in plasma using a MPS platform (Safe-SeqS). Log-rank test was used to compare survival curves and Wilcoxon matched pairs test was used to compare paired plasma samples. Results: Preliminary data is available on 19 pts in this ongoing study. Using our initial panel at least 1 mutation was found in 16 of 19 (84.2%) tumors (7 KRAS, 3 TP53, 3 BRAF, 2 APC and 1 PIK3CA), with matching ctDNA found for each pt. For the remaining 3 cases a further panel of mutations is being analyzed. Median D1 cell free DNA (cfDNA) and ctDNA levels were 1.98 ng/ul (0.15 – 57.18) and 523 mutant fragments (frag)/ml (0.4 – 109,876), respectively. Pts with D1 cfDNA of ≥ 2.5 compared with < 2.5 had shorter median overall survival (OS; 6.9 v 12.2 months, p = 0.0086), with a trend for shorter progression-free survival (PFS; 3.6 v 8.2 months, p = 0.3477). A surge of ctDNA level from D1 to D3 was typically observed (median increase: 91.2 frag/ml, p = 0.0313), followed by a drop (median decrease from D1 to C2D1: 208.8 frag/ml, p = 0.0098). All pts with a decrease in ctDNA at C2D1 had a reduction in tumor size at 8 weeks. Conclusions: In all cases of mCRC where tumor mutation was identified, matching ctDNA was detected in plasma. Circulating DNA is a promising marker of prognosis. Early changes in DNA levels may be a useful marker of tumor response.
Real-time clinical application of next-generation sequencing (NGS): Results from a multicenter program.

Aaron Richard Hansen, Andrew M. K. Brown, Philippe L. Bedard, Sebastien J. Hotte, Eric Winquist, Glenwood D. Goss, Dimitrios Vergidis, Hal W. Hirte, Stephen Welch, Tong Zhang, Lincoln D. Stein, Vincent Ferretti, Stuart Watt, Wei Jiao, Karen Ng, Teresa Petrocelli, Lillian L. Siu, John D. McPherson, Suzanne Kamel-Reid, Janet Dancey; Princess Margaret Cancer Center, University Health Network, Division of Medical Oncology & Hematology, Department of Medicine, University of Toronto, Toronto, ON, Canada; Ontario Institute for Cancer Research, Toronto, ON, Canada; Juravinski Cancer Centre, Hamilton, ON, Canada; London Health Sciences Centre, London, ON, Canada; The Ottawa Hospital Cancer Center, Ottawa, ON, Canada; Thunder Bay Regional Health Sciences Center, Thunder Bay, ON, Canada; London Regional Cancer Program, London, ON, Canada; University Health Network, Department of Pathology and Laboratory Medicine, Toronto, ON, Canada; Princess Margaret Cancer Center, Toronto, ON, Canada

Background: NGS techniques enable the identification of actionable mutations in clinical tumor samples. The objective of this study is to assess feasibility and explore the impact of real-time targeted NGS on therapeutic decision-making. Methods: Patients (pts) with advanced solid tumors underwent a biopsy of a metastatic lesion. The first phase was performed with Sequenom MassARRAY somatic genotyping and Pacific Biosciences RS-targeted NGS. The second phase broadened genomic coverage in both Sequenom and Illumina MiSeq. All pts had a molecular profiling (mp) report issued after identified actionable mutations were verified by Sanger sequencing in a CLIA-lab and reviewed by an expert panel. “Actionability” was defined as having prognostic, predictive or diagnostic implications on patient management. Details of clinical outcomes and subsequent matched therapy, if applicable, were captured. Referring physicians were surveyed on the impact of mutation results on their treatment recommendations. Results: These are summarized in the Table. Conclusions: Broader mp platforms resulted in more identified actionable mutations which required a longer time for verification prior to reporting, but may yield a greater impact on clinical decision-making. However, the matching of pts to drugs based on their molecular profiles depends highly on drug access. For mp to be clinically relevant, it must be coupled with access to approved drugs or to investigational agents on clinical trials. Clinical trial information: NCT01345513.

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<thead>
<tr>
<th>Elements</th>
<th>Phase I</th>
<th>Phase II</th>
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<tr>
<td>Platforms</td>
<td>Sequenom 19 genes, 238 mutations</td>
<td>Sequenom 23 genes, 280 mutations</td>
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<tr>
<td></td>
<td>Pacific 19 genes, 63 amplicons</td>
<td>MiSeq 54 genes, 660 amplicons</td>
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<tr>
<td>No. of pts</td>
<td>89 enrolled, all profiled</td>
<td>56 enrolled, 49 profiled</td>
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<tr>
<td>Age range (median)</td>
<td>25 to 80 yrs (54 yrs)</td>
<td>28 to 85 yrs (58 yrs)</td>
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<tr>
<td>% with actionable mutations</td>
<td>37%</td>
<td>55%</td>
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<tr>
<td>Time from consent to report (median)</td>
<td>20 days</td>
<td>29 days</td>
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<tr>
<td>Verified mutations</td>
<td>41 in 33 patients: 1 AKT, 1 BRAF, 1 CDK4, 5 EGFR, 3 KIT, 16 Kras, 11 PIK3CA, 1 NRAS, 1 HRAS, 1 PDGFR</td>
<td>35 in 27 patients: 1 APC, 2 BRAF, 1 CDH1, 2 EGFR, 1 ERBB2, 13 Kras, 2 PIK3CA, 1 PTEN, 12 TP53</td>
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<tr>
<td>Fresh and archival mutation concordance</td>
<td>46/51 pairs (90%)</td>
<td>11/17 pairs (65%)</td>
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<tr>
<td>Matched treatment</td>
<td>10/33 (30%)</td>
<td>3/27 (11%)</td>
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<tr>
<td>Treatment impacted</td>
<td>19/89 pts (21%)</td>
<td>12/49 pts (24%)</td>
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A survey of thousands of tumor exomes and transcriptomes to expand clinical opportunities for crizotinib.

Emma Bowden, Sean Eddy, Paul Williams, Nikolay Khazanov, Armand Bankhead, Dinesh Cyanam, Peter Wyngaard, Dan Rhodes; Compendia Bioscience, Ann Arbor, MI

**Background:** Cancer driver events occur as a result of chromosomal rearrangements. There are several examples where targeted inhibition of the resulting fusion produces dramatic clinical response. For example, the EML4-ALK fusion in non-small cell lung cancer (NSCLC). Other ALK fusions have been described in NSCLC and other diseases including the NPM-ALK fusion in anaplastic large cell lymphoma (ALCL). The efficacy of crizotinib and other ALK inhibitors are being investigated in these diseases. ALK is also subject to activation via mutation and sensitivity to crizotinib is reported in ALK mutation positive neuroblastoma. Finally, crizotinib has activity not only against ALK, but also against ROS1, MST1R and MET. ROS1 fusions have been found in NSCLC and glioblastoma, and MET amplification events in gastric adenocarcinoma identify additional settings that may benefit from crizotinib treatment. **Methods:** To further understand the full therapeutic potential of Crizotinib, we undertook a genomic survey of ALK, ROS1, MET and MST1R across 1,000’s of patients from the The Cancer Genome Atlas (TCGA) and Oncomine. **Results:** We confirmed the presence of EML4-ALK fusions in both lung and colorectal cancer (CRC), and also identified a novel ALK fusion in CRC. ALK hotspot mutations and focal amplifications were confined to neuroblastoma, as previously described. Our survey of ROS1 identified rare novel fusions in NSCLC and glioblastoma, and high-level amplifications in liposarcoma (2%) and rarely in breast cancer (0.2%). No fusions were identified for MET, however high-level amplifications were observed in 1-5% of papillary renal cell carcinoma, the intestinal subtype of gastric adenocarcinoma, oligodendroglioma, glioblastoma and lung adenocarcinoma. Hotspot mutations were frequently observed in squamous head and neck (11%), and more rarely in hepatocellular carcinoma, small cell lung and ovarian cancers. **Conclusions:** These results leverage all available genomic profiling data to provide a broadened scope of therapeutic opportunity for inhibitors like crizotinib. With the growing availability of next-generation sequencing, such surveys can support hypothesis-driven development of targeted therapies.
Fusion transcript discovery in formalin-fixed paraffin-embedded human breast cancer tissues and its relation to tumor progression.

Yan Ma, Ranjana Ambannavar, James Stephans, Jennie Jeong, Andrew Dei Rossi, John Morlan, Mei-Lan Liu, Samuel Levy, Joffre Baker, Dominick Sinicropi, Kunbin Qu; Genomic Health, Inc., Redwood City, CA

Background: While several recently discovered gene fusions already play an important role in personalized cancer treatment, many cancer gene fusions remain to be discovered. Next generation sequencing has enabled identification of many rare gene fusion events in fresh or frozen solid tumors. There is a need to detect gene fusions in transcriptomes of formalin-fixed paraffin-embedded (FFPE) tumor tissue, for which there is long-term clinical outcome data. We therefore sought to develop bioinformatics methods to detect fusion transcripts in FFPE tissue and to characterize their association with clinical outcomes.

Methods: RNA sequencing libraries were created and sequenced from tumor biopsy tissues (Plos One 2012 7(7): e40092) of two ER+/H11001 breast cancer cohorts consisting of 136 and 77 patients, for which clinical outcomes were available. The fusion junctions were nominated by the RNA-seq aligner GSNAP and further filtered to consider discontinuous expression patterns at exon/intron levels.

Results: A total of 108 candidate fusion transcripts were detected and RT-PCR assays confirmed 89% of the top ranking fusion transcript candidates. The majority (82%) of identified fusion gene partners are listed in the COSMIC database of known cancer sequence variations. Of note, several patients expressed multiple fusion transcripts that are significantly associated with tumor progression (P<0.001), including genes associated with cell proliferation and cellular metabolism. Furthermore, these patients also harbored inter-chromosomal gene fusions. It is noteworthy that several gene fusions were present in multiple patients. In one of these recurrent fusions the estrogen receptor gene acts as the fusion pair donor.

Conclusions: Novel bioinformatics approaches developed here demonstrate the ability to detect fusion transcripts as biomarkers from archival FFPE tissues that associate with breast cancer progression. Some gene fusions were common in multiple patients and deserved further study.
Sensitivity for detecting PIK3CA mutations in early-stage breast cancer with droplet digital PCR.

Julia A. Beaver, Sasidharan Balukrishna, Danijela Jelovac, Michaela Jane Higgins, Stacie Jeter, Vered Stearns, Antonio C. Wolff, Jill Kessler, Dustin VanDenBerg, Patricia Valda Toro, Pedram Argani, Ben Ho Park; The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, MD; Christian Medical College, Tamil Nadu, India; Massachusetts General Hospital Cancer Center, Boston, MA; Johns Hopkins School of Medicine, Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD

Background: PIK3CA is mutated in up to 30% of breast cancers. Classically somatic mutations are identified by Sanger sequencing of the primary tumor specimen. However, third generation droplet digital PCR technologies offer a novel platform for quantitative mutation detection with improved sensitivity.

Methods: Thirty stage I-III breast cancer patients were consented on an IRB-approved prospective repository study at Johns Hopkins for collection of their primary breast tumor specimen. Formalin-fixed paraffin embedded (FFPE) samples were analyzed by standard sequencing for three PIK3CA hotspot mutations. The DNA from these samples was then analyzed using the RainDrop digital PCR platform with TaqMan probes in a triplex format to simultaneously detect and quantitate hotspot mutations and genome equivalents. Results are expressed as a percentage of mutant to wild-type PIK3CA molecules for each sample.

Results: Standard sequencing of all tumors (n=30) identified seven PIK3CA Exon 20 mutations (H1047R) and three Exon 9 mutations (E545K). Samples were scored as PIK3CA mutation positive by digital PCR if the tumor DNA contained at least 5% mutant molecules. All ten mutations identified by sequencing were verified by digital PCR with quantities of mutant molecules ranging from 20.3-55.6% in a given sample. Digital PCR identified additional PIK3CA mutations that were wild type by standard sequencing including three mutant Exon 20 samples, two mutant Exon 9 samples and one sample with an Exon 20 and Exon 9 mutation. Quantities of mutant molecules in these additional samples ranged from 5-28.9%.

Conclusions: RainDrop digital PCR offers improved sensitivity and quantification for detecting PIK3CA mutations in FFPE samples using nanograms of DNA. Additional mutations identified by digital PCR may reflect genetic heterogeneity or possibly tissue contamination. The clinical utility of identifying a small proportion of mutations is unknown but may impact eligibility for targeted therapies and clinical trials. Ongoing studies will also address whether the identification of solid tumor mutations in circulating cell-free plasma DNA by digital PCR can improve diagnostics and aid in therapeutic decisions.
Use of next-generation sequencing (NGS) to identify actionable genomic alterations (GA) in diverse solid tumor types: The Foundation Medicine (FMI) experience with 2,200+ clinical samples.

Vincent A. Miller, Jeffrey S. Ross, Kai Wang, Siraj M. Ali, Geoff Otto, John Curran, Norma Alonzo Palma, Roman Yelensky, Sean Downing, Phil Stephens, Doron Lipson, Gary A. Palmer; Foundation Medicine, Inc., Cambridge, MA; Foundation Medicine, Cambridge, MA

Background: ST oncology has been transformed by the linkage of GA with targeted therapeutics. Unfortunately, most STs still have no target detected by clinically available assays. More comprehensive testing platforms are needed to determine GA in ST and thus broaden treatment options. We developed a ST NGS diagnostic assay, optimized for routine clinical FFPE specimens including core and fine needle biopsies and malignant effusions, and analyzed > 2,200 patients’ tumors in a CLIA-certified lab (Foundation Medicine).

Methods: Hybridization capture of 3,320 exons from 182 cancer-related genes and 37 introns of 14 genes commonly rearranged in cancer was applied to 50ng of DNA extracted from 2,200+ consecutive FFPE tumor specimens and sequenced to high unique coverage. GA (base substitutions, small indels, rearrangements, copy number alterations) were categorized as “actionable” if directly linked to a clinically available targeted treatment option or a mechanism-driven clinical trial. Results: 2,112/2,221 (95%) of specimens (most common 1° sites: lung 18%, breast 14%, colon 7%, other 34%) were successfully profiled (mean coverage 1134X). Alterations were reported in 155/182 (85%) of genes. Seventy-six percent of cases harbored ≥1 actionable GA, mean 1.6 (range 0-16); sixty-two percent harbored at least one actionable GA not assayed by available tumor-type specific tests or hotspot panels. This approach has led to novel insights into advanced cancer including: 13 novel, potentially druggable kinase gene fusions; alterations in known drug targets (e.g. ALK, EGFR, ERRB2, KIT, MET, PDGFR α and β, RAF1 and RET) in novel tumor types and new mechanisms of resistance to approved targeted therapies. Several patients demonstrated dramatic responses to treatment with targeted therapies directed against these alterations.

Conclusions: Comprehensive NGS genomic profiling was successful in profiling >2,200 unselected clinical cases, identified actionable alterations in 76% of cases and provided additional treatment options for 62% of patients targeting alterations in genes not assayed by available hotspot panels.
Protein tyrosine kinase 6 (PTK6, BRK) amplification in HER2+ breast cancer as a mechanism of HER2 resistance.

Tatyana A. Grushko, Maria J. Gomez-Vega, Aleix Prat, Jeffrey Mueller, Mariann Coyle, Charles M. Perou, Rodrigo Santa Cruz Guindalini, Elias Obeid, Hanna Irie, Olufunmilayo I. Olopade; The University of Chicago Medical Center, Chicago, IL; Translational Genomics Group at Vall d’Hebron University Hospital, Barcelona, Spain; The University of North Carolina at Chapel Hill, Chapel Hill, NC; Mount Sinai School of Medicine, New York, NY; The University of Chicago, Chicago, IL

Background: PTK6 gene on chromosome 20q13 encodes the intracellular non-receptor tyrosine kinase. Studies in vivo and in vitro revealed a role for PTK6 in cell proliferation and survival, particularly in HER2+ breast cancer cells suggesting that PTK6 may associate with the HER2 pathway and confer resistance to HER2-targeted therapy. PTK6 protein is frequently overexpressed in breast cancer, however, the mechanism(s) underlying PTK6 overexpression and its role in cancer remains unclear. To address this problem, we analyzed the frequency of PTK6 gene copy number variation (CNV) and expression in association with breast cancer subtypes.

Methods: Retrospective paraffin samples of invasive tumor and normal epithelium, and matching DCIS and metastases were mounted on TMA. PTK6 CNV was determined using PTK6:CEP20 FISH assay. Tumor subtypes were defined using the five-marker IHC classifier. The correlation between PTK6 CNV and mRNA expression and association of both with the intrinsic PAM50 tumor subtype were studied using TCGA database (547 cases) and publicly available seven breast cancer data sets (1005 cases). Data were normalized, gene median centered and standardized for the purpose of the study.

Results: By FISH, 20% of 41 invasive tumors carried PTK6 CNV: amplification (10%) and gene polysomy (10%). The proportion of PTK6 amplified cases differed by subtype, with the largest proportion in HER2-enriched (17%) and LumB (14%). Strikingly, amplified invasive cases also showed amplification in matching DCIS and metastases. Analysis of the public datasets confirmed the frequent PTK6 amplification in breast cancer. Both low and high levels of amplification were detected with the largest proportion in HER2+ tumors (HER2-enriched and LumB; p=2.05e-26). None of the basal-like tumors showed high levels of PTK6 amplification. A high correlation between PTK6 gene copies and mRNA expression was observed (p=1.13e-08). Conclusions: PTK6 gene is amplified early in breast cancer progression, particularly in HER2+ tumors. Further studies on PTK6 biology may help clinicians to understand its potential role in HER2 resistance. Supported by BREAST CANCER SPORE, NCI K12CA139160 and CTSA-ITM CS UL1 RR024999.
Trefoil factor 1 as a predictive factor of bone metastases in breast cancer.

Background: Patients with breast cancer frequently develop bone metastases, which are responsible for high morbidity and reduced quality of life. The early identification of patients with a high probability of relapsing in this site could be used to select candidates for tailored therapy with bone-specific drugs such as bisphosphonates or RANK-L inhibitors. We aimed to identify a pattern of tissue markers in primary breast cancer that could predict bone metastatization. Methods: Expression of different markers was retrospectively analyzed in frozen breast cancer tissue samples from 90 patients comprising 30 cases with no evidence of disease (NEDP), 30 with bone metastases (BMP) and 30 with visceral metastases (VMP). Eight transcripts were analyzed by Quantitative Real time PCR: trefoil factor 1 (TFF1), bone sialoprotein (IBSP), heparanase (HPSE), secreted protein acidic and rich in cysteine (SPARC), connective tissue growth factor (CTGF), B2 microglobulin (B2M) and receptor activator of Nf-κB (RANK). Immunohistochemistry of TFF1 was performed on a part of the case series. Results: Marker expression analysis in the 3 different subgroups showed at least twofold higher median values of all markers in NEDP and VMP subgroups than in BMP. In particular, TFF1, B2M and CXCR4 levels showed statistically significant values. Median TFF1 value in BMP patients was 430.64 compared to 115.83 and 32.79 in VMP and NEDP, respectively (p<0.004). Considering markers as dichotomous variables, TFF1 expression in BMP reached 59% compared to 21% and 23% in NEDP and VMP, respectively (p=0.002). Univariate analysis confirmed that TFF1 predicted the relapse and also the site of relapse. Immunohistochemistry data on TFF1 revealed that this protein was expressed only by cancer cells. Furthermore, the accuracy of the marker did not change at RNA or protein level, thus excluding a post transcriptional control of the RNA. Conclusions: In this preliminary study we identified a gene expression pattern in primary breast cancer that can identify patients destined to relapse to the bone. In particular, TFF1 would seem to be a suitable marker for bone metastatization and a possible target for the development of new drugs.
BRCA1 like copy number profiles to predict benefit of intensified alkylating chemotherapy in breast cancer.

Philip C. Schouten, Sabine C. Linn, Sebastian Aulmann, Hans-Peter Sinn, Andreas Schneeweiss, Frederik Marme; Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam, Netherlands; University Department of Pathology, Heidelberg, Germany; University Hospital Heidelberg, Heidelberg, Germany; Department of Obstetrics and Gynecology, University Hospital Heidelberg, Heidelberg, Germany

Background: DNA copy number profiles can identify patients with a defect in BRCA1. We previously showed that patients with a BRCA1-like profile benefit from intensified alkylating chemotherapy (IA, 4 cycles 5-fluorouracil, epirubicin, cyclophosphamide + 1 cycle carboplatin, thiotepa and cyclophosphamide with autologous stem cell transplantation (ASCT)). Presumably, this is because of the defect in error free homologous recombination DNA repair. Here we present an independent study of BRCA1-like profiles to predict benefit of IA chemotherapy (2 cycles induction chemotherapy consisting of 2500 mg/m2 ifosfamide and 40 mg/m2 epirubicin, followed by 12 g/m2 ifosfamide, 900 mg/m2 carboplatin, 180 mg/m2 epirubicin with ASCT) versus adriamycin-cyclophosphamide or cyclophosphamide-methotrexate-5-fluorouracil regimens in high risk breast cancer.

Methods: We isolated tumor DNA from 117 patients of a case-control study of high risk breast cancer patients to apply a marker by treatment interaction study design to assess overall survival. We generated copy number profiles and classified them to be BRCA1-like or non-BRCA1 like. We used Fisher Exact tests to calculate correlations and performed Kaplan-Meier and Cox regression analyses to investigate whether patients with a BRCA1-like profile benefit from IA chemotherapy compared to an AC/CMF regimen. Results: 16 of 117 (14%) patients had a BRCA1-like profile. BRCA1-like status was associated with high tumor grade (p=0.03), triple negative status (p=0.0005) and high N stage (p=0.03). When corrected for hormone receptor and HER2 status, size, positive lymph nodes, and grade we found that BRCA1 like patients had a 6-fold decreased chance of death (HR: 0.15, 95% confidence interval: 0.03-0.80, p: 0.03) compared to non-BRCA1 like patients (HR: 0.94, 95% confidence interval: 0.53-1.67, p: 0.84). The interaction test between IA chemotherapy and marker status was significant (p: 0.04).

Conclusions: We provide independent validation of the previous finding that breast cancer patients with a BRCA1 like copy number profile benefit highly from intensified alkylating chemotherapy.
Olaparib monotherapy in patients with advanced cancer and a germ-line BRCA1/2 mutation: An open-label phase II study.

Bella Kaufman, Ronnie Shapiro-Frommer, Rita K. Schmutzler, M. William Audeh, Michael Friedlander, Judith Balmaña, Gillian Mitchell, Georgeta Fried, Karin Bowen, Anitra Fielding, Susan M. Domchek; Chaim Sheba Medical Center, Tel Hashomer, Israel; Ella Institute for Research and Treatment of Melanoma, Sheba Medical Center, Affiliated to Sackler Faculty of Medicine Tel Aviv University, Tel Hashomer, Israel; Center for Familial Breast and Ovarian Cancer and Center of Integrated Oncology, Cologne, Germany; Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA; Prince of Wales Hospital, Sydney, Australia; Vall d’Hebron University Hospital, Barcelona, Spain; Peter MacCallum Cancer Center, Melbourne, Australia; Institute of Oncology, Haifa, Israel; AstraZeneca, Macclesfield, United Kingdom; Abramson Cancer Center of the University of Pennsylvania, Philadelphia, PA

Background: The oral PARP inhibitor olaparib has shown antitumor activity as monotherapy in patients (pts) with breast and ovarian cancer with gBRCA1/2 mutations. This multicenter non-comparative study evaluated whether tumors in gBRCA1/2 mutation carriers are responsive to olaparib regardless of tumor type (NCT01078662). Methods: Heavily pretreated pts with advanced cancer refractory to standard therapy (98% of breast cancer pts had ≥3 lines of prior chemotherapy for metastatic disease) and with a gBRCA1/2 mutation, received olaparib 400 mg bid (capsule) until disease progression. Primary objective: tumor response by RECIST 1.1. Secondary objectives: PFS, OS and safety. Results: 298 pts received treatment and were evaluable. Enrollment is complete, 33 pts remain on study. Median duration of treatment in this heavily pretreated population was 5.5 months (range 1–28.5 months). Most common AEs (generally grade 1/2) were fatigue (59%), nausea (59%) and vomiting (37%). Grade 3 AEs were reported for 162 pts (54%); most common was anemia (17%). 11 pts (4%) had AEs that led to treatment discontinuation. Conclusions: The observed tumor response rates indicate antitumor activity of olaparib monotherapy in gBRCA mutated pts with advanced cancer refractory to standard therapy. A clinical benefit was seen in prostate and pancreatic cancer and activity in ovarian and breast cancer was confirmed. Prolonged responses to olaparib across all tumor types support the hypothesis that therapy directed against a genetically-defined target has activity regardless of anatomic organ of origin. Olaparib was generally well tolerated with toxicities consistent with prior studies. Clinical trial information: NCT01078662.

Fibroblast growth factor receptor 1 (FGFR1) and SRY-related HMG-box (SOX2) amplification in squamous cell carcinoma (SCC) of the lung.

Amaya Gasco, Pedro Mendez, Jose Luis Ramirez, Santiago Viteri Ramirez, Teresa Moran, Enric Carcereny Costa, Carlota Costa, Ana Gimenez Capitan, Irene Sansano, Maria Perez, Montserrat Tierno, Monica Botia, Miquel Taron, Harry J.M. Groen, Rafael Rosell, Spanish Lung Cancer Group; Pangaea Biotech, Clinical Unit, Barcelona, Spain; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Institut Catala d’Oncologia, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Pangaea Biotech, Laboratory of Translational Oncology, Barcelona, Spain; University Medical Center Groningen, Groningen, Netherlands; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Pangaea Biotech, Cancer Therapeutics Innovation Group, USP Institut Universitari Dexeus, Barcelona, Spain

Background: SOX2 is a key transcription factor that is amplified in lung SCC. FGFR1 is a receptor tyrosine kinase that promotes cell proliferation, survival and apoptotic resistance through the PLCγ/PKC, RAS/MAPK and PI3K-AKT pathways, respectively. FGFR1 is amplified in 15-25% of lung SCC. Pre-clinical studies of targeted inhibitors showed a growth dependency on FGFR1 amplification both in vitro and in vivo. A European, multicenter clinical trial of second-line treatment with BIBF1120, an FGFR1 inhibitor, will be performed in patients with lung SCC with FGFR1 amplification.

Methods: We have examined FGFR1 and SOX2 gene copy number (GCN) in 76 lung SCC patients by multiplex ligation-dependent probe amplification (MLPA). Genomic DNA (gDNA) was isolated from enriched tumor cells by laser capture microdissection from formalin-fixed paraffin embedded (FFPE) tumor tissue. 50-100 ng of gDNA was analyzed in each of three independent replicates per tumor sample. Two independent probe sets were used for each gene analyzed. For inter-patient GCN comparisons, the results from each patient were normalized against the GCN values derived from FFPE peripheral blood leukocytes. In order to study intra-tumor heterogeneity (TH), we examined FGFR1 and SOX2 GCN in different areas of 4 tumors. In 2 patients, TH was examined in serial tumor biopsies and/or resections obtained at different points of disease progression.

Results: High FGFR1 amplification was detected in 13/76 (17.10%) patients. High SOX2 amplification was observed in 38/63 (60.32%) patients. 46.15% of the FGFR1-amplified tumors were also co-amplified for SOX2. Intra-TH was observed in 24 tumors. Survival according to FGFR1 and SOX2 GCN will be presented. In addition, GCN changes in FGFR1, SOX2, PIK3CA, PDGFRA, KDR, EGFR and MET over 10 years of follow-up will be presented for one surgically resected SCC lung patient. Conclusions: FGFR1 and SOX2 co-amplification could represent a novel therapeutic target and warrants further research.
Genetic and molecular biomarker characterization of KRAS mutant non-small cell lung carcinoma (NSCLC) tumors.

Li Liu, Yuan Liu, Ademi Santiago-Walker, Hong Shi, Vivian Zhang, Ashley Hughes, Carmen P. Arenas-Elliott, Lori Roadcap, Anne-Marie Martin, Tona M. Gilmer; GlaxoSmithKline, Oncology, Collegeville, PA; GlaxoSmithKline, Collegeville, PA; GlaxoSmithKline, Research Triangle Park, NC

Background: Preclinical studies demonstrated Brahma related gene 1 (BRG1) mutations or loss of expression, and mutations of LKB1 may be associated with lack of sensitivity for MEK inhibitor trametinib in a subset of KRAS mutant NSCLC lines. This study aimed to evaluate the frequency of KRAS, LKB1 and BRG1 mutations in NSCLC tumors; and determine whether KRAS mutations in corresponding plasma samples could be detected by evaluating circulating cell-free DNA (cfDNA). Methods: Human NSCLC FFPE tumor tissue and matched plasma samples were procured from Indivumed GmbH. KRAS mutation status of 101 NSCLC tumors and matched plasma were determined by direct sequencing of genomic DNA (gDNA) from tissue and/or BEAMing on tissue gDNA or plasma cfDNA. Genetic mutations of LKB1 and BRG1 were determined by direct sequencing. Additional mutations were determined using the Ion Torrent AmpliSeq Cancer Panel. BRG1 protein expression was evaluated by IHC. Results: By direct sequencing and BEAMing we found 27/101 (28.4%) NSCLC tissue and/or plasma samples harbored KRAS mutations: G12V (37.0%), G12C (29.6%), G12D (18.5%), G12S, G13C, G13D and Q61H (3.7% each). The KRAS mutation status concordance (mutant or wild-type) between tumor gDNA and plasma cfDNA was 79-81%. Among the KRAS mutant tumors, LKB1 and BRG1 mutations were detected in 10/26 (38%) and 1/26 (3.8%) tumors respectively by direct sequencing. By IHC, loss of BRG1 expression was detected in 1/21 KRAS mutant tumors. The mutation frequency and variants for KRAS and LKB1 in patient samples were comparable with KRAS mutant NSCLC cell lines and COSMIC database. However the frequency of BRG1 mutation and protein loss were much lower in patient tumors. In a subset of 15 KRAS mutant tumors, Ion Torrent confirmed KRAS and LKB1 mutations and provided additional mutations found in TP53, FGFR2, FGFR3, GNAS, KDR, KIT and MET. Conclusions: This study demonstrates that KRAS mutant NSCLC tissues have high frequency of LKB1 mutations along with other mutations. It also supports the feasibility of detection of KRAS mutations in cfDNA from blood of NSCLC patients using BEAMing technology, providing an alternative to invasive biopsy.
ROR1 mRNA expression in EGFR-mutant non-small-cell lung cancer (NSCLC) patients (p) with the T790M mutation: A potential therapeutic target.

Niki Karachaliou, Ana Drozdowskyj, Carlota Costa, Miguel Angel Molina-Vila, Ana Gimenez Capitan, Alain Vergnenegre, Bartomeu Massuti, Teresa Moran, Margarita Majem, Enriqueta Felip, Enric Carcereny Costa, M. Rosario Garcia-Campelo, Santiago Viteri Ramirez, Cordula Nicole Heidecke, Roger Estrada-Tejedor, Jordi Teixido, Trever Grant Bivona, Petros Giannikopoulos, Mayumi Ono, Rafael Rosell, Spanish Lung Cancer Group; Pangaea Biotech, Laboratory of Translational Oncology, Barcelona, Spain; Pivotal, Madrid, Spain; Cluzeau Hospital, Limoges, France; Alcante University Hospital, Alicante, Spain; Institut Catala d’Oncologia, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Hospital de Sant Pau, Oncology Service, Barcelona, Spain; Vall d’Hebron University Hospital, Barcelona, Spain; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Complexo Hospitalario A Coruña, A Coruña, Spain; Pangaea Biotech, Clinical Unit, Barcelona, Spain; Institut Quimic Sarria, Molecular Engineering Group, Barcelona, Spain; University of California, San Francisco, San Francisco, CA; Cancer Therapeutics Innovation Group, New York, NY; Kyushu University, Department of Medical Biochemistry, Fukuoka, Japan; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Pangaea Biotech, Cancer Therapeutics Innovation Group, USP Institut Universitari Dexeus, Barcelona, Spain

Background: Progression-free survival (PFS) is short in NSCLC driven by EGFR mutations treated with erlotinib alone, due to crosstalk with other signaling pathways that can cause secondary dependency. ROR1 knockdown inhibited the growth of NCI-H1975 cells (with EGFR L858R and T790M mutations). A pro-survival function for ROR1/MEK/ERK signaling has been demonstrated, with cooperation with AKT. In a subset of 95 p in the EURTAC trial (clinicaltrials.gov NCT00446225), 65% had pre-treatment T790M mutations. We have assessed ROR1 expression in 45 of these 95 p.

Methods: The T790M mutation was determined by Taqman with a PNA to inhibit amplification of the wild-type (wt) allele. Tumor samples were run in octuplicates; this method can detect 1 mutated allele among 10,000 wt alleles. ROR1 mRNA expression was examined by quantitative RT-PCR and categorized by terciles. p were classified as having low/intermediate or high ROR1 expression. The impact of ROR1 expression on outcome was examined in all 45 p and in a subset of 15 p with concomitant T790M mutations. Results: Median age 65; 68.9% female; 57.8% never-smokers; 95.6% ECOG PS <2; 91.1% adenocarcinoma; 68.9% exon 19 deletion. No differences in baseline characteristics were observed according to ROR1 expression levels. 24 p (53.3%) were treated with erlotinib and 21 p (46.7%) with chemotherapy. 10 (41.7%) erlotinib-treated p and 6 (28.6%) chemotherapy-treated p had ROR1 mRNA levels in the top tercile. Among erlotinib-treated p, response rate was 40% for p with high ROR1 levels vs 71.4% for p with low/intermediate levels (P=0.0918). Among chemotherapy-treated p, only p with low ROR1 levels responded (6.7%). PFS was 11.8 months (m) for erlotinib-treated p with low/intermediate ROR1 levels vs 5.8 m for p with high levels. PFS for chemotherapy-treated p was 5.6 and 9 m, respectively (P=0.033). Among 15 erlotinib-treated p with concomitant T790M mutations, PFS was 10.8 m for p with low/intermediate ROR1 levels vs 2.7 for p with high levels (P=0.0174). Conclusions: HighROR1 expression significantly limits PFS in p with T790M mutations. ROR1-directed therapies can enhance the efficacy of erlotinib in EGFR-mutant NSCLC p overexpressing ROR1. Clinical trial information: NCT00446225.
Components of homologous recombination and translesion synthesis (TLS) in pemetrexed/cisplatin-treated non-small-cell lung cancer (NSCLC) patients (p).

Guillermo Lopez-Vivanco, Thomas Marti, Ilya N. Kotov, Imane Chaib, Santiago Ponce-Aix, Rosario García Campelo, Jose Miguel Sanchez, Angel Artal, Isabel Bover, Miquel Taron, Maria Sanchez-Ronco, Christian Diego Rolfo, Bartomeu Massuti, Rolf A. Stahel, Rafael Rosell, Spanish Lung Cancer Group; Department of Medical Oncology, Hospital de Cruces, Barakaldo, Spain; University Hospital Zurich, Zurich, Switzerland; Catalan Institute of Oncology, Laboratory of Molecular Biology, Badalona, Barcelona, Spain; Hospital 12 de Octubre, Madrid, Spain; Oncology Service, Complejo Hospitalario Universitario A Coruña, A Coruña, Spain; Hospital La Princesa, Madrid, Spain; Hospital Universitario Miguel Servet, Zaragoza, Spain; Son Llatzer University Hospital, Mallorca, Spain; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Alcala de Henares University, Madrid, Spain; Universitair Ziekenhuis Antwer, Senior Staff Member Oncology, Antwerp, Belgium; Hospital General de Alicante, Alicante, Spain; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Pangaea Biotech, Cancer Therapeutics Innovation Group, USP Institut Universitari Dexeus, Barcelona, Spain

Background: REV3, the catalytic subunit of the TLS polymerase, can continue replication past DNA adducts. Depletion of REV3 sensitizes A549 lung cancer cells to cisplatin. REV3 expression is part of a gene signature that predicted pemetrexed sensitivity in 17 NSCLC cell lines. Homologous recombination and TLS pathways have non-redundant functions in response to cisplatin. We hypothesized that low REV3 mRNA expression – alone or in combination with low expression levels of genes involved in homologous recombination – could correlate with better outcome to cisplatin/pemetrexed in NSCLC. Methods: REV3, BRCA1, RAP80, TS and AEG1 mRNA was examined by quantitative RT-PCR and categorized by terciles. Expression of each gene was correlated with outcome in 47 cisplatin/pemetrexed-treated NSCLC p. Results: 63.8% male; 47% smokers; 80.9% ECOG PS 1; 80.8% adenocarcinoma. Overall response rate was 51%, with no differences according to expression levels of any of the genes. Progression-free survival (PFS) for p with low, intermediate and high BRCA1 levels was 13.4, 5.5 and 3.9 months (m), respectively (P=0.005). Similar differences in PFS were observed according to TS (P=0.003) and AEG1 (P<0.001) expression. Hazard ratio (HR) for PFS for p with high BRCA1 levels was 4 (P=0.002). Overall survival (OS) for p with low, intermediate and high BRCA1 levels was 29.7, 7.4 and 6.3 m, respectively (P=0.05). Similar differences in OS were observed according to TS (P=0.005) and AEG1 (P=0.001) expression. HR for OS for p with high BRCA1 levels was 3.6 (P=0.004). There were no differences in PFS or OS according to REV3 or RAP80 levels. However, the joint effect of BRCA1 and REV3 was significant for predictive modeling. PFS for p with low, intermediate and high levels of both genes was 14.9, 7.2 and 2.8 m, respectively (P=0.001). OS for p with low, intermediate and high levels of both genes was 29.7, 7.8 and 6.3 m, respectively (P=0.04). Conclusions: Low BRCA1 expression predicts longer PFS and OS in pemetrexed/cisplatin-treated NSCLC p. Low TS and AEG1 levels have similar predictive value. Analysis of these genes could be useful for customizing pemetrexed/platinum chemotherapy.
Nondisruptive mutations of TP53 and overall survival (OS) in advanced non-small-cell lung cancer (NSCLC) patients (p).

Enric Carcereny Costa, Jordi Bertran-Alamillo, Miguel Angel Molina-Vila, Radj Gervais, Bartomeu Massuti, Laura Bonanno, Teresa Moran, Margarita Majem, Enriqueta Felip, M. Rosario Garcia-Campelo, Alain Vergnenegre, Santiago Viteri Ramirez, Amaya Gasco, Luciano Wannesson, Clara Mayo-de las Casas, Laia Pujantell-Pastor, Adolfo G. Favaretto, Niki Karachaliou, Rafael Rosell, Spanish Lung Cancer Group; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Pangaea Biotech, Laboratory of Translational Oncology, Barcelona, Spain; Centre François Baclesse, Caen, France; Hospital General de Alicante, Alicante, Spain; Istituto Oncologico Veneto, Medical Oncology, Padova, Italy; Institut Catala d’Oncologia, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Hospital de Sant Pau, Oncology Service, Barcelona, Spain; Thoracic Tumors Group, Vall d’Hebron Institute of Oncology, Barcelona, Spain; Complejo Hospitalario A Coruña, A Coruña, Spain; Cluzeau Hospital, Limoges, France; Pangaea Biotech, Clinical Unit, Barcelona, Spain; IOSI (Oncology Institute of Southern Switzerland), Bellinzona, Switzerland; Istituto Oncologico Veneto, Padua, Italy; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Pangaea Biotech, Cancer Therapeutics Innovation Group, USP Institut Universitari Dexeus, Barcelona, Spain

**Background:** The tumor suppressor TP53 is the most commonly mutated gene in lung cancer. Early-stage NSCLC p with TP53 mutations may have worse prognosis and be more radio/chemoresistant. However, few studies have addressed the issue of TP53 mutations in advanced NSCLC. We have retrospectively examined the influence of TP53 mutations on OS in 241 advanced NSCLC p. **Methods:** 99 EGFR-wild-type (wt), chemotherapy-treated p from 5 European hospitals and 142 EGFR-mutated (mut), erlotinib-treated p from two clinical studies were included. Exons 5, 6, 7 and 8 of TP53 were analyzed by high resolution melting (HRM). All mutated samples were reconfirmed by sequencing. Mutations were classified as disruptive or non-disruptive based on their predicted effects on the function of the p53 protein. **Results:** TP53 mutations were detected in 31% of EGFR-wt and 24% of EGFR-mut p. OS results are available for 57 EGFR-wt p and 90 EGFR-mut p. Among EGFR-wt p, OS was 22 months (m) for p without TP53 mutations, 20m for p with disruptive TP53 mutations, and 9 m for p with non-disruptive TP53 mutations (P=0.09). Among EGFR-mut p, OS was 31 m for p without TP53 mutations, not reached for p with disruptive TP53 mutations, and 15 m for p with non-disruptive TP53 mutations (P=0.05). **Conclusions:** Non-disruptive mutations in the TP53 gene are associated with shorter OS in advanced NSCLC p, both in EGFR-wt p treated with chemotherapy and in EGFR-mut p treated with erlotinib. These p could benefit from treatment to reactivate mutant p53.
Angiogenic marker associated with resistance to neoadjuvant chemoradiotherapy in rectal cancer.

Hanjo Kim, Min Young Lee, Tae Sung Ahn, Jina Yun, Kyoungha Kim, Se Hyung Kim, Hyun Jung Kim, Sang Cheol Lee, Sang-Byung Bae, Chan Kyu Kim, Namsu Lee, Seong Kyu PARK, Kyu Taek Lee, Jong-Ho Won, Dae Sik Hong, Hee Sook Park, Suk-Young Park, Moon Soo Lee, Moo Jun Baek; Soonchunhyang University Hospital Cheonan, Cheonan, South Korea; Soonchunhyang University College of Medicine, Seoul Hospital, Seoul, South Korea; Soonchunhyang University College of Medicine, Cheonan Hospital, Cheonan, South Korea; Soonchunhyang University Bucheon Hospital, Bucheon, South Korea; Soonchunhyang University Hospital Seoul, Seoul, South Korea; Soonchunhyang University Bucheon Hospital, Gyeonggi, South Korea; Soonchunhyang University College of Medicine, Bucheon Hospital, Bucheon, South Korea; The Catholic University of Korea, Daejeon St. Mary’s Hospital, Daejeon, South Korea; Department of Surgery, Soonchunhyang University, Cheonan, South Korea

Background: The ability to achieve pathologic down staging after neoadjuvant chemoradiotherapy (CRT) is correlated with improved survival. However, there is no effective method of predicting which patients will respond to neoadjuvant CRT. Neoadjuvant CRT can change the expression of angiogenic factors. However, little is known about its possible changes in response to preoperative CRT. We examined the expression of angiogenic factors in rectal cancer tissues before preoperative CRT and after surgery. Methods: Fifty five patients with locally advanced rectal cancer were studied. All patients were given preoperative CRT of 5040 cGy for 5-6 weeks with concurrent administration of 5-fluorouracil and leucovorin. Surgical resection was performed 6–8 weeks later in all patients. Immunohistochemical staining for angiogenic markers (vascular endothelial growth factor [VEGF], placenta growth factor [PLGF], hypoxia inducible factor 1α [HIF 1α], stromal cell derived factor [SDF 1α]) were performed on specimens obtained before preoperative CRT and after surgery. A semiquantitative-immunohistochemical score established from the extension and intensity of the angiogenic factors was used for analysis. Results: The positive expression rate of VEGF, PLGF, SDF 1α, and HIF 1α was 56.4% (31/55), 65.5% (36/55), 70.9% (39/55), and 47.3% (26/55), respectively. The expression rate of VEGF, PLGF, SDF 1α, and HIF 1α was increased by 3.6% (2/55), 7.3% (4/55), 30.9% (17/55), and 1.8% (1/55) after neoadjuvant CRT, respectively. Expression of VEGF, PLGF, and HIF 1α protein was downregulated after neoadjuvant CRT in the rectal cancer tissues (P < 0.001, P = 0.001, P = 0.044, respectively). However, SDF 1α was upregulated after neoadjuvant CRT (P < 0.001). And also, upregulated expression of SDF 1α after neoadjuvant CRT was significantly associated with resistance to CRT (P = 0.035). However, SDF 1α showed no correlation with other clinical factors (age, sex, clinical stage). Conclusions: Expression of SDF-1α was increased in the rectal cancer tissue after neoadjuvant CRT, as well as has been associated with CRT resistance. Our data suggests that SDF 1α should be evaluated as new target for antiangiogenic therapy.
Phase I study (A8471004) in Asian patients of PF-03446962, a fully human mab against ALK-1 receptor involved in tumor angiogenesis: Safety, pharmacokinetics (PK), and pharmacodynamics (PD).

Kyung-Hun Lee, Toshihiko Doi, Tae Min Kim, Atsushi Ohtsu, Tae Yong Kim, Masafumi Ikeda, Kiyotaka Yoh, Corrado Gallo-Stampino, Tomoko Hirohashi, Akiyuki Suzuki, Fujii Yosuke, Yung-Jue Bang; Department of Internal Medicine, Seoul National University College of Medicine, Seoul, South Korea; National Cancer Center Hospital East, Kashiwa, Japan; Department of Internal Medicine, Seoul National University Hospital, Seoul, South Korea; Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Japan; Pfizer Oncology, Milan, Italy; Pfizer, Tokyo, Japan; Pfizer Japan Inc, Tokyo, Japan; Seoul National University Hospital, Seoul, South Korea

Background: Activin receptor like kinase 1 (ALK-1) is a member of the TGF-βRI family selectively expressed in proliferating endothelial cells, and plays an important role in regulating tumor initiation and metastasis. PF-03446962 is a fully human IgG2 mAb anti ALK-1 evaluated within two phase 1 studies in Western and Asian pts. Herein we report the preliminary safety, PK and PD data of the Phase I study.

Methods: Primary objective is to identify the maximum tolerated dose (MTD) in Asian cancer pts; secondary objectives include the safety profile, PK, antitumor activity, and potential PD markers in blood and tumor samples. PF-03446962 is administered IV on Day 1, 29 and then q 2 weeks. Results: Study A8471004 consists of two parts: a 3+3 dose escalation (Part 1) and a dose expansion (Part 2) at 2 dose levels. In Part 1, 16 pts have been enrolled at 3 dose levels (4 pts at 4.5 mg/kg, 3 pts at 7.0 mg/kg, and 9 pts at 10 mg/kg). No DLTs occurred in Part 1 and 10 mg/kg was confirmed as MTD in the Asian population. The observed AUC$_{0-28day}$ for the 4.5, 7 and 10 mg/kg doses, were 12960, 22190 and 28030 µg·h/mL and Cmax were 97.1, 131.5 and 179.8 µg/mL, respectively. Drug exposure (mean Cmax and AUC) increased in a nearly dose proportional manner in Asians. In Part 2, expansion cohorts at doses of 7.0 mg/kg (10pts) and 10.0 mg/kg (8pts) of pts previously treated with VEGF inhibitors (VEGFi) have been enrolled, and the most common drug related adverse events observed (>10%) being thrombocytopenia, pyrexia, epistaxis, and telangiectasia (an anti-ALK-1 mediated toxicity) similarly in the 2 dose levels. Telangiectasia was observed in 1 CRC and 3 HCC patients. 4 patients who progressed after VEGFi treatment (RCC, sarcoma, 2 HCC patients) presented a SD lasting for 290, 248, 247 and 208 days, respectively, suggesting the ALK-1 could serve as mechanism of escape for VEGF. Conclusions: PF-03446962 is a first in class mAb targeting ALK-1. Treatment with PF-03446962 is well tolerated in the Asian pts and preliminary observation of clinical activity supports ALK-1 as a viable target. Update of study results and potential PD effects obtained on blood and tumor samples will be presented. Clinical trial information: NCT01337050.
Elevated CSF-1 serum concentrations to predict lymph node metastasis and overall survival in women with early breast cancer.

Christian F. Singer, Seyedhossein Aharinejad, Mohamed Salama, Karin Zins, Patrick Paulus, Andreas Berger; Medical University of Vienna, General Hospital, Vienna, Austria; Medical University of Vienna, Vienna, Austria; MUW, Vienna, Austria

Background: Colony-stimulating factor-1 (CSF1) is a key modulator of tissue macrophages and contributes to the physiological function of the mammary gland. It has, however, also been associated with breast cancer since expression of its receptor CSF-1R is a strong predictor of poor outcome in early breast cancer and results in tumor cell invasiveness and pro-metastatic behavior in vitro. Methods: We have prospectively measured circulating CSF-1 with ELISA in 572 women with early breast cancer and in 688 women with benign breast lesions; and correlated these values with overall survival, nodal status and other clinical and histological parameters. Results: Serum CSF-1 concentrations were significantly elevated in patients with early breast cancer when compared to those with benign tumors (p<0.0001). Within breast cancer patients, CSF-1 was higher in women with involved axillary lymph nodes (p=0.04). CSF-1 concentrations were correlated with tumor size (p=0.002), age (p<0.001), and Ki67 expression (p=0.006). Log CSF-1 serum concentrations were predictive of poor survival in both univariate (HR: 3.77, CI: 1.65-8.65, p=0.002) and multivariate analyses (HR: 3.1, CI: 1.03-9.33, p=0.04). Post- but not premenopausal women with CSF-1 serum concentrations >873 pg/ml experienced a significantly poorer outcome (p=0.004 log rank test). Conclusions: CSF1 serum concentrations are elevated in women with malignant breast tumors. In early breast cancer, elevated serum CSF-1 is associated with nodal involvement, and in postmenopausal women also with poor overall survival.
Differential luminal breast cancer (LBC) reprogramming in response to BIBF1120 (BIBF) or bevacizumab (B).

Miguel Quintela-Fandino, Paloma Navarro, Tamara Mondejar, Jesus Sanchez, Elena Garcia, Gonzalo Gomez, David Gonzalez-Pisano, Ramon Colomer; Centro Nacional de Investigaciones Oncologicas, Madrid, Spain

**Background:** FVB MMTV PyMT is a great mouse model for studying LBC due to its stochastic nature, immune system preservation, natural growth kinetics and ER/HER2 status. We approached the transcriptional (T), proteomic (P), phospho-proteomic (P-P) and metabonomic (M) layers of regulation of the tumor phenotype attempting to unravel mechanisms of resistance to antiangiogenic drugs (ADs) and find new targets. **Methods:** Treatments started when tumors measured 0,1cm$^3$; with the VEGFR/PDGFR inhibitor BIBF 85mg/kg/day; a murine analog of B 10mg/kg/biweekly; or vehicle (V). Tumors were harvested at time 0, week 1 (T1), or at sacrifice (Tend, 1cm$^3$). Cd31 and pimimidazole measured vascularization and O2. T; P/P-P; and M layers were interrogated with sure print 3G arrays; titanium separation - HPLC fractionation - orbitrap spectrometer run; and GC/MS and LC/MS. Gene ontology pathways were discovered with GSEAS; kinase networks by consensus domain analysis; metabolic pathways rearrangement with in-house built bioinformatic tools. Pairwise comparisons included >15 mice/condition. All results: p or false-discovery ratio<0,05. **Results:** Total mice in study:2,327. BIBF-treated showed: early (T1 vs. 0) ↓ glycolysis (55%) and ↑ pyruvate diversion to Krebs mediated by ↓ 800% phosphorylation of PDH, ↑ ketones uptake and degradation (220%), mediated by 2700% ↑ AMPK activity; late (Tend) 900% ↑ lipid uptake and aerobic degradation mediated by >800% ↑ ERK/B-adrenergic/PKA and PPARg (1600%) signaling. All changes supported by GSEAS. Adding blockade of AMPK, ERK, B-adrenergic or PPARg (ShRNAs or compounds) to BIBF yielded at least 50% growth delay (Anova). Tend B-treated tumors showed 210% ↑ degradation of tryptophan to immunosuppressive (IS) kynurenine, and >200% ↑ levels of IS/vasodilator prostaglandins. Clescostib added to B delayed growth 90%. B and V induced similar ↑ of hypoxic/necrotic areas (T0-1-end: 5%-15%-52%, Poisson), no change in CD31. BIBF avoided necrosis/hypoxia despite >30% ↓ in CD31. **Conclusions:** Our strategy identified differential mechanisms of action of ADs in LBC. BIBF induces a switch from Warburg to aerobic metabolism; B reprograms the immune response. Regulators of these processes constitute targets.
LAMC2 as a prognostic marker that promotes metastasis of lung adenocarcinoma through epithelial-mesenchymal transition (EMT).

Yongwha Moon, Kang-seo Park, John Kim, Sami Sarfaraz, Donna Voeller, Pham Trung, Yisong Wang, Giuseppe Giaccone; National Cancer Institute, Bethesda, MD; Johns Hopkins University, Baltimore, MD

Background: Metastasis is the main cause of death in non-small cell lung cancer (NSCLC) patients. Genes responsible for NSCLC metastasis are unknown. LAMC2 is one of the 3 chains (α3, β3, γ2) of laminin 332, an important component of basement membranes, and LAMC2 involvement in metastasis is unclear. Methods: We have established a metastasis model in nude mice by repeated intracardiac injection of A549 lung adenocarcinoma cells. After 3-4 rounds of intracardiac injections, 100% metastasis penetrance was obtained. Microarray analysis was performed to identify genes differentially expressed between parental (A549P) and round-3 (A549R3) cells. In vitro migration/invasion and in vivo metastasis assays were performed in LAMC2-overexpressed A549P, LAMC2-knockdown A549R4, PC9, H358, and H322 cells. Public RNA microarray data of human NSCLC (GSE8894, GSE3141; n=249) and LAMC2 immunohistochemistry (IHC) of stage I NSCLC TMA samples (n=250) were analyzed to correlate LAMC2 and prognosis. Results: We identified LAMC2 as a putative metastasis marker of NSCLC through gene expression profiling of A549 cells enriched for metastasis in mice. Ectopic LAMC2 expression increased migration/invasion, whereas LAMC2 knockdown decreased migration/invasion in vitro. Conditioned media containing secreted LAMC2 promoted cell migration/invasion, which were blocked by LAMC2 knockdown or LAMC2 neutralizing antibody. Ectopic LAMC2 expression induced mesenchymal but decreased epithelial markers, indicating EMT, whereas LAMC2 knockdown elicited the opposite. A549R4 LAMC2 knockdown cells showed less metastatic activity than A549R4shRNA control cells in mice. In public microarray data high LAMC2 mRNA predicted high risk of recurrence (GSE8894, P=0.022; GSE3141, P=0.029) in adenocarcinoma (AC) but not squamous carcinoma (SC). Our IHC study showed that high LAMC2 predicted high risk of recurrence (hazard ratio=1.8; P=0.040) and death (hazard ratio=1.9; P=0.028) in AC but not SC by multivariate analysis. Conclusions: LAMC2 promotes metastasis through activation of EMT pathways, and is a potential prognostic marker and therapeutic target of metastasis in lung adenocarcinoma.
Association of downregulation of toll-like receptor 3 (TLR3) expression with aggressive breast cancer (BC).

Elias Obeid, Rita Nanda, Terri Li, Bifeng Zhang, Jeffrey Mueller, Olufunmilayo I. Olopade; The University of Chicago Medical Center, Chicago, IL; University of Chicago, Chicago, IL; The University of Chicago, Chicago, IL

Background: Inflammation in the tumor microenvironment plays a key role in human breast cancer. We previously showed that a high proportion of alternatively-activated, tumor-associated macrophages (M2) are associated with increased microvessel density (MVD) and worse outcomes in BC. The anti-tumor effect of TLR3 is well recognized. To our knowledge, there are no studies evaluating TLR3 expression and characteristics of inflammation in primary breast tumors. Methods: 48 breast tumors were obtained from the University of Chicago Breast Cancer SPORE tissue bank under IRB approved protocols. Tissue microarrays were constructed and molecular subtypes assigned based on immunohistochemical (IHC) staining into: luminal A, luminal B, HER2-like, and basal-like. Macrophage density was determined using double staining with CD68/CD163. MVD was measured by IHC using anti-CD34. Staining quantification was done by a pathologist. To evaluate the association between M2 macrophages, TLR3 and MVD, Spearman’s rho correlation coefficients were calculated. Survival comparison analysis was done using Wilcoxon statistic. Results: Of the tumors studied, 46% were LumA, 38% basal-like, 4% HER2-like, 2% LumB. We found a significant association between high MVD and high M2 content in tumors (p<0.000). Tumors with high MVD exhibited shorter survival (Wilcoxon statistic 4.845, p=0.028). Spearman’s rho nonparametric correlations showed low TLR3 expression correlated with higher MVD (p=0.014), advanced pathologic tumor stage (p=0.046) and a trend for nodal disease (p=0.069). There was no statistically significant association between TLR3 expression and number of M2 macrophages (p=0.21). Conclusions: Neovascularization is promoted by tumor-associated macrophages and in turn poorer prognosis in BC. Our results suggest that reduced TLR3 expression is associated with more aggressive breast cancer. Future studies are needed to elucidate a mechanism by which TLR3 is downregulated in highly angiogenic BC. Thus, activation of TLR3 with TLR3-ligands such as poly (A:U) can potentially be coupled with anti-angiogenic therapies in patients with BC exhibiting low TLR3 expression and high MVD to improve treatment outcomes.
Correlation of Src activation with response to dasatinib, capecitabine, oxaliplatin, and bevacizumab in advanced solid tumors.

John H. Strickler, Shannon McCall, Andrew B. Nixon, Herbert Pang, Christel Rushing, Christy Arrowood, Sherri Haley, Kellen Meadows, Herbert Hurwitz; Duke University Medical Center, Durham, NC; Cancer and Leukemia Group B Statistical Center, Durham, NC; Duke University, Durham, NC

**Background:** Src inhibition may augment sensitivity to chemotherapy, but in unselected patients (pts) with advanced solid tumors, src inhibitors have shown limited clinical activity. Biomarkers to predict benefit from src inhibitors in advanced solid tumors are not yet known. **Methods:** 22 pts (dose escalation cohort = 12 pts; colorectal cancer [CRC] expansion cohort = 10 pts) were enrolled in a phase I study to determine the safety and tolerability of the src inhibitor dasatinib with capecitabine, oxaliplatin, and bevacizumab (*J Clin Oncol* 29: 2011 [suppl; abstr 3586]). Src activation (src-a) was assessed in tumors from 16 evaluable pts. Src-a was measured by immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded tumor samples using an antibody that selectively recognizes the active conformation of src (clone 28). A GI pathologist who was blinded to pt outcomes graded membranous src-a using a standard semi-quantitative method. The endpoint of this exploratory analysis was objective response rate ([ORR] = PR + CR). 2-sided Fisher’s Exact test was used to evaluate the association between ORR and src-a. **Results:** Across all tumor types, 8 tumors had no/faint src-a (IHC = 0/1); 8 tumors had moderate/strong src-a (IHC ≥ 2). Benign colonic epithelium had no src-a (IHC = 0). The ORR was 75% (6/8) for pts with moderate/strong src-a versus (vs) 0% (0/8) for pts with no/faint src-a (p = 0.007). In the CRC expansion cohort, the ORR was 83% (5/6) for patients with moderate/strong src-a vs 0% (0/2) for pts with no/faint src-a (p = 0.107); progression free survival range was 7.9-24.4 months for pts with moderate/strong src-a. **Conclusions:** In this small phase I study, src-a is associated with benefit from the combination of dasatinib and oxaliplatin-based chemotherapy. Further evaluation of dasatinib in patients whose tumors demonstrate high levels of src-a may be warranted. Clinical trial information: NCT00920868.
A loss of microRNA expression as a characterization of synchronous peritoneal secondary localizations of epithelial ovarian cancer as compared to primary tumors.

Delia Mezzanzanica, Loris De Cecco, Daniela Califano, Simona Losito, Marina Bagnoli, Renato Franco, Giosuè Scognamiglio, Gennaro Chiappetta, Domenica Lorusso, Francesco Raspagliesi, Silvana Canevari, Sandro Pignata; Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; Fondazione G. Pascale Istituto Nazionale Tumori, Naples, Italy; MITO and Istituto Nazionale Tumori di Napoli, Napoli, Italy

Background: Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy and one of the most challenging areas of cancer research being a highly heterogeneous disease difficult to diagnose and treat. EOC has a peculiar dissemination process due to the sloughing-off of cells from primary tumors and their spread throughout the peritoneal cavity. A better characterization of the mechanism involved in tumor spreading might help in design new therapeutic intervention. Methods: Forty-four couples of chemo naïf primary tumors and synchronous secondary peritoneal localizations, obtained at primary surgery from MITO2 clinical trial, have been profiled for microRNA (miRNA) expression on an Agilent Platform. Total RNA was extracted from formalin-fixed paraffin embedded tissues. An independent validation set of samples with similar characteristics, has been collected at INT Milan. Results: By class comparison analysis, imposing a false discovery rate <10%, 45 miRNAs were identified as differentially expressed: 32 down-modulated and 13 up-modulated in secondary localizations compared to primary tumors. Among the miRNAs down-modulated in the secondary localizations we detected most of the miRNA belonging to the Xq27.3 cluster, whose low expression we previously described to be associated with EOC early relapse, and a number of miRNAs related to epithelial/mesenchimal transition (EMT) whose modulation could be related to dissemination of the disease and response to drug treatment. In particularly loss of has-miR-506 resulted associated to platinum resistance since its ectopic expression in EOC cell lines increased their sensitivity to the drug. Furthermore preliminary data indicated that has-miR-506 regulated N-cadherin linking its modulation to EMT. Conclusions: To our knowledge, the present study is the first attempt to characterize a miRNA signature differentially expressed between EOC primary tumors and synchronous secondary peritoneal localizations. The validation of the miRNA profile as well as of target genes might help in elucidating EOC dissemination mechanisms and in defining possible new therapeutic targets.
NOTCH1 as a potential prognostic biomarker for anti-VEGF therapy in patients with metastatic colorectal cancer.

Tadeu Ferreira Paiva, Alexandre Andre Balieiro Anastacio da Costa, Flavio Augusto Ismael Pinto, Victor Hugo Fonseca Jesus, Raul A. Marques, Patricia Peresi, Mariana Petaccia Macedo, Ludmilla T. D. Chinen, Benedito Mauro Rossi, Maria D. Begnami, Vladmir C. Lima; Hospital A.C. Camargo, São Paulo, Brazil; Barretos Cancer Hospital, Barretos, Brazil

Background: There are no validated biomarkers for clinical response or survival benefit in patients treated with bevacizumab (Bv) in advanced metastatic colorectal cancer (mCRC). The aim of this study was to evaluate the predictive value of putative biomarkers in mCRC. Methods: One hundred and five mCRC patients who received Bv combined with FOLFOX or FOLFIRI were retrospectively evaluated for clinical and pathological characteristics. VEGFR1, VEGFR2, VEGFR3, PIGF, DLL4 and NOTCH1 expression were assessed by immunohistochemistry on formalin-fixed, paraffin-embedded neoplastic tissue of either primary or metastatic tissue in a tissue microarray. High levels of expression were defined as less than or equal to or more than the median. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test. For multivariate analysis the Cox proportional hazards model was used.

Results: Grade 1 or 2 (p=0.01), non-mucin-producing histology (p=0.04) and presence of liver metastasis (p=0.001) were associated with a higher response rate. There was no difference between the expression of markers and the response rate. ECOG 0 or 1 (p=0.002), grade 1 or 2 (p=0.02), liver metastasis (p=0.003), no lymph node metastasis (p=0.01) no peritoneal metastasis (p=0.02) and resection of metastasis (p<0.001) were correlated with higher progression-free survival (PFS). There was also a strong correlation between ECOG 0 or 1 (p=0.001), grade 1 or 2 (p=0.006), no lymph node metastasis (p=0.004), liver metastasis (p<0.001) and resection of metastasis (p<0.0001) with better overall survival. There was a trend between high expression of NOTCH1 (p=0.06) and worst PFS. High expression of VEGFR2 (p=0.07) was slightly associated with a better overall survival, while high expression of NOTCH1 was associated with a worse overall survival (p=0.01). Using multivariate analysis, NOTCH1 proved to be an independent variable for adverse overall survival (HR 2.01, IC 1.07 – 3.77, p=0.02). Conclusions: High NOTCH1 expression assessed by immunohistochemistry is capable of predicting poor survival in advanced colorectal cancer patients treated with bevacizumab.
Effect of nonmalignant accessory cells from the metastatic microenvironment on breast cancer cell resistance to antiestrogens.

Eugen Dhimolea, Constantine S. Mitsiades; Harvard Medical School, Boston, MA; Dana-Farber Cancer Institute, Boston, MA

Background: To address the role of the local metastatic microenvironment in the antiestrogen (AE) resistance exhibited by disseminated breast cancer (BrCa), we assembled heterotypic in vitro three-dimensional (3D) tissue cultures comprised of estrogen receptor-positive (ER+) BrCa cells and non-malignant accessory cells from organs frequently targeted by metastatic disease, to model the composition and architecture of metastatic lesions. Methods: MCF7 cells expressing luciferase, cultured alone or with accessory cells, were exposed to dose-ranges of hydroxytamoxifen (4-OHT), fulvestrant or raloxifene. MCF7 cells were also injected s.c. in nude mice either alone or with HS-5 bone marrow stromal cells (BMSCs). Tumor spheroid viability or xenograft growth were measured by bioluminescence. Results: MCF7 response to AEs in 3D conditions (collagen type I and/or Matrigel) was marked by acinar differentiation of tumor spheroids that resembles normal breast epithelium. Co-culture of MCF7 and immortalized accessory cells from the bone (BMSCs; HOBIT and hFOB osteoblasts), brain (SVGp12 astrocytes), liver (THLE3 hepatocytes), and lung (MRC9 fibroblasts) in 3D (but not 2D) conditions, conferred resistance to 4-OHT, raloxifene, and fulvestrant at clinically relevant doses. Heterotypic xenografts (MCF7/BMSCs) had reduced response to tamoxifen compared with monotypic xenografts (MCF7 alone). BMSCs induced in MCF7 cells downregulation of TGFβ2; upregulation of a transcriptional signature enriched for genes associated with high-grade tumors, including genes involved in interferon response, signaling through EGFR superfamily members, NFkB and other antiapoptotic pathways; and elevated c-Myc protein levels. Exogenous TGFβ2 or neutralizing antibodies against INFγ partially re-sensitized MCF7 cells to 4-OHT in co-cultures. Conclusions: Our results indicate that accessory cells representing the microenvironment of metastatic sites confer AE resistance to BrCa cells in 3D tissue cultures and xenograft models. The mechanisms of stroma-induced AE resistance may reveal new therapeutic targets for refractory BrCa patients with metastatic disease.
Targeted next-generation sequencing (NGS) of circulating tumor cells (CTCs) in hormone-sensitive prostate cancer (HSPC).

Stephen V. Liu, Paul W. Dempsey, William Strauss, Yucheng Xu, Tong Xu, Jacek K. Pinski, Tanya B. Dorff, David I. Quinn, Timothy J. Triche, Jessamine Winer-Jones, Andre De Fusco, Janine McMurdie, Amir Goldkorn; University of Southern California Norris Comprehensive Cancer Center, Los Angeles, CA; Cynvenio Biosystems, Inc, Westlake Village, CA; Life Technologies, Los Angeles, CA

Background: Recently a succession of new hormonal therapies has emerged, highlighting the need for biomarkers to guide the management of HSPC. Biomarker development in HSPC has been hampered by the absence of primary tumor tissue in men who undergo radiation or present with metastatic disease. CTCs can address this challenge by providing real-time cancer tissue for biomarker analysis in HSPC. To test this approach we conducted a pilot of CTC capture and targeted NGS in HSPC. Methods: Under IRB approval, blood samples from patients with HSPC were labeled with EpCAM ferrofluid and placed into the LiquidBiopsy platform (Cynvenio Biosystems, Inc.), an immunoaffinity-based microfluidic device tailored to query genomic events. CTCs were identified by CK, CD45 and DAPI expression. A matched WBC pellet served as a control representing germline sequence. Amplicon libraries were generated using Life Technologies AmpliSeq 2.0 and sequenced on an Ion Torrent platform. Somatic single nucleotide variants (SNV) present in CTCs but not in WBC were identified. Results: CTCs were detected in all 8 patients with HSPC (CTC median 64.5, range 17-217). Germline variants were consistently detected in matched CTC and WBC samples. Significant SNVs (occurring in > 1% of DNA in a sample) were found in 4 of 8 CTC samples (range 1-5 SNVs/sample, frequency 1.2%-11.9% with 620X-14,422X coverage). Notably, 3 patients had biochemical recurrence only (no clinical metastases) yet still yielded CTCs associated with SNVs in KIT, APC, RET, SMAD4 and PTEN. One patient who had untreated metastatic disease had the highest number of CTCs which harbored 4 SNVs. Conclusions: This pilot demonstrates the feasibility of using CTCs as real-time disease relevant substrate for NGS to identify personalized genomic targets in HSPC. A high number of CTCs were detectable in all patients and CTC germline variants correlated with matched WBC controls. Encouragingly, even with a relatively narrow, primary tumor-derived AmpliSeq platform, cancer relevant SNVs were detected in half of the patients including those with only biochemical recurrence, making targeted NGS of CTCs a promising approach for biomarker discovery and validation in HSPC.
Circulating tumor cells as a prognostic marker in metastatic non-small-cell lung cancer patients receiving chemotherapy.

Yuichi Sakamori, Young Hak Kim, Hiroshige Yoshioka, Masataka Hirabayashi, Koichi Onaru, Motonari Fukui, Toshiki Hirata, Michiaki Mishima; Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan; Department of Respiratory Medicine, Kurashiki Central Hospital, Kurashiki, Japan; Hyogo Prefectural Amagasaki Hospital, Hyogo, Japan; Department of Respiratory Medicine, Sakai City Hospital, Sakai, Japan; Division of Respiratory Medicine, Respiratory Disease Center, Kitano Hospital, Osaka, Japan; Center of Respirology, Iseikai Hospital, Osaka, Japan

Background: Radiographic response remains the gold standard for assessment of the chemotherapy effect and has been used as a surrogate endpoint in clinical trials. Recently, circulating tumor cells (CTC) have emerged as a novel prognostic marker in many types of cancer; however, their significance has not been fully examined in patients with non-small-cell lung cancer (NSCLC). Methods: We conducted a prospective study to evaluate the clinical significance of CTC in metastatic NSCLC patients treated with chemotherapy. Peripheral blood samples were collected for CTC analysis before chemotherapy, after 1 cycle of chemotherapy, and after 2 cycles of chemotherapy. CTC analysis was performed using CellSearch (Veridex). Results: One hundred and forty-eight patients were enrolled between August 2009 and January 2012, and 121 patients were eligible for the analysis. CTC was positive (CTC ≥1) in 30.6% (37/121) before chemotherapy, in 21.0% (26/118) after 1 cycle of chemotherapy, and in 21.6% (24/111) after 2 cycles of chemotherapy, respectively. CTC counts were higher in patients with N3 lymph node metastases (vs. N0-2, p = 0.0001), M1b status (vs. M1a, p = 0.0081) or ≥2 metastasis sites (vs. 1 metastasis site, p = 0.0342). Although not statistically significant, a positive trend was observed between the radiographic response and the dynamic change of CTC counts (p = 0.0734). In multivariate analysis, including the radiographic response (responder vs. non-responder), baseline CTC was a significant negative predictive factor for PFS (HR = 1.867; p = 0.0080) and OS (HR = 2.753; p = 0.006). Considering pre- and post-treatment time points (before and after 2 cycles of chemotherapy) together, CTC-positive patients at either time point experienced significantly worse PFS (HR = 1.747; p = 0.0143) and OS (HR = 2.031; p = 0.0123) than those who were CTC negative at both time points. Conclusions: CTC was an independent prognostic factor in patients with metastatic NSCLC who was treated with chemotherapy.
Evaluation of prevalence, number, and temporal changes of circulating tumor cells as assessed after 2 and 5 years of follow-up in patients with early breast cancer in the SUCCESS A study.

Emanuel C. A. Bauer, Julia Katharina Neugebauer, Ulrich Andergassen, Bernadette Jaeger, Julia Kathrin Jueckstock, Peter A. Fasching, Lothar Haeblerle, Thomas W. P. Friedl, Iris Schrader, Andreas Lorenz, Hans Tesch, Mahdi Rezai, Elisabeth Thurner-Herrmanns, Andreas Schmeeviss, Matthias W. Beckmann, Klaus Pantel, Wolfgang Janni, Brigitte Kathrin Rack; Department of Gynecology and Obstetrics, University Ulm, Ulm, Germany; Department of Gynecology and Obstetrics, Klinikum der Ludwig-Maximilians-Universitaet, Munich, Germany; Department of Gynecology and Obstetrics, Ludwig-Maximilians-University, Munich, Germany; Department of Gynecology and Obstetrics, University of Ulm, Ulm, Germany; Frauenklinik Innenstadt Munich University, Munich, Germany; Department of Obstetrics and Gynecology, University Erlangen, Erlangen, Germany; Department of Biometry/Epidemiology, Erlangen, Germany; Department of Gynecology and Obstetrics, Universitaetsklinikum Ulm, Ulm, Germany; Gynaekolog-Okolog. Gem.Praxis, Hannover, Germany; Fachartzpraxis Lorenz, Hildburghausen, Germany; Onkologische Gemeinschaftspraxis am Bethanien-Krankenhaus, Frankfurt/Main, Germany; Breast Center Duesseldorf, Louis Hospital, Dusseldorf, Germany; RoMed Klinikum Rosenheim Klinik F. Gynaeologie, Rosenheim, Germany; University Hospital Heidelberg, Heidelberg, Germany; Department of Gynecology and Obstetrics, University Hospital Erlangen, Erlangen, Germany; Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

**Background:** Recent studies revealed that temporal changes in circulating tumor cells (CTC) prevalence assessed before and immediately after adjuvant chemotherapy (CT) might indicate treatment response in early breast cancer (EBC). However, there is limited knowledge on CTC status one or more years after chemotherapy treatment. Here we present descriptive data on CTC status prospectively evaluated 2 and 5 years after primary diagnosis in the German SUCCESS A study. **Methods:** The SUCCESS A trial is a large, randomized, open-label, 2x2 factorial design Phase III study comparing disease free survival (DFS) in patients with EBC treated with 3 cycles of Epirubicin-Fluorouracil-Cyclophosphamide (FEC) followed by either 3 cycles of Docetaxel (D) or 3 cycles of Gemcitabine-Docetaxel (DG), and comparing DFS in patients treated with 2 years or 5 years of Zoledronate. CTC status at various time points was assessed using the FDA-approved CellSearch System (Veridex, USA). **Results:** Data on CTC status both at 2 years and at 5 years after primary diagnosis were available for 983 (26.2%) out of 3754 randomized patients. After 2 and 5 years, CTCs were found in 132 (13.4%; median 1; range 1 – 99) and 88 (9.0%; median 1; range 1 – 60) patients, respectively. The majority of patients (n = 779; 79.2%) had no CTCs at any of the two time points. CTCs were found at 2 years but not at 5 years after primary diagnosis in 116 (11.8%) patients, at 5 years but not at 2 years of follow-up in 72 (7.3%) patients, and both at 2 and at 5 years of follow-up in 16 (1.6%) patients. **Conclusions:** CTCs in peripheral blood were detected in a subset of early breast cancer patients without relapse up to five years after primary diagnosis. These CTCs may indicate the presence of occult “dormant” micrometastases.
Persistence of HER2 overexpression on circulating tumor cells in patients after systemic treatment for HER2-positive breast cancer: Follow-up results of the German Success B trial.

Julia Katharina Neugebauer, Brigitte Kathrin Rack, Bernadette Anna Sophia Jaeger, Ulrich Andergassen, Aurelia Pestka, Peter A. Fasching, Thomas W. P. Friedl, Doraid Mouarrawy, Michael R. Clemens, Gabriele Ziemendorff, Georg Heinrich, Ekkehard von Abel, Andreas Schneeweiss, Matthias W. Beckmann, Klaus Pantel, Wolfgang Janni; Department of Gynecology and Obstetrics, Klinikum der Ludwig-Maximilians-Universitaet, Munich, Germany; Department of Gynecology and Obstetrics, Universityhospital Ulm, Ulm, Germany; Department of Gynecology and Obstetrics, Klinikum der Ludwig-Maximilians-Universität, Munich, Germany; Department of Gynecology and Obstetrics, Ludwig-Maximilians-University, Munich, Germany; Department of Obstetrics and Gynecology, University Erlangen, Erlangen, Germany; Department of Gynecology and Obstetrics, Universitätsklinikum Ulm, Ulm, Germany; Klinikum Bremerhaven-Reinkenheide, Bremerhaven, Germany; Klinikum Mutterhaus, Trier, Germany; Klinikum Ludwigsburg, Ludwigsburg, Germany; Gynäkologisch-Onkologische Schwerpunktpraxis, Fürstenwalde, Germany; Klinikum Schwäbisch-Gmünd, Schwäbisch-Gmünd, Germany; National Center for Tumor Diseases, University of Heidelberg, Heidelberg, Germany; Frauenklinik der Universität Erlangen, Erlangen, Germany; Institute of Tumor Biology, Campus Forschung, University Hospital Hamburg-Eppendorf, Hamburg, Germany; Universitaetsklinikum Ulm, Ulm, Germany

Background: The discordance between HER2-expression on circulating tumor cells (CTC) in peripheral blood and the primary tumor has already been shown by our study group for early breast cancer patients with HER2-positive tumors. Here, we compare the results to CTC prevalence and HER2-status of CTC after adjuvant chemotherapy. Methods: The SUCCESS B trial compares FEC-Docetaxel vs. FEC-Docetaxel-Gemcitabine and HER2-targeted therapy as adjuvant treatment for patients with early, HER2-positive, node positive or high risk node negative primary breast cancer. We prospectively analyzed 23ml peripheral blood before and after chemotherapy. CTC and HER2-status were assessed with the CellSearchSystem (Veridex, USA). After immunomagnetic enrichment with an anti-Epcam-antibody, cells were labeled with anti-CK 8/18/19, anti-CD45 antibodies as well as a fluorescein conjugate antibody for HER2-phenotyping. Cutoff for CTC positivity was $\geq 1$ CTC. HER-positivity of CTC was assigned if at least one CTC showed strong HER2 staining (3+). Results: CTCs and their HER2-status both before and after chemotherapy were available for 392 patients. In 179 (45.7%) patients no CTC were detected before and after chemotherapy. CTC status changed from positive before to negative after chemotherapy in 104 (26.5%) patients and from negative before to positive after chemotherapy in 69 (17.6%) patients, while 40 (10.2%) patients had a consistently positive CTC status. Patients were significantly more likely to change their CTC status from positive to negative than from negative to positive (p = 0.01). Of the 40 patients with CTC both before and after chemotherapy, 14 (35%) patients had HER2-positive CTC before and after therapy, and 9 (22%) patients had HER2-negative CTC at both time points. 7 (18%) patients had HER2-positive CTC before but not after chemotherapy, while 10 (25%) patients showed the reverse pattern (p = 0.63). Conclusions: Cytotoxic treatment does not seem to influence the HER2-status on CTC. Follow-up data within the Success B trial will analyze the relevance of the HER2-expression of CTC to predict the efficacy of targeted treatment.
Characterization and identification of specific EGFR mutations in circulating tumor cells (CTCs) isolated from non-small cell lung cancer patients using antibody independent method, ApoStream.

Hai T. Tran, Vladislava O. Melnikova, Anne S. Tsao, Frank V. Fossella, Faye M. Johnson, Vassiliki Papadimitrakopoulou, Miguel Garza, Chris Neal, Dave Hasegawa, Amy Kruempel, Grant Wu, Katherine Richardson, Marcia E. Lewis, Benjamin Jr Legendre, Kenna Lynn Anderes, Darren W. Davis, John Heymach; Department of Thoracic Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX; ApoCell, Inc., Houston, TX; The University of Texas MD Anderson Cancer Center, Houston, TX; Transgenomic, Inc, Omaha, NE; Transgenomic, Inc., Omaha, NE; Transgenomic, Inc., New Haven, CT

Background: A variety of methods for isolation of CTCs of epithelial origin are available; most employ antibodies to epithelial cell adhesion molecule (EpCAM). Using classic phenotypic definition, a CTC is nucleated, cytokeratin CK(+), CD45(-) cell. However, some CTCs may elude capture as they originate from primary tumor cells which have undergone epithelial-mesenchymal transition (EMT). We report here the use of ApoStream, a novel dielectrophoresis field-flow-assisted, antibody-free method to isolate CTCs from blood. Methods: Blood was collected from consented NSCLC patients and processed using ApoStrea. For CTC enumeration comparison, CellSearch FDA-approved kit was used. Isolated cells were evaluated with multiplexed immunofluorescent assay and laser scanning cytometry analysis were applied to identify multiple combinations of positive and/or negative staining for CK/CD45/DAPI and EpCam. To determine specific EGFR mutations from captured CTCs, samples were analyzed using Improved and Complete Enrichment with CO-amplification at Lower Denaturation temperature (ICE COLD-PCR). Results: Blood samples from 32 NSCLC patients and 3 healthy volunteers were processed. ApoStream isolated 0 to 65 CK(+)/CD45(-) CTCs(n=32) and CellSearch isolated 0 to 13 EpCAM(+)/CK(+)/CD45(-) CTCs(n=7). Additionally, ApoStream™ recovered 37-3536 CK(-)/CD45(-) and 4-10702 CK(+)/CD45(+) cells. EpCAM expression was detected in 7-100% of CK(+)/CD45(-) and 0-5% of CK(-)/CD45(-) cells, and 18-100% of CK(+)/CD45(+) cells. EGFR mutations [exon 19 deletion and exon 21 L858R] were determined and found to be concordant when compared to tumor tissue analysis by Sanger sequencing. Conclusions: The ApoStream platform enriched EpCAM(+) and EpCAM(-) CTCs from the blood of NSCLC patients demonstrating utility in recovering cancer cells with multiple phenotypes. From recovered CTCs, detection of EGFR mutations was possible indicating the clinical relevance and potential utility of CTCs as an alternative to tissue biopsy. Complete mutation analysis will be presented.
mRNA expression profiles in circulating tumor cells (CTCs) of patients with metastatic breast cancer (MBC) treated with aromatase inhibitors (AI).

Esther Anneke Reijm, Anieta M. Sieuwerts, Joan Bolt-de Vries, Bianca Mostert, Wendy Onstenk, Dieter Peeters, Luc Yves Dirix, Caroline Seynaeve, A. Jager, Felix E. de Jongh, Paul Hamberg, Anne M. van Galen, Jaco Kraan, Maurice P. H. M. Jansen, Jan-Willem Gratama, John A. Foekens, John W. M. Martens, Els M. J. Berns, Stefan Sleijfer; Erasmus MC, Department of Medical Oncology and Cancer Genomics Netherlands, Rotterdam, Netherlands; Translational Cancer Research Unit, University of Antwerp and GZA Hospitals Sint-Augustinus, Antwerp, Belgium; TCRG-A/Oncology Centre, St. Augustinus Hospital, Antwerp, Belgium; Erasmus MC-Daniel den Hoed Cancer Center, Rotterdam, Netherlands; Department of Internal Medicine, Ikazia Hospital, Rotterdam, Netherlands; Department of Internal Medicine, Sint Franciscus Gasthuis, Rotterdam, Netherlands

Background: Enumeration of CTCs can be used to assess prognosis in MBC and to evaluate treatment response. Besides enumeration, molecular CTC characterization is a promising tool to develop a more personalized treatment approach. Here, we evaluated the association between mRNA expression of currently known CTC-specific genes and response to first-line AI in MBC patients with estrogen receptor (ER)+ primary tumors. Methods: CTCs were isolated and enumerated from blood of 25 MBC patients before first-line therapy with an AI. Fourteen patients received a non-steroidal AI (8 letrozole, 6 anastrozole) and 11 patients were treated with exemestane. mRNA expression levels of 96 genes were measured by quantitative RT-PCR as previously described (Sieuwerts et al. Clin Cancer Res. 17:3600-3618, 2011). Expression levels of these genes were studied for their association with time to progression (TTP) after start of first-line AI. Results: Median TTP was 338 (range 14–1,239) days. Median baseline CTC count for the 25 patients was 14 (range 0–753). In this relatively small cohort, the clinically relevant cut-off level of ≤5 CTCs in association with TTP did not reach statistical significance (Hazard Ratio [HR] 4.76, 95% Confidence Interval [CI]: 0.59–38.22, P=0.14). For type of AI, when comparing steroidal with non-steroidal AI, the measures in Cox univariate regression analysis were HR 2.54 (95% CI: 0.67–9.64), P=0.17. A 10-gene CTC profile was constructed based on the Wald statistics of the contribution of the individual genes in univariate Cox regression analysis of TTP. To identify patients with good and poor outcome, the Wald corrected sum of the 10 genes was used to dichotomize the continuous 10-gene predictor (HR 12.87 [95% CI: 1.60–103.56], P=0.016). In multivariate analysis, corrected for the clinically relevant variables type of AI and CTC count, only the 10-gene CTC profile was an independent factor associated with TTP (HR 12.46 [95% CI: 1.29–120.08], P=0.029). Conclusions: A 10-gene CTC predictor was constructed which distinguishes good and poor outcome to first-line AI in MBC patients. This profile is currently being validated in an independent group of patients.
Low cytokeratin- and low EpCAM-expressing circulating tumor cells in pancreatic cancer.

Daniel Adams, Susan Tsai, Olga V. Makarova, Peixuan Zhu, Shuhong Li, Platte T. Amstutz, Cha-Mei Tang; Creatv MicroTech, Inc., Rockville, MD; Medical College of Wisconsin, Milwaukee, WI; Creatv MicroTech, Inc., Chicago, IL; Creatv MicroTech, Inc., Potomac, MD

Background: To date, detecting circulating tumor cells (CTCs) in the peripheral blood of pancreatic patients using standard immuno-capture techniques has met with limited success. As pancreatic cancer is prone to metastasize at distant sites, and therefore should have high numbers of CTCs, it is possible that immuno-capture methods are not suitable for this disease. Using a microfiltration approach, we show that CTCs are present in the peripheral blood in over 75% of pancreatic cancer patients, and that two distinct subtypes can be identified. Methods: Pancreatic patient samples were provided by Medical College of Wisconsin, Milwaukee, WI. CellSieve microfilters, with precision 7 micron diameter pores distributed in uniform arrays were employed. 7.5 mL of whole blood was diluted in pre-fixation solution and filtered through CellSieve microfilters. CTCs collected by this size exclusion technique were fixed, permeabilized, and stained with DAPI, an antibody cocktail against cytokeratin 8, 18 and 19 (FITC), EpCAM (PE), and CD45 (Cy5). CTCs, defined as cytokeratin positive and CD45 negative, were found in two distinct subtypes. One subtype had the “classic” characteristics of a CTC, with high EpCAM and cytokeratin expression, identifiable cytokeratin filamentation, and a cancer-like nuclear structure. The second subtype is indicative of a CTC undergoing epithelial-mesenchymal transition (EMT), with low or no EpCAM, weak cytokeratin expression, and a smooth oval nuclear structure. Results: The “classic” CTCs were found in ~20% (n=40) of patient samples. The EMT-like CTCs were found in ~75% of the same patient cohort. Neither cell was present in any healthy subjects (n=30). EMT-like CTCs consistently lacked EpCAM expression and commonly presented as multi-cell clusters, or microemboli, in ~40% of the cases. Conclusions: We show that two distinct CTC subtypes circulate in the blood of most pancreatic patients. The low expression of cytokeratin and EpCAM of the EMT-like subtype implies that immuno-capture based CTC isolation methods have limited utility for pancreatic cancer. Further, this subtype provides a useful strategy for tracking pancreatic CTCs over the course of treatment.
Sequential monitoring of androgen receptor expression and copy number variation in castration-resistant prostate cancer (CRPC).

Mitchell E. Gross, David B. Agus, Tanya B. Dorff, Jacek K. Pinski, David I. Quinn, Angel E Dago, Asya Stepansky, Anders Carlsson, Natalie Felch, Madelyn Luttgen, Anand Kolatkar, James B Hicks, Peter Kuhn; University of Southern California, Los Angeles, CA; University of Southern California Keck School of Medicine, Beverly Hills, CA; University of Southern California Norris Comprehensive Cancer Center, Los Angeles, CA; The Scripps Research Institute, La Jolla, CA; Skyline Genomics, New York, NY; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Background: The high-definition circulating tumor cell (HD-CTC) assay provides for an enrichment-free approach to identify and characterize CTCs. Here, we utilized the HD-CTC assay to study androgen receptor (AR) expression combined with single-nucleus sequencing for genome-wide analysis of copy number variation (CNV) in sequential samples obtained from patients with CRPC treated with abiraterone acetate (AA). Methods: Patients were approached for participation in a study to provide peripheral blood at baseline, at 2-5 weeks, and at 9-12 weeks (or at progression). In each sample, 10^6 cells (defined with a DAPI-intact nucleus) were quantitatively examined for the presence of cytokeratin and AR (CTCs) and CD45 (leukocytes). Initial results are available from 9 subjects treated with AA as standard of care. Results: At baseline, the median (range) CTC was 7.8 (1.1-57.2) cells/ml. Using a definition of AR positive (AR+) as >6 standard deviations over mean signal observed in leukocytes, the median (range) of AR+ and total CTCs observed at baseline were 3.1(0.33.8) and 7.8 (1.1-57.2) cells/ml, respectively. Detailed single-nucleus CNV analysis was performed in sequential samples in a single subject (Table). Complex genomic rearrangements were observed including AR amplification and 8p deletion in both AR+ and AR- cells at baseline. During AA treatment, the frequency of AR+ CTCs decreased along with changes in the CNV pattern including loss of AR amplification. At 10 weeks, disease progression occurred coincident with re-emergence of an AR+ CTC population exhibiting AR amplification and a novel CNV pattern only distantly related to that of the baseline CTCs. While multiple complex abnormalities were noted, MYC amplification was observed at higher frequency in cells present at progression. Conclusions: Overall, our results demonstrate the feasibility of monitoring of CTCs for treatment emergent changes in protein which may be used to better monitoring and predict therapeutic responses in patients with metastatic cancer.

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<th>PSA (ng/ml)</th>
<th>CTC/ml</th>
<th>%AR+ CTC</th>
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<td>57.2</td>
<td>59</td>
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<td>10 wks AA</td>
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Detection and genomic interrogation of invasive circulating tumor cells (iCTCs) derived from men with metastatic castration resistant prostate cancer (mCRPC).

Terence W. Friedlander, Vivian K. Weinberg, Ritu Roy, Vy Ngo, Huang Dong, Shaun Doty, Qiang Zhao, Gayatri Premasekharan, Elizabeth Gilbert, Charles J. Ryan, Wen-Tien Chen, Pamela Paris; University of California, San Francisco, San Francisco, CA; UCSF Medical Center, San Francisco, CA; Stony Brook University, Stony Brook, NY

Background: Isolation, enumeration, and genomic profiling of CRPC CTCs offers the potential to discover genetic changes that occur in advanced disease. The Vitatex VitaCap platform captures CTCs based on their ability to invade a collagenous matrix (CAM), allows for capture of invasive CTCs (iCTCs) independent of EpCAM status, and yields viable cells suitable for comprehensive genomic study. Here we sought to compare CTC yields between the CAM and CellSearch platforms, to determine the utility of prostate-specific membrane antigen (PSMA) as an iCTC biomarker, to identify iCTC clusters and iCTCs expressing stem-like markers, and to explore the feasibility of iCTC epigenomic analysis. Methods: CTCs were isolated and enumerated simultaneously using the CellSearch and CAM platforms in 23 men with mCRPC. CAM-isolated iCTCs were defined as EpCAM⁺/H11001⁺PSMA⁺/H11001⁻ and were enumerated immunocytochemically (ICC) and by flow cytometry. iCTC clusters were enumerated by ICC. The Illumina Infinium Human-Methylation27 BeadChip was used to determine whole genome methylation status for CAM isolated cells. Results: 35 samples were collected for CAM analysis. A median of 27 (range 0-800) and 23 (range 2-390) iCTCs/ml were detected by ICC and flow respectively. In a subset of 20 samples, a median of 7 CTCs/ml (range 0-85) were detected by the CellSearch platform. CTCs were detectable by either CAM or CellSearch in >95% of samples. iCTC clusters were observed in 23% of samples with a median 7 clusters/ml (range 1-200). iCTCs expressing stem-like markers CD44 and Seprase were detected in 70% and 97% of samples by ICC and flow respectively, with a median of 9/ml (range 1-264) by flow. The iCTC methylation profile highly resembled mCRPC. Conclusions: The CAM and CellSearch platforms yield comparable CTC counts. iCTC clusters and iCTCs expressing stem-like markers are detectable using the CAM platform, and the iCTC methylome closely resembles that of mCRPC. Correlation with clinical data may yield further insight into the functional significance of these findings.
Evaluation of UGT1A1 genotyping for predicting individual toxicity of irinotecan plus platinum analog regimens: Interim safety analysis of a prospective observational study.

Masashi Takano, Kaichiro Yamamoto, Tsutomu Tabata, Yuji Minegishi, Takuma Yokoyama, Eiji Hirata, Takeshi Ikeda, Junzo Kigawa, Kouzo Yamada, Satoshi Morita, Yuichi Ando, Yukihiro Okutani, Masahiro Sugihara, Toru Sugiyama, Yuh Sakata; Department of Obstetrics and Gynecology, National Defense Medical College, Tokorozawa, Japan; Faculty of Medicine, Kinki University, Sakai Hospital, Osaka, Japan; Mie University Graduate School of Medicine, Mie, Japan; Nippon Medical School, Tokyo, Japan; Kyorin University School of Medicine, Tokyo, Japan; Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan; Naga District Hospital, Wakayama, Japan; Tottori University Hospital, Yonago, Japan; Department of Thoracic Oncology, Kanagawa Cancer Center, Yokohama, Japan; Yokohama City University Medical Center, Kanagawa, Japan; Clinical Oncology and Chemotherapy, Nagoya University Hospital, Nagoya, Japan; Daiichi Sankyo Co., Ltd., Tokyo, Japan; Iwate Medical University, Morioka, Japan; Department of Biostatistics, School of Public Health, University of Tokyo, Tokyo, Japan; Misawa City Hospital, Misawa, Japan

Background: Irinotecan (IRI)+platinum (Pt) therapy works synergistically and is widely used for solid carcinomas. Although UGT1A1 genotyping has been implemented in daily practice since long, recommended doses of IRI+Pt therapy according to UGT1A1 genotyping remain undetermined. Risk factors for severe toxicities of IRI+Pt regimens remain uncertain. We conducted a prospective observational study to examine the correlation between UGT1A1 genotyping and toxicity/efficacy in IRI+Pt regimens (NCT01040312). Methods: During October 2009 and March 2012, 321 patients were enrolled. Eligible patients had histologically confirmed SCLC, non-SCLC, cervical, ovarian, or gastric cancer; had PS 0-2; and were receiving IRI+Pt (cisplatin, 63%; carboplatin, 20%; nedaplatin, 17%) regimen, following UGT1A1 genotyping: hetero (*1/*6, *1/*28) and homo (*6/*6, *6/*28, *28/*28). Primary endpoint was to evaluate overall toxicity profile during first 3 cycles of treatment. In this interim analysis, incidences of grade 3/4 toxicities were compared among UGT1A1 phenotypes, and logistic regression models were used to identify independent risk factors for these toxicities. Results: At the time of abstract writing, toxicity data from 137 patients were available. There were 110 (80%) hetero and 27 (20%) homo genotypes. Combination therapy of IRI (57.2 ± 12.2 mg/m² for average initial dose) and Pt analogs (cisplatin, 63%; carboplatin, 20%; nedaplatin, 17%) was administered. Incidences of grade 3/4 toxicities during first 3 cycles of treatment in hetero and homo were as follows: leukocytopenia, 38% and 59%; neutropenia, 55% and 67%; thrombocytopenia, 7% and 22%; and diarrhea, 9% and 7%. Overall grade 3/4 hematological toxicity was 50% (55/110) in hetero and 78% (21/27) in homo; the adjusted odds ratio was 3.379 (p = 0.0185) in homo compared with hetero. Conclusions: A higher rate of grade 3/4 hematological toxicity in homo than in hetero was confirmed with IRI+Pt therapy. Thus, UGT1A1 genotyping could be a potential biomarker of toxicity with individualized chemotherapy using low-dose IRI regimens. At the presentation, analysis of >250 patients’ data will be shown. Clinical trial information: NCT01040312.
Fecal metabolome and microflora differences between colorectal cancer patients and healthy adults.

Tiffany Weir, Robert Frederick Marschke, Regina J. Brown, Joanne O'Malia, Erica Dickson, Marlon Bazan, Amy Sheflin, Adam Heuberger, Erica Borresen, Stefan Pettine, Elizabeth Ryan; Department of Food Science and Human Nutrition, Graduate Program in Cell and Molecular Biology, Fort Collins, CO; Department of Oncology Research, Poudre Valley Hospital System, Fort Collins, CO; Cancer Center of the Rockies, Fort Collins, CO; Oncology Research Department, PVHS - UCH-North, Fort Collins, CO; Colorado State University, Fort Collins, CO; College of Veterinary Medicine and Biological Sciences, Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO; Northern Colorado Surgical Associates, Fort Collins, CO

Background: High throughput genomic technologies such as 454 pyrosequencing and metabolomics platforms are now available to explore the relationships between gastrointestinal microflora, metabolism and colorectal cancer (CRC). Recent efforts to characterize the colorectal cancer microbiome have led to the identification of numerous bacteria whose presence or absence is associated with diseased tissue. Methods: Stool samples were collected from 10 healthy adults and 11 colorectal cancer patients prior to surgery at the University of Colorado Health-Poudre Valley Hospital in Fort Collins, CO. Fecal samples were processed for isolation of microbial DNA and sequenced using the 454 pyrosequencing platform. Metabolites were extracted using acidified water for short chain fatty acids (SCFA) and 3:2:2 isopropanol:acetonitrile:water to obtain global metabolite profiles utilizing Gas Chromatography-Mass Spectrometry (GC-MS). Results: There were no significant differences in the overall microbial community structure associated with disease state, but several bacterial genera, particularly butyrate-producing species, were under-represented in the CRC samples, while a mucin-degrading species, Akkermansia muciniphila, was about 4-fold higher in CRC (p<0.01). Consequently, the chemoprotective SCFA, butyrate, was significantly lower in CRC samples than in those from healthy adults (p<0.0001) and GC-MS profiling revealed that there were higher levels of amino acids in stool samples from CRC patients and higher poly and monounsaturated fatty acids in stool from healthy adults (p<0.01). Conclusions: This systems biology approach may allow us to identify functional groups of gastrointestinal bacteria and their associated metabolites as novel therapeutic and chemopreventive targets. The Colorado Agricultural Experiment Station, Shipley Foundation and the NIH R03CA150070 supported this work.
Prognostic significance of ATP-binding cassette (ABC) and solute carrier (SLC) transporters in pancreatic ductal adenocarcinoma (PDAC).

Beatrice Mohelnikova-Duchonova, Veronika Brynychova, Martin Oliverius, Jan Hlavsa, Eva Honsova, Jan Mazanec, Bohuslav Melichar, Zdenek Kala, Pavel Soucek; Department of Oncology, Palacky University Medical School and Teaching Hospital, Olomouc, Olomouc, Czech Republic; Department of Toxicogenomics, National Institute of Public Health, Prague, Prague, Czech Republic; Department of Transplantation Surgery, Institute of Clinical and Experimental Medicine, Prague, Prague, Czech Republic; Department of Surgery, Masaryk University Hospital and Faculty of Medicine, Brno Bohunicce, Brno, Czech Republic; Department of Clinical and Transplantation Pathology, Institute of Clinical and Experimental Medicine, Prague, Prague, Czech Republic; Department of Pathology, Masaryk University Hospital and Faculty of Medicine, Brno Bohunicce, Brno, Czech Republic; Palacky University Medical School and Teaching Hospital, Olomouc, Czech Republic; Department of Surgery, The University Hospital Brno, Brno, Czech Republic

Background: Resistance against anticancer drugs significantly limits the clinical use and efficacy. One of the most important mechanisms of the multidrug resistance is low accumulation of the drug in cancer cells caused by an increased efflux (mediated mainly by ABCs) or by a decreased uptake (mediated by some members of SLCs). The aim of the present study was to investigate prognostic importance of 13 anticancer drug-relevant SLCs and all 49 human ABCs in PDAC patients in association with clinical and pathological characteristics and the tumor KRAS mutation status. Methods: Tumors and adjacent non-neoplastic pancreatic tissues were obtained from 50 patients with histologically verified PDAC. The transcript profile of ABCs and SLCs was assessed using quantitative real-time PCR. KRAS mutations in exon 2 were assessed by high-resolution melting analysis and sequencing. Associations of transcript levels with clinical parameters were assessed by non-parametric Mann-Whitney tests. The Kaplan-Meier method with the log-rank test and Cox regression were used for analysis of overall survival (OS). Results: Most transporters investigated were deregulated in PDAC and 14 ABCs or SLCs were associated with clinical characteristics. Up-regulation of 7 drug resistance-associated ABC efflux transporters and down-regulation of 5 drug uptake-relevant SLC transporters was observed in tumors vs. non-neoplastic tissues. Moreover, expression levels of SLC28A1 and SLC22A1 were associated with OS of all patients (P=0.001). High transcript levels of SLC29A3 and SLC22A3 significantly predicted longer OS in chemotherapy-treated patients (P=0.004 and P=0.038, respectively). No association of ABC and SLC expression with KRAS mutation status was observed. Conclusions: Up-regulation of numerous drug resistance-associated ABCs and down-regulation of SLCs implicated in drug uptake may explain the generally very poor response of PDAC to cytotoxic agents. This study also identified some ABCs and SLCs as potential prognostic and predictive factors in PDAC. Supported by the Czech Science Foundation grant P301/12/1734 and CZ.1.05/2.1.00/03.0076 European Regional Development Fund.
Clinical significance of \textit{BIM} deletion polymorphism in non-small cell lung cancer with epidermal growth factor receptor mutation.

Kazutoshi Isobe, Yoshinobu Hata, Kyohei Kaburaki, Hiroshi Kobayashi, Keishi Sugino, Susumu Sakamoto, Naobumi Tochigi, Yujiro Takai, Kazutoshi Shibuya, Keigo Takagi, Sakae Homma; Department of Respiratory Medicine, Toho University Omori Medical Center, Tokyo, Japan; Department of Chest Surgery, Toho University Omori Medical Center, Tokyo, Japan; Department of Pathology, Toho University Omori Medical Center, Tokyo, Japan

\textbf{Background:} Germ line alterations in the proapoptotic protein BCL2-like 1 (\textit{BIM}) can have a crucial role in how a tumor responds to treatment. To clarify the clinical usefulness of detecting \textit{BIM} deletion polymorphism in non-small cell lung cancer (NSCLC) with positive epidermal growth factor receptor (\textit{EGFR}) mutation we examined the prognosis in patients with or without the \textit{BIM} changes.

\textbf{Methods:} Seventy NSCLC patients with positive \textit{EGFR} mutation treated with EGFR-tyrosine kinase inhibitor (EGFR-TKI) between January 2008 and January 2013 were enrolled in this study. \textit{BIM} deletion polymorphism was analyzed by PCR in 58 peripheral blood samples, 24 formalin-fixed paraffin-embedded (FFPE) slides of surgical specimens (lung tissue in 20, brain tissue in 4), and 12 samples that included both blood and tissue specimens. We performed retrospective analyses on clinical characteristics, response rate and toxicity of EGFR-TKI, and estimated the prognosis in patients with or without \textit{BIM} deletion polymorphism.

\textbf{Results:} \textit{BIM} deletion polymorphism was present in 13 of 70 patients (18.6\%) with homozygous deletion in 1 and heterozygous deletion in 12. There was no discordance between the two types of samples among the 12 patients. There were no significant differences between patients with or without \textit{BIM} deletion polymorphism on clinical characteristics, response rate to EGFR-TKI, and incidence of EGFR-TKI toxicity. Patients with \textit{BIM} deletion polymorphism showed significantly shorter PFS that in patients without \textit{BIM} deletion polymorphism (median: 216 days vs. 430 days, \(p<0.001\)). There was no significant difference in OS. In multivariate analyses using the Cox regression model, \textit{BIM} deletion polymorphism (hazard ratio = 4.2, \(p<0.001\), 95\% CI = 2.026-8.777) was identified as an independent factor for shorter PFS. \textbf{Conclusions:} \textit{BIM} deletion polymorphism could be detected by PCR on peripheral blood samples and FFPE slides of surgical specimens. \textit{BIM} deletion polymorphism has emerged as an independent prognostic factor for shorter PFS. Therefore, new treatment strategies should be established for patients with \textit{BIM} deletion polymorphism.
A genome-wide association study (GWAS) of docetaxel-induced peripheral neuropathy in CALGB 90401 (Alliance).

Daniel Louis Hertz, Kouros Owzar, Susan Halabi, William Kevin Kelly, Hitoshi Zemmbutsu, Chen Jiang, Jai Narendra Patel, Dorothy Watson, Ivo Sherev, Deanna L. Kroetz, Paula N. Friedman, John Francis Mahoney, Michael Anthony Carducci, Michael J. Kelley, Eric Jay Small, Phillip G. Febbo, Yusuke Nakamura, Michiaki Kubo, Mark J. Ratain, Howard L. McLeod; University of Michigan, Ann Arbor, MI; Duke University Medical Center, Durham, NC; Alliance Statistical and Data Center/Duke University Medical Center, Durham, NC; Kimmel Cancer Center of Thomas Jefferson University, Philadelphia, PA; University of Tokyo, Tokyo, Japan; University of North Carolina Institute for Pharmacogenomics and Individualized Therapy, Chapel Hill, NC; Department of Biostatistics and Bioinformatics, Duke University, Durham, NC; University of California, San Francisco, San Francisco, CA; Cancer and Leukemia Group B, Chicago, IL; Carolinas Hematology-Oncology Associates, Charlotte, NC; Johns Hopkins School of Medicine, Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD; Durham VA Medical Center/ Duke University Medical Center, Durham, NC; University of California, San Francisco Helen Diller Family Comprehensive Cancer Center, San Francisco, CA; Human Genome Center, Institute of Medical Sciences, University of Tokyo, Tokyo, Japan; Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN, Yokohama, Japan; The University of Chicago, Chicago, IL; University of North Carolina Eshelman School of Pharmacy, Chapel Hill, NC

**Background:** There are currently no effective methods for predicting, preventing, or treating chemotherapy-induced peripheral neuropathy. We performed a genome-wide association study in a clinical trial of castration-resistant prostate cancer (CRPC) to discover variants that may be useful for identifying patients at high risk of neuropathy during docetaxel treatment. **Methods:** Treatment and toxicity data were collected prospectively on the Cancer and Leukemia Group B (CALGB) 90401 trial of chemotherapy naive CRPC patients treated with docetaxel and prednisone ± bevacizumab. Genotyping was performed by the RIKEN Institute using the Illumina HumanHap610-Quad platform. Genetically defined European subjects were included in the discovery analysis of all single nucleotide polymorphisms (SNPs) that passed quality control. The primary endpoint was the cumulative dose level triggering a grade 3+ sensory neuropathy. The inference was conducted within the framework of a competing risk model accounting for early treatment termination induced by death or progression, or other toxicities. SNPs that were highly associated with neuropathy were assessed for a broader taxane effect in a cohort of paclitaxel-treated patients from a breast cancer clinical trial, CALGB 40101. **Results:** 623 Caucasian patients and 498,022 SNPs were included in the discovery analysis. The incidence of grade 3 neuropathy was 8%. One intergenic SNP (rs11017056) was associated with increased risk of neuropathy (HR=2.83, p=4.7x10⁻⁷). This association surpassed the genome-wide significance threshold after covariate adjustment (p=7.2x10⁻⁸). However, none of the 7 SNPs selected for replication were associated with neuropathy in the paclitaxel-treated breast cancer cohort. **Conclusions:** Using a prospectively enrolled prostate cancer patient cohort we identified multiple SNPs that may identify risk of docetaxel-induced peripheral neuropathy, but not paclitaxel-induced neuropathy. However, since it is unknown whether the genetic factors that affect taxane neuropathy are drug-specific, further replication studies in docetaxel-treated cohorts are of great interest.

Raquel Nunes, Lynette Wray, Mihraye Mete, Thomas Godwin, Pia Maarit Herbolzheimer, Karen L. Smith, Lana Bijelic, Marc E. Boisvert, Sandra M. Swain; Washington Cancer Institute, MedStar Washington Hospital Center, Washington, DC; MedStar Health Research Institute, Hyattsville, MD; MedStar Washington Hospital Center, Washington, DC

Background: Molecular profiling of breast cancer (BC) identifies intrinsic subtypes with distinct gene expression and clinical characteristics. In the US, BC is less frequent in African-American females (AAF); however mortality is higher, particularly among younger women. Unfavorable subtypes of BC seem to be more frequent in premenopausal AAF. Methods: Tumor gene expression in AAF presenting with early stage or locally advanced BC was performed using the Symphony platform on fresh and paraffin-embedded tissue (Agendia inc), a microarray-based method which classifies tumors according to prognosis (Mammaprint, MP), molecular subtype (Blueprint, BP) and estrogen receptor (ER), progesterone receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) mRNA levels (TargetPrint, TP). Genomic information is correlated with clinical and pathologic characteristics and Oncotype DX recurrence score (RS) when available. We plan to enroll 100 patients. Results: Results available in 46 patients. Median age 62 years (range 24-100), 20 stage I, 15 stage II, 11 stage III disease. There was no significant association between MP risk and stage, but MP risk was significantly associated with grade 3 disease (p=.006). 9 cancers were triple negative by IHC; using BP, 8 of these were Basal-type and 1 HER2-type. Basal-type was the most common subtype in patients ≤ 40 years old (p < .001). In the 6 cases ER positive by IHC but negative by TP, 3 were Basal-type and 3 were HER2-type. 14 patients had Oncotype RS results available: 2 were High Risk by Oncotype and MP; 3 had intermediate RS, 2 of which were High Risk by MP; 9 had a low RS, 4 of which were High Risk by MP. Conclusions: African-American women with stage I to III BC often present with High-Risk disease irrespective of stage. BP classified all young patients ≤ 40 years old as Basal-type. Molecular subtyping confirmed the biologic heterogeneity in triple negative and hormone positive tumors. Oncotype RS and MP offered different prognostic information. Follow up will be needed to determine correlation with outcome. Funding: MP, BP and TP test provided by Agendia. Biostatistical support by GHUTTCS-CTSA.

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<td>HER2</td>
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The usefulness of UGT1A1 polymorphism testing before starting irinotecan-based chemotherapy.

Taishi Harada, Haruhiro Saito, Makiko Sugiura, Shuji Murakami, Tetsuro Kondo, Fumihiro Oshita, Kouzo Yamada; Department of Thoracic Oncology, Kanagawa Cancer Center, Yokohama, Japan

Background: A few studies have revealed an association between UGT1A1 genotype and irinotecan-induced neutropenia. However, the usefulness of UGT1A1 polymorphism testing before starting irinotecan-based chemotherapy is controversial, even now. We assessed the clinical usefulness of UGT1A1 polymorphism testing before chemotherapy. Methods: 136 lung cancer patients were treated with nedaplatin and irinotecan combination chemotherapy as initial chemotherapy. Except for the patients with low enzyme activity of UGT1A1, 70 patients were treated after UGT1A1 polymorphism testing. (test group) 66 patients were treated without UGT1A1 polymorphism testing. (non-test group) We retrospectively analyzed adverse events and compared the test group with non-test group. Results: We could not confirm any reduction in hematologic or non-hematologic toxicity statistically in the test group. In 9 patients with non-hematologic toxicity of grade 4 and 5, 6 patients had febrile neutropenia (FN). All patients with FN were older than 70 years old. Adverse events in elderly patients were significantly more frequent than in the non-elderly. Conclusions: In patients treated with nedaplatin and irinotecan combination chemotherapy, UGT1A1 polymorphism testing before starting chemotherapy did not reduce adverse events. With UGT1A1 polymorphism testing only, it was difficult to predict the onset of severe adverse events. Therefore, it is more important to manage adverse events carefully, especially in elderly patients.
Induction of an aggressive phenotype and poor survival in early-stage lung adenocarcinoma and epigenetic inactivation of microRNA-34b/c.

Ernest Nadal, Marc Gallegos, Guoan Chen, Ramon Palmero, Gabriel Capella, Felipe Cardenal, David G. Beer; University of Michigan Comprehensive Cancer Center, Ann Arbor, MI; Catalan Institute of Oncology, Translational Research Laboratory, L’Hospitalet de Llobregat, Spain; University of Michigan, Section of Thoracic Surgery, Ann Arbor, MI; Catalan Institute of Oncology, Section of Medical Oncology, L’Hospitalet de Llobregat, Spain; Translational Research Laboratory, Institut Català d’Oncologia-IDIBELL, L’Hospitalet de Llobregat, Spain; Catalan Institute of Oncology, Section of Medical Oncology, Barcelona, Spain

**Background:** The microRNA-34b/c (miR-34b/c) is a transcriptional target of TP53 that is frequently mutated in primary lung adenocarcinoma (AC). We investigated the clinical implications of miR-34b/c methylation in early stage lung AC patients and the functional role of miR-34b/c re-expression in lung AC cell lines. **Methods:** DNA methylation of miR-34b/c promoter was assessed by real-time PCR temperature dissociation in 15 lung AC cell lines and 140 early stage lung AC resected at the Bellvitge Hospital (Barcelona) and at the University of Michigan (Ann Arbor). Patient characteristics: 65% males, 88% smokers, 68.5% stage I and 31.5% stage II. Expression of miR-34b/c was determined by TaqMan RT-PCR. Two lung AC cell lines (NCI-H1838 and SK-LU-1) were transfected with an expression vector containing both miRs and the effects upon cell proliferation, migration and invasion were determined. **Results:** MiR-34b/c was methylated in 40% of cell lines and in 46% of primary tumors with significant association with higher tumor stage (P<0.033), recurrence (P=0.017) and death (P=0.005). In the training set (n=58), patients with higher levels of miR-34b/c methylation had significantly shorter median DFS (28.5 months) compared to low to medium levels (not reached, log-rank P=0.016). In the test set (n=82), higher levels of miR-34b/c methylation were also associated to shorter median DFS (19 months) compared to patients with low to medium levels (not reached, log-rank P=0.005). MiR-34b/c methylation remained an independent prognostic marker for DFS after adjusting by age, gender and stage. Tumors harboring TP53 mutations and miR-34b/c methylation expressed significantly lower levels of miR-34b/c (P=0.001). Stable cells expressing miR-34b/c had lower proliferation rate relative to cells transfected with empty vector (P≤0.001). Expressing miR-34b/c in SK-LU-1 cells reduced migration and invasion ability. **Conclusions:** Epigenetic inactivation of miR-34b/c by DNA methylation is an independent prognostic marker in early stage lung AC. Ectopic expression of miR-34b/c generated a less aggressive phenotype in lung AC cell lines suggesting a potential for therapeutic targeting.
Effect of BRM promoter variants on survival outcomes of stage III-IV non-small cell lung cancer (NSCLC) patients.

Sinead Cuffe, Lu Cheng, Abul Kalam Azad, Yonathan Brhane, Dangxiao Cheng, Zhuo Chen, Xin Qiu, Kevin Boyd, Marjan Emami, Natasha B. Leighl, Sandy Der, Wei Xu, Frances A. Shepherd, Ming Sound Tsao, David Reisman, Geoffrey Liu; Department of Medical Oncology and Hematology, Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada; Princess Margaret Hospital, Ontario Cancer Institute, Toronto, ON, Canada; Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada; Biostatistics, Princess Margaret Hospital, University of Toronto, Canada; Princess Margaret Hospital, Toronto, ON, Canada; Princess Margaret Hospital, University Health Network, University of Toronto, Toronto, ON, Canada; University of Florida, Gainesville, FL

Background: BRM, a key catalytic subunit of the SWI/SNF chromatin remodeling complex, is a putative tumor susceptibility gene in NSCLC. Loss of BRM expression occurs in 15% of NSCLC, and has been linked to adverse outcome. Recently, our group has shown that variants of two novel BRM promoter insertion polymorphisms (BRM-741, BRM-1321) lead to loss of BRM expression by recruiting histone deacetylases; individuals carrying homozygous variants for both polymorphisms have doubled NSCLC risk; pharmacological reversal of these epigenetic changes is a potentially viable therapeutic strategy. We thus evaluated the effect of BRM promoter variants on survival outcomes of advanced NSCLC patients, where initial clinical trials are likely to be focused. Methods: 564 stage III-IV NSCLC patients were genotyped for the BRM promoter variants using Taqman. Association of BRM variants and overall (OS) and progression-free survival (PFS) were assessed using Cox proportional hazard models adjusted for prognostic variables. Results: Among our patients, 73% were Caucasian, 52% male, median age 63yrs, 55% stage IV disease, and 67% adenocarcinoma. Median OS was 1.6yrs; median follow up, 3.6yrs. The frequency of homozygosity was BRM-741, 23%; BRM-1321, 21%; both, 12%. Homozygous variants of BRM-741 were strongly associated with worse OS (adjusted HR [aHR] 2.3 [p=2x10E-8]) and PFS (aHR 2.0 [p=2x10E-7]) compared to the wild types. Similar findings were observed for BRM-1321 homozygous variants (aHR for OS 1.8 [p=8x10E-5] and aHR for PFS 1.6 [p=2x10E-4]). Carrying homozygous variants of both BRM-741 and BRM-1321 was associated with substantially worse OS (aHR 2.3 [p=1x10E-5]) and PFS (aHR 2.2 [p=3x10E-6]), with similar associations seen among the stage III (aHR for OS 2.3 [p=6x10E-6]) and stage IV (aHR for OS 2.5 [p=5x10E-6]) patients. Conclusions: The same two homozygous BRM promoter variants that are associated with increased risk of NSCLC are also strongly associated with adverse OS and PFS in this cohort of stage III-IV NSCLC patients. Validation of results in a clinical trial dataset is underway, and will better elucidate the prognostic significance of these BRM promoter variants.

Impact of IVS14+1G>A and 2846A>T DPYD polymorphisms on toxicity outcome of patients treated with fluoropyrimidine-containing regimens.

Marzia Del Re, Fotios Loupakis, Cecilia Barbara, Tiziana Latiano, Enrico Vasile, Elena Zafarana, Stefano Sergio Cordio, Luisa Toffolatti, Miriam Ricasoli, Angela Prestifilippo, Filippo Venturini, Samantha Di Donato, Federico Grifalchi, Antonio Rinaldi, Alfredo Butera, Evaristo Maiello, Salvatore Siena, Alfredo Falcone, Federico Cappuzzo, Romano Danesi; Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy; U.O. Oncologia Medica 2, Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy; Department of Medical Oncology, Ospedale Civile di Livorno, Livorno, Italy; Medical Oncology, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; U.O. Oncologia Medica 2, Azienda Ospedaliero-Universitaria Pisana, Istituto Toscano Tumori, Pisa, Italy; Medical Oncology Unit, Ospedale Misericordia e Dolce, Prato, Italy; Oncology Unit - Garibaldi Hospital, Catania, Italy; Medical Oncology Unit, Azienda Ospedaliero Vimercate, Monza, Italy; Medical Oncology Unit, USL 3, Pistoia, Italy; Istituto Oncologico del Mediterraneo, Viagrande, Catania, Italy; Dipartimento Oncologico, Ospedale Niguarda Ca’ Granda, Milano, Italy; Medical Oncology Unit, Ospedale MG Vannini, Roma, Italy; DH Oncology Hospital Castellaneta, Castellaneta, Italy; Medical Oncology Hospital Agrigento, Agrigento, Italy; IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; Department of Oncology, Ospedale Niguarda Ca’ Granda, Milano, Italy; Medical Oncology Unit 2, University Hospital and Tuscany Tumor Institute, Pisa, Italy; Istituto Toscano Tumori, Department of Medical Oncology, Civil Hospital of Livorno, Livorno, Italy

Background: DPD deficiency is an inherited syndrome resulting from loss-of-function mutations within the DPYD gene. The IVS14+1G>A variant is associated with DPD deficiency as a result of a 165-bp deletion in the DPD mRNA. A rare mutation, 2846A>T, is characterized by a change of the acidic aspartic acid to the aliphatic valine with potential impairment of enzyme activity (Amstutz et al., 2011). In this study, we describe the spectrum of toxicities of 5-FU and capecitabine in patients carrying the IVS14+1G>A and 2846A>T variants. Methods: Data were collected from 450 patients with gastrointestinal, breast and pancreas cancers. They were evaluated for DPD genotype upon development of grade ≥2 non-hematological and ≥3 hematological toxicities (CTCAE v. 4) secondary to standard fluoropyrimidine-containing regimens in combination with other cytotoxic agents and/or EGFR and VEGF antibodies. DNA was extracted from blood and IVS14+1G>A and 2846T>C DPD variants were screened on a Real-Time Life Sciences 7900 HT platform. The study was approved by the local Ethics Committee. Results: A total of 23 IVS14+1GA, four 2846AT, one IVS14+1AA and one 2846TT subjects were identified. Toxicities in all subjects were G3/4 diarrhea (100%), G3/4 mucositis (48%), febrile neutropenia (45%), G3/4 thrombocytopenia (38%), G3/4 anemia (24%), G2/3 hand-foot syndrome (14%), G3 dermatitis (7%) and G2/4 alopecia (7%). The homozygous IVS14+1AA patient survived because she was given a reduced 5-FU 250 mg/sqm test dose without folates, while the 2846TT patient deceased after the first cycle of FOLFOX4 treatment. Conclusions: Patients carrying the deleterious IVS14+1G>A and 2846T>C variant alleles display severe toxicities which is fatal in homozygous variant subjects. This finding suggests the usefulness of pre-treatment screening of DPD in patients candidates to fluoropyrimidine treatment. Acknowledgments. This study was supported by the Italian Association for Cancer Research (AIRC, Milano) and the Istituto Toscano Tumori (ITT, Firenze, Italy). Reference. Amstutz U, Froehlich TK, Largiadèr CR. Pharmacogenomics 2011;12:1321-36.
Patient segmentation assay for MAPK pathway mutations.


Background: Mutational status of solid tumors is increasingly important for identifying the best treatment options. Tumors harboring activating mutations in RAS and RAF result in constitutive activation of the RAS/MAPK signal transduction pathway and do not respond well to MAPK pathway inhibitors. Scientific attention is mostly focused on the major mutational hotspots in these genes (e.g., KRAS codons 12, 13 and 61, BRAF codon 600). However, there is increasing evidence that other mutations can be tumorigenic. Thus, there is a need for highly sensitive and specific assays to detect these mutations. To address this need, we developed and analytically validated an assay that detects 33 mutations that activate the MAPK pathway.

Methods: We constructed a set of multiplexed single nucleotide primer extension (SNPE) assays that detect 33 activating mutations in KRAS, NRAS, or BRAF. The assays were analytically validated using a set of 60 formalin fixed paraffin embedded (FFPE) tissue samples from various tumor types, cell lines, and oligos with specific mutations. Performance was compared to dideoxy sequencing. Discordant calls were resolved with next generation sequencing (NGS). The limit of detection was determined by serial dilution of mutant DNA into wildtype DNA. Results: The developed multiplexed assay requires only 15 ng genomic DNA, relies on established technology, is cost effective, is amenable to high throughput, and can yield a patient eligibility decision in a CLIA lab in 4 days, making it a practical alternative to a NGS-based assay. Since low allele frequency mutations may be critical to patient survival, we devised a replicate strategy to increase the specificity and sensitivity of the assay. We showed that analysis of technical triplicates relative to no replicates increased the sensitivity from 97 to 100% and the specificity from 91 to 100%. Limit of detection varied from 2 -12%, depending on the mutation. Conclusions: We developed a selective and sensitive SNPE assay capable of detecting 33 hotspots in the MAPK pathway. Our approach reduced false positive and false negative calls of low allele frequency samples. The assay has clinical applicability for the selection of patients for early phase clinical studies.
Modulation of breast cancer cell-free DNA with surgical resection.

Howard B. Urnovitz, Julia Beck, Ekkehard Schütz, Gopal Singh, William M. Mitchell, Dalliah M. Black, Gildy Babiera, Isabelle Bedrosian, Henry Mark Kuerer, Gordon B. Mills, Funda Meric-Bernstam; Chronix Biomedical, Göttingen, Germany; The University of Texas MD Anderson Cancer Center, Houston, TX; Department of Pathology, Vanderbilt University, Nashville, TN

Background: Recently we demonstrated the ability to detect the presence of invasive breast cancer by next generation sequence (NGS) analysis of cell-free DNA (cfDNA) in serum without prior knowledge of the specific gene perturbations of the neoplasm (J. Clin. Oncology 2010; 28 (15S):10505). We hypothesized that cfDNA levels would hold promise as a marker to monitor therapeutic efficacy in breast cancer. In this study, we sought to determine whether cfDNA decreases after surgery in patients with operable invasive breast cancer. 

Methods: Serum was collected before and 1-4 weeks after surgery from 16 breast cancer patients and also from 24 age and gender matched controls. cfDNA was isolated from the serum, amplified by Chronix proprietary method and sequenced on an Illumina HiSeq platform. The breast cancer derived cfDNA sequences from each patient were compartmentalized in 250kbp bins, normalized, and compared to the mean (± 3SD) of the controls to identify regions of chromosomal number imbalance (CNI). Genomic DNA was isolated from the primary tumor and blood WBC buffy coat, pre-amplified, sequenced and subjected to comparative analysis with cfDNA. 

Results: Analysis of pre- and post surgery serum demonstrate that CNIs are present in the pre-surgery serum cfDNA that correlated with the associated tumor CNI and was not observed in WBC genomic DNA. The number of tumor associated CNI DNA biomarkers ranged from as low as 3 to as high as 865. In 13 of 16 patients (81%) analyzed, the post-surgery cfDNA was free of detectable tumor related-CNI DNA. In the remaining 3 patients, only a partial reduction in tumor specific CNI DNA serum biomarkers was observed, since some markers were still detectable after surgery. 

Conclusions: cfDNA from primary breast carcinomas accurately reflects tumor CNI. Removal of the primary tumor results in the elimination of tumor cfDNA in the majority of patients. Thus cfDNA may be of clinical value in evaluation of therapeutic efficacy on a real time basis. Further work is needed to determine if residual tumor CNI in cfDNA after surgery is a marker of minimal residual disease which can be pursued as a prognostic marker.
PIK3CA mutations in primary HER2-positive and triple negative breast cancer.

Sibylle Loibl, Carsten Denkert, Sherene Loi, Fabrice Andre, Berit Mueller, Andreas Schneweiss, Jens U. Blohmer, Christian Jackisch, Guo Sanxing, Stephan Gade, Peter A. Fasching, Christian Schem, Christos Sotiriou, Michael Untch, Gunter Von Minckwitz; German Breast Group, Neu-Isenburg, Germany; Charité-Universitätsmedizin Berlin, Institute of Pathology, Berlin, Germany; Peter MacCallum Cancer Center, Melbourne, Australia; Institut Gustave Roussy, Villejuif, France; Charité-Universitätsmedizin Berlin, Berlin, Germany; National Center for Tumor Diseases, University of Heidelberg, Heidelberg, Germany; Brustzentrum Sankt-Gertrauden-Krankenhaus, Berlin, Germany; Klinikum Offenbach, Offenbach, Germany; Frauenklinik des Universitaetsklinikums Erlangen, Erlangen, Germany; University of Kiel, Kiel, Germany; Jules Bordet Institute, Brussels, Belgium; Helios Klinikum Berlin-Buch, Berlin, Germany

Background: Phosphatidylinositol 3-kinase (PI3K)/AKT pathway aberrations are common in breast cancer (BC). PIK3CA mutations being the most common. Mutations are frequently found in hot-spots located in the helical and kinase domains (exons 9 and 20). Reported data is discrepant with regard to prognostic or predictive value of PIK3CA mutations especially in HER2+ve BC. We therefore investigated the frequency and prognostic associations of PIK3CA mutations in HER2+ve and triple negative (TN) primary BC by treated with neoadjuvant therapy. Methods: We prospectively evaluated PIK3CA mutations in the 595 participants of the neoadjuvant GeparSixto study (NCT01426880). The study investigates the effect of adding carboplatin to a liposomal doxorubicin/taxane combination for the treatment of patients with HER2+ve and TN primary BC. All HER2+ve patients received trastuzumab and lapatinib, the TN patients received bevacizumab. HER2, hormone receptors (HR), and Ki67 were centrally assessed. PIK3CA was genotyped in tumor material from formalin-fixed, paraffin embedded core biopsies taken before therapy using classical Sanger sequencing of exon 9 and 20. Results: From 09/2011 to 11/2012, 595 patients with HER2+ve or TN primary BC have been randomized in the GeparSixto study. Median age was 47 years (range 21-78); most tumors were cT2 (65%); cN0 (57%); ductal invasive (93%), grade 3 (65%); within the HER2+ve group 28% were HR positive. So far, PIK3CA genotype was evaluated in 395 randomized patients - 176 with HER2+ve and 219 with TN disease. Overall, 11.1% were found to have at least one mutation, in HER2+ve: 18.2% and TN BC: 5.5%. An exon 9 mutation was detected in 6.3% of the HER2+ve and 2.7% of the TNBC cases and an exon 20 mutation in 11.9% of the HER2+ve and 3.6% of the TN cases. A mutation in both exons was detected in 2 TN cases. PIK3CA mutations were more frequent in the HER2+ve/HR+ve compared to the HER2+ve/HR-ve group: 22.8% vs 10.6% respectively (p=0.047). Conclusions: PIK3CA mutations are more frequent in HER2+ve then in TN BC which is in line with previous reports. Results on all 595 patients and the correlation with response to therapy (pCR) will be presented at the meeting. The project has been funded within the EU-FP7 project RESPONSIFY No 278659. Clinical trial information: NCT01426880.
Background: KRAS is mutated in about 40% of colorectal cancers; it is the only validated predictive marker used in patients with metastatic disease. ERCC1 is a critical enzyme in nucleotide excision repair and is associated with response, progression free and overall survival in patients with NSCLC, colorectal, and gastric cancer treated with platinum based chemotherapy. We tested whether ERCC1 mRNA expression correlates with KRAS and BRAF mutation status in patients with colorectal, pancreatic, and lung cancer.

Methods: Formalin fixed paraffin embedded tumor specimens from 1,514 patients (573 colorectal; 91 pancreatic; 850 lung) were microdissected; DNA and RNA were extracted. Specifically designed primers and probes were used to detect 7 different base substitutions in codons 12 and 13 of KRAS and the V600E BRAF mutation. ERCC1 mRNA expression levels were measured by quantitative RT-PCR in a CLIA approved laboratory.

Results: Mt KRAS tumors had significantly lower ERCC1 mRNA levels relative to wt KRAS tumors in both colorectal (0.89 vs. 1.06; p < 0.001) and pancreatic (1.20 vs. 1.80; p = 0.006) groups. While mt KRAS lung tumors showed decreased ERCC1 expression (1.25 vs. 1.39), the trend did not meet significance (p = 0.069). Within the colorectal subset, mt BRAF cancers trended towards less ERCC1 expression than tumors harboring both wt KRAS/BRAF genes (0.96 vs. 1.06; p = 0.25).

Conclusions: This is one of the first reports linking KRAS mutation status with ERCC1 gene expression in colon, pancreatic and lung cancer. Colorectal tumors with either the BRAF or KRAS mutation have decreased ERCC1 expression, relative to those that carry both wildtype genes. This may explain the poor sensitivity to oxaliplatin based regimens in these patients. Furthermore, this suggests that ERCC1 expression may be driven by MAPK activation given the uniform ERCC1 downregulation amongst mt KRAS and mt BRAF cancers. Thus, MEK inhibitors may improve the efficacy of oxaliplatin based chemotherapy. Prospective biomarker driven studies should validate these principles, guide clinical decision making and improve outcomes.
Ribonucleotide reductase subunit-2 (RRM2) and thymidylate synthase (TS) to predict shorter survival in patients (pts) with resected stage I-III non-small cell lung cancer (NSCLC).

Francesco Grossi, Maria Giovanna Dal Bello, Graziana Savarino, Sandra Salvi, Roberto Puzone, Ulrich Pfeffer, Vincenzo Fontana, Erika Rijavec, Giulia Barletta, Carlo Genova, Claudio Sini, Giovanni Battista Ratto, Mauro Truini, Domenico Franco Merlo; Lung Cancer Unit, National Institute for Cancer Research, Genova, Italy; Department of Pathology, National Institute for Cancer Research, Genova, Italy; Clinical Epidemiology Division, National Institute for Cancer Research, Genova, Italy; Functional Genomics, National Institute for Cancer Research, Genova, Italy; Unit of Epidemiology and Biostatistics, National Institute for Cancer Research, Genova, Italy; Department of Thoracic Surgery, National Institute for Cancer Research, Genova, Italy; National Institute for Cancer Research, Genoa, Italy

Background: Tumor biomarkers can help to identify pts with early-stage NSCLC with high risk of relapse and poor prognosis. The aim of this study was to investigate the prognostic value of 7 biomarkers involved in DNA synthesis and repair. Methods: Tumour tissues from 82 radically resected, stage I-III NSCLC pts were consecutively collected to investigate the following biomarkers: excision repair cross-complementation group 1 (ERCC1), breast cancer 1 (BRCA1), ribonucleotide reductase subunit 1 (RRM1), ribonucleotide reductase subunit 2 (RRM2), subunit p53R2, thymidylate synthase (TS) and class III beta-tubulin (TUBB3) using immunohistochemistry (IHC) and quantitative real time-polymerase chain reaction (qRT-PCR). Expression levels of these genes were also investigated in a large publicly available NSCLC microarray dataset (Director Challenge Consortium, DCC). Results: RRM2 expression (p = 0.031), TS expression (p = 0.023), and pathologic stage (p < 0.001) were found as independent prognostic factors for shorter survival. The expression of ERCC1, RRM1, p53R2, TUBB3, BRCA1, as well as other clinical characteristics, failed to show any statistically significant association with the survival. Despite the lack of statistical significance, patients with lower RRM2 expression (i.e., ≤ 140) survived longer than pts with higher RRM2 levels (p = 0.069). There was a trend towards longer survival for BRCA1-, ERCC1-, RRM1- and TS-negative pts and for p53R2- and TUBB3-positive pts. For all of the biomarkers except TUBB3, the OS trends relative to the protein expression levels were in agreement with those relative to the respective gene expression levels, although the differences were not statistically significant. In the larger DCC dataset, TS (p = 0.005), and BRCA1 (p = 0.021) were identified as prognostic markers for OS, independent of tumour stage. Conclusions: This study has shown that high RRM2 and TS protein levels are negative prognostic factors for resected, stage I-III NSCLC pts. The data obtained by qRT-PCR confirmed these results. Analysis of the DCC microarray dataset detected TS and BRCA1 as independent prognostic markers of OS.
Exploration of a plucked hair gene signature as a potential pharmacodynamic marker for the dual PI3K/mTOR inhibitor VS-5584.

David T. Weaver, Daniel Paterson, Gino Miele; Verastem, Inc., Cambridge, MA; Epistem, Manchester, United Kingdom

**Background:** The PI 3-kinase/mTOR pathway plays a central role in cancer cell proliferation, survival, and cancer stem cell mechanisms. Depending on the mode of pathway activation, different PI3K isoforms and mTOR complexes have been shown to play essential roles in oncogenesis. VS-5584 inhibits PI3K/mTOR signaling with equipotency against all four human Class I PI3K isoforms and the mTOR kinase. VS-5584 has been shown to induce broad and robust antitumor activity with corresponding inhibition of PI3K/mTOR downstream targets in human xenograft models. Considering the inhibitory activity of VS-5584 against multiple targets within the PI3K/mTOR pathway, multiple clinical pharmacodynamic biomarkers need to be assessed.

**Methods:** Due to its vascularization, ease of sampling and epithelial origin, anagen hair represents a practical surrogate tissue for monitoring drug effects in patients. Human anagen hair from volunteer donors were treated ex vivo with VS-5584 and another PI3K/mTOR dual inhibitor, BEZ235. Following 6, or 24 hour exposure to various drug concentrations, RNA was extracted, amplified and analyzed on Affymetrix PrimeView microarrays.

**Results:** Transcriptomic profiles indicated a robust and dose-dependent response to VS-5584. A VS-5584-specific 91 gene signature was identified by 3-way ANOVA, and a Connectivity map evaluation indicated a strong positive correlation with other agents targeting the PI3K/mTOR pathway (wortmannin, LY-294002 and sirolimus). Likewise, VS-5584 exposure _ex vivo_ in human anagen hair follicles resulted in a dose responsive suppression at both 6 and 24 hrs with an alternative PI3K/mTOR dual inhibitor 115 gene signature trained on BEZ235. A seven gene subset was consistently reduced by both VS-5584 and BEZ235 at 6 hrs and 24 hrs.

**Conclusions:** Gene signatures from plucked anagen hair may provide a non-invasive approach to assess pharmacodynamic effects of VS-5584 in cancer patients.
Association of IGF2 DMR0 hypomethylation in esophageal squamous cell carcinoma with poor prognosis.

Yoshifumi Baba, Masayuki Watanabe, Asuka Murata, Hironobu Shigaki, Shiro Iwagami, Takatsugu Ishimoto, Hideo Baba; Kumamoto University, Kumamoto, Japan

Background: The insulin-like growth factor 2 gene (IGF2) is normally imprinted. Loss of imprinting (LOI) of IGF2 in humans is associated with an increased risk of cancer and is controlled by CpG-rich regions known as differentially methylated regions (DMRs). Specifically, the methylation level at IGF2 DMR0 is correlated with IGF2 LOI and is a suggested surrogate marker for IGF2 LOI. A relationship between IGF2 DMR0 hypomethylation and poor prognosis has been shown in colorectal cancer. However, no study has examined the relationships among the IGF2 DMR0 methylation level, LOI and clinical outcome in esophageal squamous cell carcinoma (ESCC). Methods: The IGF2 imprinting status was screened using ApaI polymorphism, and IGF2 protein expression was evaluated by immunohistochemistry with 30 ESCC tissue specimens. For survival analysis, IGF2 DMR0 methylation was measured using a bisulfite-pyrosequencing assay with 216 ESCC tissue specimens. The Cox proportional hazards model was used to calculate mortality hazard ratios (HR) adjusted for clinical, epidemiologic, and pathological variables. The term “prognostic marker” is used throughout this article according to the REMARK Guidelines. Results: Twelve (40%) of 30 cases were informative (i.e., heterozygous for ApaI), and 5 (42%) of 12 informative cases displayed IGF2 LOI. IGF2 LOI cases exhibited lower DMR0 methylation levels (mean, 23%) than IGF2 non-LOI cases (37%). The IGF2 DMR0 methylation level was significantly associated with IGF2 protein expression. Among 202 patients eligible for survival analysis, IGF2 DMR0 hypomethylation was significantly associated with higher cancer-specific mortality (log-rank P=0.013; univariate HR=2.03, 95% confidence interval: 1.14–3.58, P=0.017; multivariate HR=2.34, 95% CI: 1.29-4.22, P=0.0054). Conclusions: The IGF2 DMR0 methylation level in ESCC was associated with IGF2 LOI and IGF2 protein expression. In addition, IGF2 DMR0 hypomethylation was associated with a shorter survival time, suggesting its potential role as a prognostic biomarker.
Micro-RNA signature differences in lung adenocarcinoma with specific driver alterations.

Lorenza Landi, Pierluigi Gasparini, Stefania Carasi, Carmelo Tibaldi, Luciano Cascione, Greta Ali', Armida D’Incecco, Jessica Salvini, Gabriele Minuti, Antonio Chella, Gabriella Fontanini, Federico Cappuzzo, Carlo M. Croce; Istituto Toscano Tumori, Department of Medical Oncology, Civil Hospital of Livorno, Livorno, Italy; The Ohio State University, Comprehensive Cancer Center, Department of Molecular Virology, Immunology and Medical Genetics, Columbus, OH; Department of Molecular Virology, Immunology and Medical Genetics, Ohio State University Medical Center and Comprehensive Cancer Center, Columbus, OH; Azienda Ospedaliera Universitaria Pisana, Pisa, Italy; Azienda Ospedaliera Universitaria Pisana, Pisa, Italy; Department of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University Comprehensive Cancer Center, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, Columbus, OH

Background: Oncogenic driving alterations define types of lung adenocarcinoma with different prognosis and sensitivity to targeted agents. MicroRNAs (miRNAs) are a new class of non-coding RNAs involved in gene expression regulation. How miRNAs are dysregulated in lung cancer with ALK translocation, EGFR or KRAS mutation is unknown. In this study we aimed to identify miRNA signatures according to the presence of specific driver and to correlate miRNAs deregulation with patient outcome. Methods: The study was conducted in a cohort of 70 lung cancer patients (pts) including 18 ALK+/H11001 tumors, 11 ALK-/EGFR mutation+/H11001, 15 ALK-/KRAS mutation+/H11001, 26 ALK-/EGFR and KRAS wild-type and defined as triple negative. Matched normal lung tissue from 18 cases representative of the entire cohort were also included onto the analysis. RNA was isolated from formalin-fixed paraffin-embedded tissue (FFPE), using the Recover ALL kit (Ambion). NanoString nCounter system platform was used to generate the miRNA profile. We used Limma to test for differential expression analysis of data. The miR-515 family expression between tissues was validated by RT-qPCRs, analyzed using the parametric t-test (unpaired, 2-tailed for validation). Results: miRNA expression profile clusters distinctly ALK+ pts from ALK- and normal lung tissue. Within the ALK- group we found specific miRNAs subsets able to sub-stratify KRAS versus EGFR careers clustering sharply triple negative versus EGFR mutation+ and triple negative versus KRAS mutation+. miRNAs belonging to the miR-515 family seems to be the most deregulated in the ALK+ versus ALK-. Although their expression is stably high in normal tissues and ALK+ class, they are highly downregulated in KRAS mutated versus EGFR mutated and versus triple negative (p-value < 0.001 for all comparisons). Conclusions: miRNAs profile significantly differs in pts with ALK translocation, EGFR mutations and KRAS mutations. Analysis of miR-515 family members is ongoing in order to correlate their expression levels with pts’ outcome. In vitro modulation of miR-515 family expression levels, together with drugs treatment are ongoing in order to find possible chemo-resistance/chemo-sensitivity miRNA dependent, in ALK+ and ALK- model.
Integrated genomic analysis of EGFR-mutant non-small cell lung cancer immediately following erlotinib initiation in patients.

Trever Grant Bivona, Petros Giannikopoulos, Carlota Costa, Niki Karachaliou, Santiago Viteri, M. Rosario Garcia-Campelo, John St. John, Andrew V. Uzilov, Anne S. Wellde, William Reilly Polkinghorn, Margarita Majem, Enriqueta Felip, Enric Carcereny, Cordula Nicole Heidecke, Bartomeu Massuti, George W. Wellde, Jonathan S. Weissman, Rafael Rosell, Cancer Therapeutics Innovation Group; University of California, San Francisco, San Francisco, CA; Cancer Therapeutics Innovation Group, New York, NY; Pangaea Biotech, Laboratory of Translational Oncology, Barcelona, Spain; Pangaea Biotech, Clinical Unit, Barcelona, Spain; Complexo Hospitalario A Coruña, A Coruña, Spain; Memorial Sloan-Kettering Cancer Center, Cancer Therapeutics Innovation Group, New York, NY; Hospital de Sant Pau, Oncology Service, Barcelona, Spain; Thoracic Tumors Group, Vall d’Hebron Institute of Oncology, Barcelona, Spain; Medical Oncology, Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Spain; Alicante University Hospital, Alicante, Spain; University of California, San Francisco, Howard Hughes Medical Institute, Cancer Therapeutics Innovation Group, San Francisco, CA; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Pangaea Biotech, Cancer Therapeutics Innovation Group, USP Institut Universitari Dexeus, Barcelona, Spain

Background: Major obstacles limiting the clinical success of EGFR TKI therapy in non-small cell lung cancer (NSCLC) patients are heterogeneity and variability in the initial anti-tumor response to treatment. The underlying molecular basis for this heterogeneity has not been explored in patients immediately after initiation of therapy. Methods: We conducted CT-guided core needle biopsies immediately prior to erlotinib treatment initiation and at 6 days post erlotinib initiation in a patient with histologically confirmed NSCLC harboring an established activating mutation in EGFR. DNA from the paired frozen biopsies and matched normal tissue was analyzed by whole exome sequencing and RNA from the biopsies was analyzed by whole transcriptome sequencing. High-resolution CT images were obtained at the time of each biopsy to compare the degree of molecular and radiographic response observed. Results: Two established activating somatic mutations were identified in EGFR (p.G719A and p. R776H). Selective depletion of each EGFR mutant allele, but not the EGFR wild type allele, was observed upon erlotinib treatment. Gene expression analysis of the paired transcriptomes revealed that erlotinib treatment resulted in significant upregulation of proapoptotic genes including BAD, BAX, BID, CASP3 and growth inhibitory genes including CDKN1A, GADD45B, GADD45G and downregulation of growth-promoting genes including CCNB1 and CCND3. Several unexpected and novel molecular biomarkers were identified by transcriptome analysis and the complete dataset will be presented. High-resolution CT scans revealed no interval radiographic changes in the target lesion and no clinical complications were encountered. Conclusions: This study is the first reported integrated genomic analysis of EGFR-mutant NSCLC immediately following EGFR TKI initiation. We documented the feasibility, safety and utility of this strategy to establish initial drug efficacy at the molecular level prior to any radiographic evidence of response. Additional, serial integrated genomic analysis is ongoing in the index patient and others on therapy to enhance the management of NSCLC patients on targeted therapy.
Frequency of MET amplification determined by comprehensive next-generation sequencing (NGS) in multiple solid tumors and implications for use of MET inhibitors.

Norma Alonzo Palma, Gary A. Palmer, Siraj M. Ali, Philip J. Stephens, Jeffrey S. Ross, Vincent A. Miller, Doron Lipson; Foundation Medicine, Cambridge, MA; Foundation Medicine, Inc., Cambridge, MA; Albany Medical College, Albany, NY

Background: MET expression has been shown to be prognostic and possibly predictive in several tumor types and both small molecule inhibitors of MET kinase as well as monoclonal antibodies are under study as therapies for this subset of patients. However, the methods to assess MET overexpression are not well standardized. In the clinic, NGS is an advanced diagnostic method for identifying known and unknown targeted-therapy options. We therefore sought to explore the ability of NGS to detect high level MET amplifications in a general oncology population. Methods: We review here the results from analysis of 2,221 FFPE tissue samples across a range of tumor types by an NGS assay in a CLIA-certified laboratory (Foundation Medicine). We specifically report base pair substitutions, small insertions/homozygous deletions (indels), high level (> 6 copies/nucleus) amplification (amp) and select rearrangements in 189 genes, including MET. Results: MET was altered in 28/2,223 (1.2%) of patient samples. MET alterations were limited to amplifications (median 15X, range 6X-40X); no base pair substitutions, indels, or rearrangements were found. MET amp was present in 3/48 (6.2%) primary bone sarcomas, 1/20 (5.0%) myogenic sarcomas, 2/58 (3.4%) gastric, 1/38 (2.6%) kidney, 2/78 (2.5%) hepatocellular, 1/41 (2.4%) uterine, 4/188 (2.1%) unknown primary, 2/99 (2.0%) ovarian, 8/386 (2.0%) lung, 3/157 (1.9%) colorectal, and 1/109 (0.9%) pancreatic carcinomas. Conclusions: Use of NGS to characterize MET alterations in a 2,000+ patient population provides evidence for the role of NGS in identifying the future use of MET-targeted therapy in a variety of common and uncommon solid tumors. Clinical follow-up of the subset of cases treated with a MET inhibitor is ongoing.
Prognostic value of microRNAs (miRs) expression in stage I-IIIA lung adenocarcinoma (AC).

Marcin Tomasz Skrzypski, Krzysztof Goryca, Piotr Czapiewski, Ewa Jassem, Wojciech Biernat, Ryszard Pawlowski, Witold Rzyman, Jacek Jassem; Medical University of Gdańsk, Department of Oncology and Radiotherapy, Gdańsk, Poland; Department of Gastroenterology and Hepatology, Medical Center for Postgraduate Education at the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; Medical University of Gdańsk, Gdańsk, Poland; Department of Forensic Medicine, Medical University of Gdańsk, Gdańsk, Poland

Background: About 50% of NSCLC patients (pts) will develop distant metastases following pulmonary resection. Currently, apart from clinical stage at diagnosis, there are no reliable factors to select high-risk pts for adjuvant chemotherapy. We previously demonstrated prognostic value of 22 miRs in frozen samples of early squamous cell lung carcinoma, and the feasibility of their assessment in formalin fixed paraffin embedded (FFPE) samples (Skrzypski et. al. J Clin Oncol 2010;28;15s). In this study, we investigated the expression of 350 microRNAs in operable AC pts. Methods: FFPE tumor samples were obtained from 80 stage I-IIIA pts who underwent curative pulmonary resection, 48% of whom subsequently developed distant metastases. Median follow-up of pts who were disease recurrence-free was 5.8 years (range, 4.0-9.1 years). RNA was isolated from tumor tissue with RecoverAll kit (Ambion). Expression of 350 miRs was analyzed by qRT-PCR (Appliedbiosystems). Raw data were normalized vs. the geometric mean of U6, RNU48 and RNU44 expression. Relative miRs expression was correlated with distant metastases-free survival (MFS) and the mean expression was compared between the groups with and without relapse. Results: Expression of 41 miRs correlated with MFS in Cox regression analysis and 21 of these showed different level in pts with and without relapse in the t-Student test (both at p<0.05 level). The top prognostic miRs included miR-99a* (p=0.006), miR-1255B (p=0.005), miR-1233 (p=0.013) and miR-1303 (p=0.03); all previously shown to be prognostic in AC or NSCLC. We also found 14 new microRNAs (patent pending) potentially prognostic for relapse in AC. Conclusions: Expression of selected miRs may aid in prediction of distant relapse in AC pts undergoing pulmonary resection.
Clinical and analytical performance of non-small cell lung cancer biomarkers.

Steven M. Anderson, Li Cai, James H. Tepperberg, Hawazin Faruki; Laboratory Corporation of America, Research Triangle Park, NC

Background: A variety of biomarkers are currently used to help guide treatment decisions for patients with non-small cell lung cancer (NSCLC). These include mutation analysis for the EGFR and KRAS genes, along with gene rearrangement analysis for the ALK and ROS1 loci. In this study we have evaluated the clinical and analytical performance features of these assays in a series of formalin-fixed paraffin-embedded (FFPE) tissue samples. Methods: FFPE samples submitted for analysis of the EGFR, KRAS, ALK and ROS1 genes were evaluated using molecular and FISH assays. EGFR mutation analysis was performed using Sanger nucleic acid sequencing methods for exons 18-21. KRAS mutations were detected using allele specific PCR or pyrosequencing methods. Rearrangements involving the ALK gene were detected using break-apart FISH probes (Abbott Molecular). Genetic alterations involving the ROS1 gene were determined using FISH probes (Kreatech Diagnostics). Over 6,200 test results for these 4 markers are included in this analysis. Results: Mutations in the EGFR gene were detected in 10.1% of samples evaluated (n=3,872). A slightly higher percentage of samples from female patients (13%) had a detectable mutation compared to samples from males (7%) (chi-square p<0.0001). Deletions in exon 19 (51%) were the most common alterations detected, followed by point mutations in exon 21 (35%). KRAS mutations were detected in approximately 22% of specimens. ALK gene rearrangements were observed in 3.1% of samples (n=1,524). Specimens from individuals <50y of age were more likely to provide a positive result (11%) compared to samples from individuals >50y of age (2.5%) (chi-square p<0.0001). Gene amplification for the ALK gene was a common finding in the NSCLC samples evaluated. ROS1 alterations were detected in 2.8% of the specimens. In this cohort, no specimens were positive for both an EGFR mutation and an ALK gene rearrangement. Conclusions: Biomarker testing is well established in clinical practice for NSCLC, with results from the tests used to guide important therapy decisions. Assays for biomarkers such as EGFR, KRAS, ALK and ROS1 are robust, allowing for the analysis of multiple analytes in FFPE samples, even when the amount of tissue may be limiting.
Inflammatory phenotype of classical (CD14+ CD16-) monocytes in patients with advanced non-small cell lung cancer.

Fiona Margaret McCarthy, Cristina Ghirelli, Raphael Zollinger, Melissa Phillips, Rozita Roshani, Juliana Candido, Roisin Trehy, Michael Sheaff, Jeremy Steele, Thorsten Hagemann; Barts Cancer Institute, Queen Mary, University of London, London, United Kingdom; St Bartholomews Hospital, London, United Kingdom; St Barthomolomews Hospital, London, United Kingdom; St Bartholomew’s Hospital, London, United Kingdom

Background: Monocytes are intrinsic members of the innate immune system and play an important role in immunity and inflammation. Human monocytes are subdivided into three populations depending on cell surface CD14 and CD16 expression: Classical (CD14++ CD16−), intermediate (CD14+CD16−) and non-classical (CD14− CD16++). These populations have diverse functions and have been postulated to play both anti and pro-inflammatory roles in a variety of diseases including atherosclerosis, sarcoidosis and other rheumatological conditions. However, the prevalent monocyte populations in cancer have not as yet been identified. We aim to define the prevalent monocyte populations in non-small cell lung cancer as well as further characterising them using flow cytometry and Affymetrix technology.

Methods: Blood was obtained from 24 newly diagnosed patients with advanced non-small cell lung cancer and 12 age matched healthy donors. Monocyte subpopulations were sorted using flow cytometry. Gene expression profiling was performed using Affymetrix Human U133 Plus 2.0 array.

Results: The classical (CD14++ CD16−) monocyte population is significantly increased in non-small cell lung cancer patients when compared to healthy donors (p<0.01). The intermediate (CD14+CD16−) and non-classical (CD14− CD16++) populations are unchanged. Analysis of the gene expression profile of the classical monocyte subset identified 265 up-regulated and 261 down-regulated genes in cancer patients compared to healthy donors (p<0.05, fold change >2). These genes were assigned to biological processes with gene ontology annotation. Functional annotation reveals a strong association of regulated genes with the G.O term “inflammation” (p=0.009). Among these inflammatory genes are a cluster of chemokines including CXCL2 and CXCR4.

Conclusions: By identifying the prevalent monocyte subsets as well as characterising their function in cancer, there is the potential to target detrimental effects and promote their beneficial functions. As such, monocytes subsets may be used as a possible therapeutic target.
Cancer-testis antigens in triple-negative and locally advanced breast cancer.

Samuel John Harris, Natalie Heather Turner, Fiona J.M. Chionh, Marzena Walkiewicz, Anannya Chakrabarti, Siddhartha Deb, Jonathan S. Cebon, Shane White, Tom John; Joint Austin-Ludwig Oncology Unit, Austin Health, Melbourne, Australia; Austin Hospital, Melbourne, Australia; Austin Hospital, Melbourne, Australia; Ludwig Institute for Cancer Research/Austin Health, Melbourne, Australia; Monash Medical Centre, Melbourne, Australia; Austin Health, Melbourne, Australia; Ludwig Institute for Cancer Research, Melbourne, Australia

Background: Cancer-Testis Antigens (CTAs) are immunogenic molecules that have increased expression in triple negative breast cancers (TNBC), a phenotype that whilst associated with poorer survival, is chemosensitive. We investigated expression of the CTAs MAGE-A, MAGE-C1, and NY-ESO-1 in women with Locally Advanced Breast Cancers (LABC) and TNBC to determine the association between CTA expression, survival and response to chemotherapy.

Methods: We reviewed patient charts, treated for either TNBC or LABC between 1997 and 2011. Tissue samples were used for immunohistochemical (IHC) staining for MAGE-A, MAGE-C1 and NY-ESO-1 and compared using Fisher’s exact test. Positive expression was defined as any antigen staining above background. Clinicopathological features were correlated with IHC results and survival estimated using the Kaplan Meier method.

Results: A total of 106 cases were investigated (64 TNBC and 42 LABC). In the TNBC cohort the median age was 58 and TNM stages 1 to 3c. CTA expression occurred in 56, 51 and 43% for MAGE-A, MAGE-C1 and NY-ESO-1 respectively. CTA expression was not associated with overall survival (OS) or time to progression. In the LABC cohort the median age was 54 and consisted of stage IIIb tumors. All breast cancer subtypes were represented. CTA expression occurred in 26, 64 and 21% for MAGE-A, MAGE-C1 and NY-ESO-1 respectively. CTA expression was not associated with overall survival (OS) or time to progression. In the LABC cohort the median age was 54 and consisted of stage IIIb tumors. All breast cancer subtypes were represented. CTA expression occurred in 26, 64 and 21% for MAGE-A, MAGE-C1 and NY-ESO-1 respectively. There was no association between CTA expression and response to chemotherapy. In a univariate analysis MAGE-A expression in the LABC group was associated with poorer OS (median 25 vs 76 months, HR 3.347 95% CI 1.44 to 21.68, p=0.015). However, there were more TN patients and Grade 3 tumors in the MAGE-A positive group. Across the two groups, MAGE-A (51 vs 14% p=0.002) and NY-ESO-1 (37 vs 2% p=0.006) but not MAGE-C1 had significantly higher expression in ER -ve tumors. There was higher expression of MAGE-A (51 vs 25% p=0.025) and NY-ESO-1 (43 vs 10% p=0.002) in Grade 3 tumors. Conclusions: MAGE-A, MAGE-C1 and NY-ESO-1 are highly expressed in TNBC and high-grade subsets of early breast cancer. CTA expression in TNBC was not predictive of survival nor response to chemotherapy in LABC. However, MAGE-A expression was found to be associated with poorer overall survival in LABC.
Analysis of the prognostic impact of Treg-related genes in tumor and stroma in resectable NSCLC.

Marta Usó, Eloisa Jantus-Lewintre, Rafael Sirera, Sandra Gallach, Ana Blasco, Nieves Martínez, Cristina Hernando, Ricardo Guijarro, Carlos Camps; Fundación para la Investigación del Hospital General Universitario de Valencia, Valencia, Spain; Universidad Politécnica de Valencia, Valencia, Spain; Consorcio Hospital General Universitario de Valencia, Valencia, Spain; Universitat de Valencia, Valencia, Spain

**Background:** Immunosuppressive regulatory T lymphocytes (Tregs) have been proved to play a critical role in immune tolerance to tumor. In this study we have analyzed the expression of 11 genes related to Tregs in both tumor and stroma samples of resectable NSCLC patients. **Methods:** Primary tumor tissues of FFPE samples from 125 early-stage NSCLC patients were used in this retrospective study. The most representative areas of tumor cells and tumor stroma of each sample were carefully micro-dissected. RTqPCR using hydrolysis probes (TaqMan, Applied Biosystems) was performed to assess the expression of Treg markers such as: CD127, CD25, FOXP3, CTLA-4, IL-10, TGFB-1, LAG-3, GITR and TNF-a as well as CD4 and CD8. Relative gene expression was assessed using GAPDH and CDKN1B as endogenous controls and results were normalized against a human cDNA (Clontech) as a reference. All statistical analyses were considered significant at p<0.05. **Results:** In both tumor and stroma, we found over-expression of CD25 (5.40X and 7.95X, respectively) and down-expression of CD127 (0.28X and 0.37X, respectively). There was a tendency toward higher expression of FOXP3 (1.67X and 2.01X, respectively) and CTLA-4 (1.92X and 1.76X, respectively) as well. Paired Wilcoxon test showed significant gene expression differences between tumor and stroma in FOXP3 (p=0.006), CD25 (p<0.0001), CD4 (p<0.0001), CD8 (p=0.028), IL-10 (p<0.0001) and TGFB-1 (p<0.0001). Kruskal-Wallis test showed that FOXP3 expression was higher in adenocarcinoma than in SCC samples (2.56X vs 1.79X, p=0.002). Survival analyses revealed that patients with a “Treg profile” (↑ CD25 and ↓ CD127) had a reduced overall survival (OS) (median 29.90 vs 74.33 months, p=0.003). We also found that those patients with higher levels of the ratio FOXP3 stroma/tumor had worse time to progression (TTP) (median 32.50 vs NR, p=0.04). **Conclusions:** Treg markers in the tumor microenvironment seem to play an important prognostic role in early-stage NSCLC patients. Supported by grants PS09-01149 and RD06/0020/1024 from ISCIII.
A mouse model for cancer immunoediting with renal cell carcinoma to explore mechanisms of immune escape.

Elizabeth Bigger, Michael Quigley, Yiping Yang; Duke University, Durham, NC; Bristol-Myers Squibb, Redwood City, CA; Duke University Medical Center, Durham, NC

Background: Cancer immunosurveillance is the immune system’s ability to recognize and destroy newly arising malignant cells. Recent data suggests that the immune system functions also to apply a selective pressure, leading to growth of less immunogenic tumors, a process termed cancer immunoediting. However, direct experimental evidence to support this hypothesis has been lacking. Methods: We examined the interaction between tumor and tumor-specific CD8 T cells in vivo with a model tumor antigen, influenza hemagglutinin (HA), in a renal cell carcinoma cell line (Renca-HA) and studied the HA-specific CD8 T cell response to Renca-HA in vivo. Naïve HA-specific CD8 T cells (Thy1.1+) purified from HA-TCR transgenic mice that recognize a Kd-restricted HA epitope were transferred into congenic Thy1.2+recipients inoculated the day prior with Renca-HA or wild type Renca (Renca-WT). At subsequent time points, recipient lymphocytes and pulmonary tumors were harvested and analyzed. Results: Four days after transfer, vigorous proliferation of HA-specific CD8 T cells was detected in Renca-HA inoculated mice, but not Renca-WT inoculated mice, leading to activation and effector differentiation of HA-specific T cells. HA-specific CD8 T cell activation resulted in destruction of Renca-HA in vivo, causing a significant (p <0.001) reduction of tumor burden and prolongation of survival. However, a small fraction of Renca-HA tumor cells survived immune-mediated killing and evaded immune surveillance. Microarray technology identified upregulation of transforming growth factor β-3 (TGF-β3) expression in edited Renca-HA, but not in unedited Renca-HA, compared with Renca-WT, confirmed by real-time quantitative PCR. Functional abrogation of TGF-β3 signaling on tumor-specific CD8 T cells delayed Renca HA tumor growth in vivo. Conclusions: These data provide direct evidence for the cancer immunosurveillance and immunoediting processes, and suggest that tumor induction of TGFβ-3 during the immunoediting process suppresses tumor-specific CD8 T cell function in vivo, allowing tumor immune escape. Our results provide mechanistic insights as well as potential strategies to block cancer immune evasion.
Clinical significance of programmed death ligand-1 expression in non-small cell lung cancer (NSCLC).

Vamsidhar Velcheti, Kurt Schalper, Daniel Carvajal, Lieping Chen, Mario Sznol, Scott N. Gettinger, Roy S. Herbst, David Rimm; Yale University, New Haven, CT

Background: Programmed cell death ligand-1 (PDL1) is expressed on various human cancers and is a major mechanism for immune evasion. Preliminary evidence suggests that PDL1 expression on cancer cells predicts response to anti-PD-1 therapy. Here we report our findings using automated quantitative immunofluorescence (QIF) to determine the frequency and prognostic value of PDL1 in two independent NSCLC cohorts. Methods: Five antibodies against PDL1 were screened for sensitivity and specificity using PDL1 transfected cells, normal human placenta and tonsil. Only one monoclonal antibody (clone 5H1) met criteria for specificity. This antibody was used to assess two cohorts of NSCLC cases in TMAs. The cohorts represented 204 cases from Yale University and 340 cases from hospitals in Greece. PDL1 protein was measured using QIF analysis using AQUA method. In addition, in-situ PDL1 mRNA levels were measured in the Greek cohort using the RNAscope paired-primer assay with AQUA method. Results: PDL1 protein expression was detected in 25% and 36% of the studied cohorts, respectively. PDL1 positivity was significantly associated with the presence of tumor-infiltrating lymphocytes (TIL) in both series. Patients with PDL1 protein positivity had better outcome in both series (Log Rank: p=0.036 for Yale cohort, p=0.027 for Greek cohort). Multivariate analysis showed that PDL1 expression was associated with better outcome independent of histology, presence of TILs, and stage in the Greek cohort and marginally significant in the Yale cohort. These findings were further validated by the PDL1 in-situ mRNA measurement in the Greek cohort. PDL1 protein and mRNA levels showed a positive non-linear relationship (R²=0.14, P<0.001). Positive PDL1 mRNA was found in 53% of patients with NSCLC and was also associated with the presence of TILs and longer overall survival (31 vs. 43 months, p=0.017). Conclusions: Tumor PDL1 protein positivity is detected in 25-30% of NSCLCs and associates with increased TILs and better outcome. Elevated PDL1 mRNA occurs in a higher proportion of NSCLC cases and provides comparable prognostic information. Measurement of in situ PDL1 protein and mRNA could be of more clinical value than either method alone.
Using $^{124}$I-PU-H71 PET imaging to predict intratumoral concentration in patients on a phase I trial of PU-H71.

John F. Gerecitano, Shanu Modi, Devika Gajria, Tony Taldone, Mary Alpaugh, Erica Gomes DaGama, Mohammad Uddin, Gabriela Chiosis, Jason Stuart Lewis, Steven M. Larson, Naga Vara Kishore Pillarsetty, Komal L. Jhaveri, Brian Krichovsky, Mei Hsuan Chen, Payal Dixit, Mark Dunphy; Memorial Sloan-Kettering Cancer Center, New York, NY; Program in Molecular Pharmacology and Chemistry, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY; New York University Langone Medical Center, New York University Cancer Institute, New York, NY; Hospital for Special Surgery, New York, NY

**Background:** PU-H71 is a Heat Shock Protein 90 inhibitor that can be labeled with $^{124}$I without altering its biochemical properties. Intratumoral drug concentration can be calculated based on $^{124}$I-PU-H71 (**PU-H71**) region of interest analysis and dilution principle. A microdose pilot study has shown uptake of **PU-H71** in a variety of tumors. **PU-H71** PET is currently being used to estimate intratumoral concentrations in subjects on our phase I study. **Methods:** Patients with previously treated solid tumors or lymphoma are eligible for this phase I trial. PU-H71 is given twice-weekly for 2 weeks each 21 days at escalating dose levels. A mix of **PU-H71** and unlabeled PU-H71 is given during cycle 2 followed by serial PET imaging. Patients on the pilot study are administered a microdose of **PU-H71** alone, followed by serial PET scans. Intratumoral PUH-71 concentration is measured directly in optional pre- and post- treatment core needle tumor biopsies (CNB). **Results:** To date, 13 patients have received PU-H71 on the phase I trial. Of these, 10 have undergone **PU-H71** PET imaging. 4 imaged patients also volunteered for CNBs, with results reported in the table. Of the 10 patients who underwent **PU-H71** imaging in the phase 1 study, 5 also underwent prior **PU-H71** imaging in the microdose pilot. Intratumoral concentrations as calculated in the pilot and phase I studies were in close concordance. **Conclusions:** **PU-H71** can be used to visualize **PU-H71** uptake in a variety of solid tumors and lymphoma, and **PU-H71** PET scans can be used to estimate intratumoral concentrations of **PU-H71**. Direct intratumoral measurements of **PU-H71** correlate reasonably closely with concentrations calculated from **PU-H71** PET imaging. Further refinement of this imaging tool will allow quantitative assessment of **PU-H71** uptake in tumors during the ongoing phase I trial. Clinical trial information: NCT01393509.

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Target lesion selection as a source of variability of response classification by RECIST 1.1.

Christiane K. Kuhl, Alexandra Barabasch, Tim Dirrichs, Philipp Bruners, Lieven Kennes, Tim Bruemmendorf, Sebastian Keil; University of Aachen, RWTH, Aachen, Germany

Background: Response classification in RECIST is based on manual uni-dimensional quantification of changes of target lesion size. In RECIST 1.1, the maximum number of target lesions was reduced to 5, with a maximum of 2 per organ. We analyzed the importance of different factors (manual vs. automated size measurement, uni- vs. three-dimensional size assessment, and between-reader-variability of target lesion selection) on response categorization. Methods: 41 female patients (58.1±13.2 y) with metastatic breast cancer underwent contrast-enhanced thoraco-abdominal CT for initial staging and first follow-up after systemic chemotherapy. Data were independently and prospectively interpreted by three radiologists. In addition, response was evaluated by a CAD system that allowed automated uni- and three-dimensional assessment of target lesions. Results: Response classification differed between readers in 19/41 patients (46%). In 25/41 patients, readers chose the same target lesions. In 6 of these 25 patients (24%), readers disagreed with regards to response classification. In 16/41 patients, readers chose different target lesions. In 13 of these 16 patients, readers disagreed (81%) (p < 0.001). When dichotomizing response classification according to its therapeutic implication into progressive vs. non-progressive disease, readers disagreed in 11/41 patients (27%). In 9 of these 11 patients, readers had chosen different target lesions. Classification by manual vs. automated uni-dimensional measurement differed in 11/41 of patients (27%). Classification by uni-dimensional vs. volumetric measurements differed in 6/41 of patients (15%). Conclusions: Response classification by RECIST suffers from substantial between-reader variability. Major source of variability is not the manual or uni-dimensional measurement, but the variable choice of target lesions between readers.
The predictive role of integrated BRCA1 and HERC2 mRNA expression in advanced non-small cell lung cancer (NSCLC) patients (p) treated with platinum-based first-line chemotherapy.

Laura Bonanno, Carlota Costa, Jose Javier Sanchez, Margarita Majem, Ana Gimenez Capitan, Ignacio Rodriguez, Alain Vergnenegre, Bartomeu Massuti, Adolfo G. Favaretto, Massimo Rugge, Cinta Pallares, Miquel Taron, Rafael Rosell; Istituto Oncologico Veneto, Medical Oncology, Padova, Italy; Pangaea Biotech, Laboratory of Translational Oncology, Barcelona, Spain; Universidad Autónoma de Madrid, Madrid, Spain; Hospital de Sant Pau, Oncology Service, Barcelona, Spain; Department of Obstetrics, Gynecology and Reproductive Medicine, University Hospital Quiron Dexeus, Barcelona, Spain; Cluzeau Hospital, Limoges, France; Alicante University Hospital, Alicante, Spain; Istituto Oncologico Veneto, Padua, Italy; Second Unit of Pathology, Padova Teaching Hospital, Padova, Italy; Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain; Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Pangaea Biotech, Cancer Therapeutics Innovation Group, USP Institut Universitari Dexeus, Barcelona, Spain

Background: The use of cis- or carboplatin is the backbone of first-line treatment for advanced NSCLC p, and currently no molecular markers of platinum sensitivity are used in routine clinical practice. Preclinical data and retrospective analyses have shown that high BRCA1 expression is associated with resistance to platinum. HERC2 is an E3 ubiquitin ligase promoting DNA repair by interacting with the E3 ubiquitin ligase RNF8 to facilitate the assembly of the RNF8-UBC13 complex, leading to the recruitment of BRCA1 and other DNA repair components responsible for double-strand break repair. Methods: We retrospectively analyzed 71 tumor samples from advanced NSCLC p treated with cis- or carboplatin plus gemcitabine or pemetrexed in the first-line setting. BRCA1 and HERC2 mRNA levels were determined by real-time PCR and categorized using medians as cut-off points. Results: Overall survival (OS) for all 71 p was 10.7 months (m), and progression free survival (PFS) was 7.2 m. Expression of both genes was successfully analyzed in 53 p (74.6%). Neither gene as a single variable influenced outcome. However, the joint effect of BRCA1 and HERC2 was significant for predictive modeling. Among 30 p with low BRCA1 levels, OS was 15.3 m and PFS was 7.4 m in the 21 p with low HERC2 levels, compared to 7.4 m and 5.9 m, respectively, in the 9 p with high HERC2 levels (OS, P=0.008; PFS, P=0.01). The multivariate analyses identified the combination of low BRCA1 and low HERC2 expression as predictive of longer OS and PFS. In contrast, high levels of either gene were associated with an increased risk of death (HR=3.7, P=0.004) and of progression (HR=1.4, P=0.03). Conclusions: The integrated analysis of multiple DNA repair components can improve available predictive models in NSCLC. BRCA1 and HERC2 low expression levels can predict improved outcome to first-line platinum-based chemotherapy.

The clinical impact and prognostic value of 18F-fluorodeoxyglucose PET/CT in the initial staging non-small cell lung cancer at MD Anderson Cancer Center.

Satoshi Takeuchi, Benjapa Khiewvan, Stephen Swisher, Eric Rohren, Homer A. Macapinlac; The University of Texas MD Anderson Cancer Center, Houston, TX

Background: 18F-fluorodeoxyglucose Positron Emission Tomography/Computed Tomography (FDG-PET/CT) has an important role for Non-Small Cell lung cancer (NSCLC) management, especially in staging. Our objective was to assess stage migration, the clinical impact, and prognostic value of PET/CT in patients with NSCLC at MD Anderson Cancer Center (MDACC). Methods: We retrospectively reviewed the database from MDACC, and identified 729 NSCLC patients referred for staging between 2006 and 2011. Stage was classified using TNM classification. FDG-PET/CT and conventional imaging staging were compared with all-cause mortality and the survival rates of the respective clinical stage. The management impact of FDG-PET/CT was determined based on conventional imaging and PET/CT management plans. A change in stage was confirmed by histopathology and/or further imaging. Results: We identified 598 NSCLC patients with FDG-PET/CT and conventional imaging performed. FDG-PET/CT changed stage in 28.1% (16.4% upstaged, 11.7% downstaged). Based on FDG-PET/CT, treatment plans were modified in 38% of patients. Median progression free survival (PFS) and overall survival (OS) was significantly worse in patients with management impact of FDG-PET/CT than patients without impact (PFS, 24.9 vs 60.6 months, P < 0.001; OS, 66.7 vs 115.9 months, P < 0.001). Multivariate analysis showed that the impact of FDG-PET/CT on management was an independent prognostic factor for DFS (hazard ratio [HR] = 2.08; 95% CI, 1.63 to 2.65; P < 0.001) and OS (HR = 2.16; 95% CI, 1.56 to 2.99; P < 0.001). Stage migration from stage I (40/249 patients) showed worse outcome than those without change (PFS, 21.0 vs 60.0 months, P < 0.001; OS, 64.7 vs 115.9 months, P = 0.003). Conclusions: FDG-PET/CT has major role in NSCLC management. The added staging information provided by FDG-PET/CT as compared to conventional imaging resulted in a change in management in more than one third NSCLC patients. FDG-PET/CT is also a powerful tool for outcome prediction. Even in patients diagnosed as stage I by conventional method, FDG-PET/CT at initial diagnosis may have an impact on survival.
Molecular tumor profiling (MTP) of poorly differentiated neoplasms (PDN) of unknown primary site.

F Anthony Greco, David R. Spigel, John D. Hainsworth; Tennessee Oncology, PLLC/SCRI, Nashville, TN; Sarah Cannon Research Institute; Tennessee Oncology, Nashville, TN

Background: The inability to definitively determine the lineage of neoplasms is less common with modern immunohistochemistry (IHC) and genetic profiling. Nonetheless some PDN defy lineage classification by extensive standard pathologic evaluation. The advent of MTP may provide a new method of improving the diagnosis of these challenging cancers. Methods: A total of 30 of 751 (4%) patients (pts) seen from 2000 – 2012 with cancer of unknown primary (CUP) had PDN without a definitive lineage determined by IHC (median 18 IHC stains, range 9 – 51). From 2008 – 2012 the 30 biopsies had MTP (RT-PCR mRNA CancerTYPE ID, bioTheranostics, Inc.). Additional IHC, genetic sequencing, fluorescent in situ hybridization for specific chromosomal changes and repeat biopsies were performed when feasible to support the MTP diagnosis, and clinical features correlated. Results: MTP lineage diagnoses were made in 25 of 30 (83%), including 10 carcinomas (3 germ cell, 2 neuroendocrine, 5 others), 5 melanomas, 8 sarcomas (3 peritoneal mesothelioma, 1 PNET) and 2 hematopoietic neoplasms (1 lymphoma, 1 chloroma). Additional IHC, genetic testing [BRAF, i(12)p] or repeat biopsies confirmed the MTP diagnoses in 11 of 15 tumors, and the clinical features were consistent with the MTP diagnoses in the majority of patients. Conclusions: This MTP assay can frequently provide a diagnosis for CUP pts and PDN without a definitive lineage defined by extensive IHC. The earlier application of MTP will likely provide an expedited diagnosis, and for some neoplasms is the only test capable of defining lineage and a more specific diagnosis. Appropriate therapy, particularly for pts with germ cell tumors, melanoma, and lymphoma depends on a specific tissue of origin diagnosis.
Noninvasive measurement of prostate-specific membrane antigen (PSMA) expression with radiolabeled J591 imaging: A prognostic tool for metastatic castration-resistant prostate cancer (CRPC).

Scott T. Tagawa, Naveed Hassan Akhtar, Paul J. Christos, Joseph Osborne, Shankar Vallabhajosula, Shoaib Freedy, Stanley J. Goldsmith, David M. Nanus, Neil Harrison Bander; Weill Cornell Medical College, New York, NY; Memorial Sloan-Kettering Cancer Center, New York, NY

Background: PSMA is nearly universally expressed by PC, upregulated with increased grade and castration-resistance. Evidence points towards PSMA expression as a downstream cellular biomarker of androgen receptor (AR) activity and non-invasive measurement of PSMA expression has recently been demonstrated to be a novel biomarker of AR activity. Methods: Planar gamma camera images following radiolabeled J591 (\(^{111}\)In-J591 or \(^{177}\)Lu-J591) were semi-quantitatively scored using 2 methods by 2 independent radiologists blinded to outcome. A 5-point visual score (VS) of 0 - 4+ was assigned. Tissue Targeting Index (TTI), a novel metric designed to semi-quantitatively score images was calculated using the ratio of lesion count density (corrected for background) to whole body count density, with maximum (TTI\(_{\text{max}}\)) and mean (TTI\(_{\text{ave}}\)) scores recorded. Follow up tabulating subsequent therapies and overall survival (OS) was recorded and imaging scores were associated with OS using Cox regression analysis. Results: 130 men with metastatic CRPC underwent radiolabeled J591 imaging. 86.2% had bone metastases, 51.5% lymph node, 16.9% lung, 9.2% liver. 87.7% had accurate targeting of known sites of disease by planar imaging. CALGB (Halabi) nomogram scores were prognostic for the population. As continuous variables, TTI\(_{\text{max}}\) (p=0.013) and TTI\(_{\text{ave}}\) (p=0.002) were associated with worse survival. VS demonstrated a trend for worse survival (p=0.09). In multivariate analysis, TTI maintained independent prognostic value when controlling for Halabi score: TTI\(_{\text{max}}\) HR 1.05 [95% CI 1.01, 1.10; p=0.02], TTI\(_{\text{ave}}\) HR 1.09 [1.03, 1.16; p=0.004]. Conclusions: Level of PSMA expression measured by planar gamma-camera imaging following radiolabeled J591 is associated with OS in men with metastatic CRPC. High PSMA expression may indicate more aggressive tumor biology with increased AR pathway dysfunction. Improvements in quantitative molecular imaging techniques such as PSMA PET/CT with \(^{89}\)Zr-J591 may prove to be a valuable prognostic and predictive biomarker, particularly in the setting of AR- and PSMA-targeted therapy. Clinical trial information: NCT00195039, NCT00538668.
Background: In patients with solid tumors, the use of a waterfall plot displaying the best percentage change in sum of the longest diameters of target lesions per patient is a common way to depict anti-tumor activity for cytostatic agents (Booth 2008, Dhani 2009). This representation assumes that the best percentage change represents the maximum anti-tumor activity for each patient. Information about new lesions and/or changes in non-target lesions is not incorporated in the waterfall plot; yet these additional events were found to be significant prognostic factors for overall survival adjusting for change in the target lesions (Litiere 2012, Suzuki 2012).

Methods: We analyzed two phase III lung cancer trials of 1st and 2nd line combination chemotherapy ± ASA404 (ATTRACT-1, n=1299, ATTRACT-2, n=920). For patients whose best response in the target lesions was shrinkage, we calculated how often this best response was synchronously accompanied by non-target disease progression. Results: See Table. Conclusions: There can be substantial tumor shrinkage in target lesions synchronously with progressive disease outside the target lesions. Therefore, graphical displays of anti-tumor activity should consider incorporating new and non-target lesion information, as well as target lesion tumor burden. We propose an extended waterfall plot presenting a more complete assessment of anti-tumor activity by incorporating non-target lesion information. We illustrate its utility in an additional data set, the RECORD-1 phase III renal cell cancer trial. Clinical trial information: NCT00662597.

<table>
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<tr>
<th>Best shrinkage (%) in target lesion diameters</th>
<th>Fraction (%) of patients with non-target PD at time of best target lesion response.</th>
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<tr>
<td></td>
<td>ATTRACTION-1</td>
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<tr>
<td>≥30%</td>
<td>20/231* (9)</td>
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<td>&gt;0% (some shrinkage)</td>
<td>70/484 (14)</td>
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* 231 patients achieved at least a 30% shrinkage in the target lesion tumor burden. Of these 231, 20 patients (9%) had synchronous disease progression outside the target lesions.
Developing a molecular imaging agent for Met using onartuzumab (MetMAb).

Cara Elizabeth Wright, Elaine Jagoda, Fabiola Cecchi, Veerendra Bhadrasetty, Stephanie Histed, Mark Williams, Gabriela Kramer-Marek, Esther Mena, Lauren Rosenblum, Lixin Lang, Lawrence Szajek, Chang Paik, Peter L. Choyke, Jan Marik, Jeffrey Tinianow, Mark Merchant, Donald P. Bottaro; Urologic Oncology Branch, National Cancer Institute at the National Institutes of Health, Bethesda, MD; Molecular Imaging Program, National Cancer Institute, National Institutes of Health, Bethesda, MD; Molecular Imaging Program, CCR, National Cancer Institute, Bethesda, MD; Molecular Imaging Program, CCR, National Cancer Institute, Bethesda, MD; Molecular Imaging Program, National Cancer Institute, Bethesda, MD; Molecular Imaging Program, National Cancer Institute, Bethesda, MD; Laboratory of Molecular Imaging and Nanomedicine, NIBIB, NIH, Bethesda, MD; Radiation Oncology Branch, NCI, NIH, Bethesda, MD; Laboratory of Molecular Imaging and Nanomedicine, NIBIB, NIH, Bethesda, MD; NMD, Warren Grant Magnuson Clinical Center, National Cancer Institute, Bethesda, MD; Molecular Imaging Program, CCR, NCI, NIH, Bethesda, MD; Genentech, Inc., South San Francisco, CA; Genentech Inc., South San Francisco, CA; National Cancer Institute, Bethesda, MD

Background: Developing an imaging agent to assess Met expression would aid in diagnosis and monitoring tumor response to Met-targeted therapies. Onartuzumab (MetMAb), a Met selective humanized one-armed monoclonal antibody, has been studied in Phase I-II clinical trials in which it was generally well tolerated and has shown the most benefit in patients with MET positive tumors. Methods: Studies to assess Met-binding were executed using the human gastric carcinoma cell line MKN-45 which exhibits a high level of Met expression. Murine PET studies and biodistribution assays were performed using MKN-45 xenografts. Results: Plasma shed Met concentration is directly related to total tumor burden (p = 0.001). The absence of a positive correlation between shed Met and %ID in blood indicates that binding of tracer to shed Met present in plasma is unlikely. There are positive correlations between tumor mass, Met abundance, and phosphoMet content and uptake of $^{89}$Zr-df-onartuzumab in MKN-45 mouse xenografts. Lastly, tumor mass, Met, pMet and $^{89}$Zr-df-onartuzumab uptake were all significantly decreased by drug treatment. Conclusions: MKN-45 tumor uptake of $^{89}$Zr-df-onartuzumab correlated significantly with tumor mass and Met abundance. Blood tracer uptake did not positively correlate with the presence of plasma shed Met. The amounts of Met, pMet, as well as $^{89}$Zr-df-onartuzumab image intensity correlated significantly with tumor size (all Spearman p values < 0.001). Tumor volumes and Met content were significantly decreased in TKI treated versus control mice, which correlated with imaging results. $^{89}$Zr-df-onartuzumab has potential utility for imaging Met to identify patients for treatment with Met-targeted therapeutics and to identify the emergence of Met-driven acquired resistance to other molecularly targeted cancer therapies.
Recall rate in a semi-annual breast surveillance program for high-risk women.

Hiroyuki Abe, Rodrigo Santa Cruz Guindalini, Elias Obeid, Linda J. Patrick-Miller, Angela R. Bradbury, Marion S. Verp, Susan Hong, Kristen Wroblewski, Greg S. Karczmar, Gillian Newstead, Olufumilayo I. Olopade; The University of Chicago Medical Center, Chicago, IL; University of Pennsylvania, Philadelphia, PA; Department of Health Studies, University of Chicago, Chicago, IL; The University of Chicago, Chicago, IL

Background: High recall rate and unnecessary biopsies are a concern with screening mammogram (MMG) and magnetic resonance imaging (MRI) in young women at high risk for breast cancer. The aim of this study is to investigate the overall recall rate (ORR) and its consequences in a semi-annual MRI surveillance protocol. Methods: A multi-modality surveillance program for women at high-risk for breast cancer was initiated in 2004. Yearly MMG was supplemented with semi-annual MRI. After 5 years of this protocol, an additional 5 years of follow-up with yearly MMG and MRI were offered only to mutation carriers. Defining positive screening as BIRADS 0, 4 and 5, ORR were analyzed and biopsies findings characterized. Fisher’s exact test was performed to evaluate association between the effect of MMG density, menopausal status, mutation status, age (<50y or ≥ 50y), and previous breast cancer with recalls. Results: With a mean follow-up of 3.6 years, 226 patients (pts) underwent 1,467 MRI and 851 MMG. Among 56 abnormal MRI and 18 abnormal MMG, 62 pts (27%) were recalled for further tests (6 recalled at two screening episodes). The MRI and MMG ORR were 3.8% and 2.1%, respectively. Only six cases (8.8%) were abnormal for both tests. Overall, 50 additional breast ultrasound, 10 additional MMG, 8 additional MRI were performed. Of the 40 biopsies in 38 pts (2 pts were biopsied twice), 11 cancers were detected (ductal in situ: 3; invasive: 7 ductal and 1 lobular). Abnormal MRI findings led to 37 biopsies, of which 10 (27%) detected cancers. One high-grade ductal carcinoma in situ was exclusively detected by MMG. The first and second MRI screening rounds together showed 27/56 (66%) abnormal findings which led to 23/40 (58%) of the biopsies, and detected 5/11 (45%) of the tumors. No statistically significant predictors of recall were identified (p < 0.05). Conclusions: Semi-Annual MRI protocol did not show increased ORR when compared to annual MRI/MMG surveillance. Approximately two-thirds of all recalls were in the first two rounds of screening which is consistent with previous results. Ongoing work will evaluate clinical and MRI features associated with recall to improve practice. Clinical trial information: NCT00989638.
The CAP-IASLC-AMP molecular testing guideline for the selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors.

Marc Ladanyi, Phil T. Cagle, Mary Beth Beasley, Dhananjay Chitale, Sanja Dacic, Giuseppe Giaccone, Robert B. Jenkins, David J. Kwiatkowski, Juan-Sebastian Saldivar, Jeremy Squire, Erik Thunnissen, Neal Ian Lindeman; Memorial Sloan-Kettering Cancer Center, New York, NY; The Methodist Hospital, Houston, TX; Mount Sinai Medical Center, New York, NY; Department of Pathology, Henry Ford Health System, Detroit, MI; University of Pittsburgh, Pittsburgh, PA; National Cancer Institute, Bethesda, MD; Mayo Clinic, Rochester, MN; Dana-Farber Cancer Institute, Boston, MA; City of Hope National Medical Center, Duarte, CA; Department of Pathology and Molecular Medicine, Queen’s University, Kingston, ON, Canada; VU University Medical Center, Amsterdam, Netherlands; Brigham and Women’s Hospital/ Harvard Medical School, Boston, MA

Background: The College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) jointly initiated an effort to establish evidence-based recommendations for the molecular analysis of lung cancers required to guide EGFR- and ALK-directed therapies, addressing which patients and samples should be tested, and when and how testing should be performed. Methods: Three co-chairs without relevant conflicts of interest were selected, one from each of the sponsoring societies: CAP (P.T.C.), IASLC (M.L.), and AMP (N.I.L.). Writing and advisory panels were formed from additional experts from these societies. Unbiased literature searches were performed to capture articles up to February 2012, yielding 1,533 articles whose abstracts were screened to identify 521 pertinent articles that were then reviewed in detail for relevance. Evidence was formally graded for each of the recommendations first formulated by the co-chairs and panel members at a public meeting. Each guideline section was assigned to at least two panelists. Successive drafts were circulated for comments to the writing panel, the advisory panel, the public (online posting), and the three professional societies. Results: We generated 37 guideline items addressing 14 areas of EGFR and ALK testing. The major, evidence-based recommendations are to test for EGFR mutations and ALK fusions in all patients with advanced stage adenocarcinoma, regardless of sex, race, or smoking history, and to prioritize EGFR and ALK testing over other molecular predictive tests. Recommendations and expert consensus opinions were generated for all other key aspects of EGFR and ALK testing in lung cancer related to oncology and pathology practice and technical issues in molecular testing. Conclusions: As scientific discoveries and clinical practice outpace the completion of randomized clinical trials, evidence-based guidelines developed by expert practitioners are vital for communicating emerging clinical standards and thereby improving patient outcomes.
**BRAF** mutation testing with a novel, rapid, fully automated molecular diagnostics prototype platform.

Helen J. Huang, Benoit Devogelaere, Gerald Steven Falchook, Siqing Fu, Laura S. Angelo, David S. Hong, Sarina Anne Piha-Paul, Aung Naing, Veronica R. Holley, Apostolia Maria Tsimeridou, Vanda M. T. Stepanska, Kevin Kim, Vivek Subbiah, Jennifer J. Wheler, Ralph Zinner, Robert A. Wolff, Erwin Sablon, Geert Maertens, Razelle Kurzrock, Filip Janku; Department of Investigational Cancer Therapeutics (Phase I Program), University of Texas MD Anderson Cancer Center, Houston, TX; Biocartis NV, Mechelen, Belgium; Center for Human Immunobiology, Baylor College of Medicine, Houston, TX; Department of Investigational Cancer Therapeutics (Phase I Program), University of Texas MD Anderson Cancer Center, Houston, TX; Department of Melanoma Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX; University of California, San Diego, San Diego, CA

**Background:** Mutations in the **BRAF** gene provide actionable targets for cancer therapy in melanoma and other tumor types. Novel, fast, and accurate diagnostic systems are needed for further implementation of personalized therapy. **Methods:** The molecular diagnostics (MDx) prototype platform (Biocartis, Mechelen, Belgium) is a fully integrated real-time PCR-based system with high sensitivity (1% mutant in wild-type [wt] background) and fast turnaround time (< 90 minutes), which requires no sample preparation and <2 min hands-on time. Archival formalin-fixed paraffin-embedded tumor samples (1 to 5 shavings of 10 μm) from patients (pts) with advanced cancers previously tested for **V600 BRAF** mutations in a CLIA-certified Molecular Diagnostic Laboratory (PCR-based sequencing or Sequenom MassARRAY) were tested for **BRAF** V600 mutations using the MDx prototype platform. Concordance between methods and treatment outcomes with **BRAF/MEK** inhibitors were analyzed. **Results:** Forty-seven pts (melanoma, n=26; colorectal, n=8; papillary thyroid, n=3; other cancers, n=10) with available tissue and CLIA laboratory **BRAF** results were selected (**BRAF** V600 mutant, n=37; **BRAF** V600 wt, n=10). Of the 40 pts for whom the same tissue block was used for MDx and CLIA, **BRAF** status was concordant in 38 (95%; kappa 0.87; 95% CI 0.69-1.05) of them. **BRAF** status by MDx was discordant with CLIA in 3 of 47 cases (mutant by CLIA, but not MDx); one discrepant case contained a different mutation subtype (resp. V600E vs. V600K/R), and in another case different tissue blocks were used for MDx vs. CLIA testing. Of 34 pts with **BRAF** mutations detected by MDx, 28 were treated on protocols (on the basis of the CLIA results) with **BRAF/MEK** inhibitors and 8 (29%) had a partial (n=7) or complete response (n=1). Of interest, 1 pt with prostate cancer (V600E by CLIA, wt by MDx) received a **BRAF/MEK** inhibitor and did not respond. Detailed patient characteristics, mutation types and discrepancy analysis will be presented. **Conclusions:** The **BRAF** V600 mutation MDx prototype assay is a fast (turn-around time about 1.5 hours) and simple (<2 minutes hands-on time) test to determine **BRAF** mutation status with 95% concordance with CLIA laboratory if identical tissue blocks are used.
Tumor, skin, and plasma concentrations of protein kinase inhibitors (PKIs) in patients with advanced cancer.

Mariette Labots, Maarten Neerincx, Johannes C. Van der Mijn, Henk Dekker, Richard Honeywell, Maria Rovithi, Kristy Gotink, Margreet Voelbl-De Jong, Donald L. Van der Peet, Martijn R. Meijerink, Gerrit A. Meijer, Connie R. Jiménez, Godefridus J Peters, Henk M.W. Verheul; Department of Medical Oncology, VU University Medical Center, Amsterdam, Netherlands; Department of Surgery, VU University Medical Center, Amsterdam, Netherlands; Department of Radiology, VU University Medical Center, Amsterdam, Netherlands; Department of Pathology, VU University Medical Center, Amsterdam, Netherlands

Background: PKIs are selective for target receptors at low concentrations, but they act promiscuously at higher concentrations (PMID 18183025). This lack of selectivity may be relevant for their antitumor activity and for the development of PKI treatment selection tools. To obtain more insight in their clinical mechanism of action, we designed a pilot study to determine PKI tumor, skin and plasma concentrations in patients (pts) after 2 weeks of treatment. Results are related to cell line sensitivity data. Methods: Prior to standard palliative systemic treatment, pts were allocated to standard-dose PKI treatment (N=5 per PKI) for 10-14 days. Plasma, tumor and skin biopsies were collected within 24 hours of last dose. Sample PKI concentrations were determined by liquid chromatography – tandem mass spectrometry (LC-MS/MS); tissue concentrations in pg/mg were converted to molarity for comparison with preclinical sensitivity data (PMID 21980135). Concentrations of sunitinib (SUN), sorafenib (SOR), erlotinib (ERL), dasatinib (DAS) and everolimus (EVE) that inhibit 50% of cell proliferation (IC50) were determined by MTT assay in 1 RCC (786-O) and 8 CRC cell lines (HCT 116, HT-29, RKO, SW480, SW1398, DLD-1, COLO 205, CaCo-2). Results: Since August 2011 samples were obtained from 27 pts; 5 received SUN, 4 SOR, 4 ERL, 5 DAS and 5 EVE. After 12 ± 1 days of treatment, median tumor concentration (TC) was 9.0 μM (2.3-50.0) (range) for SUN, 8.5 μM (3.7-22.0) for SOR, 5.3 μM (0.9-10.8) for ERL and 2.1 μM (0.2-64.0) for DAS. EVE was measurable in 2 of 5 tumors: 3.5 μM (3.4-3.6). On average, PKI skin concentrations were 2.4-fold lower than TCs. SOR and ERL plasma concentrations (PCs) were in the range of TCs while SUN and DAS PCs were at least 14-fold lower than in tumors. Mean IC50 of the cell line panel was 1.3 μM (0.8-1.4) for SUN, 2.2 μM (1.4-3) for SOR, 8.2 μM (4-11.5) for ERL, 0.06 μM (0.02-1.8) for DAS and 1.2 μM (0.05-11) for EVE. Conclusions: PKI tumor concentrations may vary considerably from plasma concentrations, but are in the IC50 range of cancer cells in vitro. These results are indicative for the inhibitory concentrations of PKIs in patient tumors and should be considered for the development of individualized treatment strategies. Clinical trial information: NCT01636908.
Objective measurement of breast density, a marker of breast cancer risk, using fully automated radiation- and compression-free MRI.

Georg Wengert, Thomas Helbich, Stephan Polanec, Peter Christian Dubsky, Zsuzsanna Bago-Horvath, Rupert Bartsch, Wolf-Dieter Vogl, Michael Curda, Katja Pinker-Domenig; Medical University Vienna, Department of Radiology, Division of Molecular and Gender Imaging, Vienna, Austria; Department of Surgery, Medical University of Vienna, Vienna, Austria; Department of Pathology, Medical University of Vienna, Vienna, Austria; Department of Medicine 1, Clinical Division of Oncology and Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria; Medical University Vienna, CIR, Vienna, Austria; University of Economics and Business, Vienna, Austria

Background: Women may have contraindications to mammography (MG) or simply refuse the test due to subjective reservations usually concerning compression and/or radiation. Other than the detection of suspicious findings these women do not have standardized information concerning their breast density (BD), one of the strongest independent predictors of breast cancer (BC) risk. We developed a fully-automated quantitative magnetic resonance imaging (MRI) based BD measurement system, provided correlation to MG BD estimation and compared BD in BC patients and age-matched healthy controls. Methods: In this IRB-approved prospective study 35 healthy women and 19 BC patients age-matched to one of the healthy controls were included. BD for healthy women and BC patients was assessed using a) subjective radiologist’s review allocating 1 of 4 ACR BIRADS BD categories, b) Cumulus, a MG based semi-automatic method and c) a radiation- and compression-free MRI measurements system, which automatically calculates volume of breast (cm$^3$), % of fatty tissue and % of glandular tissue. Descriptive statistics were used to define the typical range of quantitative MRI BD readings corresponding to the qualitative four ACR BIRADS BD categories. Appropriate statistical tests were used to compare mean values of method b) and c) and BD readings of cancers and controls. Results: % MRI BD correlated well with % MG BD (r = 0.83; P < 0.001). MRI BD measurements ranged from 1.7% to 61.9% (mean 29.05%). MG BD measurements ranged from 5.42% to 74.33% (mean 29.05%). Mean BD (%) was higher in BC patients than in healthy controls: 24% (SD 16.9%) vs. 18.4% (16.6). Conclusions: MRI BD measurement strongly correlates with MG based BD readings. The data suggest that objective, radiation- and compression-free MRI BD measurement is a convenient alternative to MG for assessment of BD. MRI BD measurement confirms higher breast density in BC patients compared to healthy women. The data is entirely consistent with the fact that BD is a strong independent risk factor for BC.
Thymidylate synthase expression as predictive biomarker of pemetrexed sensitivity in advanced cancer patients.

Federico Rojo, Manuel Domine, Sandra Zazo, Cristina Chamizo, Gloria Serrano, Cristina Carames, Nuria Perez-Gonzalez, Tatiana Hernandez, Carmen Laura Auz, Nerea Carvajal, Irene Moreno, Jose Ignacio Martin-Valades, Yann Izarzugaza, Alberto Lendinez, Brezo Martinez-Amoros, Juan Luis Arranz, Juan Madoz, Ana Leon, Francisco Lobo, Jesus Garcia-Foncillas; Translational Oncology Division. Hospital Universitario Fundacion Jimenez Diaz, Madrid, Spain; Fundacion Jimenez Diaz, Madrid, Spain; Translational Oncology Division. Hospital Universitario Fundacion Jimenez Diaz, Madrid, Spain; Oncology Department and Translational Oncology Division. Hospital Universitario Fundacion Jimenez Diaz, Madrid, Spain; Translational Oncology Division Fundacion Jimenez Diaz, Madrid, Spain; Translational Oncology Division. Hospital Universitario Fundacion Jimenez Diaz, Madrid, Spain; Oncology Department. Hospital Universitario Infanta Elena, Madrid, Spain; Oncology Department. Fundación Jiménez Díaz, Universidad Autónoma de Madrid, Madrid, Spain; Oncology Department. Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain; Translational Oncology Division. Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain; Oncology Department. Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain; Oncology Department, Fundación Jiménez Díaz, Universidad Autónoma de Madrid, Madrid, Spain; Translational Oncology Division. Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain; Oncology Department. Hospital Universitario Fundacion Jimenez Diaz, Madrid, Spain; Translational Oncology Division. Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain; Oncology Department. Hospital Universitario Fundacion Jimenez Diaz, Madrid, Spain

Background: Although a high level of thymidylate synthase (TS) expression in malignant tumours has been suggested to be related to a reduced sensitivity to the antifolate drug pemetrexed, no direct evidence for such an association has been demonstrated in routine clinical samples from patients treated with this drug. The purpose of this study was to evaluate the impact of quantitative TS expression in tumor cells as predictor of the efficacy in patients with advanced non-small cell lung cancer, small cell lung cancer (SCLC) and mesothelioma treated with pemetrexed in our institution. Methods: 54 patients were included in this study: 40 stage IV NSCLC (26 adenocarcinomas, 11 large cell, and 3 squamous cell carcinoma), 3 SCLC and 11 mesothelioma. 21 patients received platins-pemetrexed as first line NSCLC, 20 pemetrexed in monotherapy as second and further lines and 3 carboplatin-pemetrexed for extensive disease SCLC. Total RNA was isolated by RNeasy FFPE procedure (Qiagen). The expression of TS was analyzed by RT-qPCR using appropriate mRNA specific primers and probes in LightCycler 480II platform at 45 cycles. TS levels was calibrated to expression in normal tissue. Results: From 54 cases, TS expression data were available in 32 cases, detecting overexpression in 23 (71.8%) and low expression in 9 (28.2%) patients. The response rate for patients with low TS expression was 0.63 compared with 0.15 in patients with overexpression (p=0.015). A significant benefit in time to progression was observed in patients with low expression (median TTP 12 vs. 2 months respectively, p= 0.002), whereas did not impact on overall survival (median OS 20 vs. 19 months respectively, p= 0.595). Conclusions: TS overexpression in tumor cells correlated with a reduced response to pemetrexed-containing chemotherapy and might be used as a predictive biomarker in advanced lung and mesothelioma cancer patients.
Molecular subtyping to predict better clinical and pathologic tumor response in operable early-stage breast cancer treated with docetaxel-capecitabine with or without trastuzumab.

**Stefan Gluck, Melanie Royce, Lisette Stork-Sloots, Femke De Snoo; Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL; University of New Mexico Cancer Center, Albuquerque, NM; Agendia, Amsterdam, Netherlands**

**Background:** Classification into molecular subtypes is important for the selection of therapy for patients with early breast cancer. Here we determine rates of pathological complete response (pCR) in early stage breast cancer to neoadjuvant capecitabine plus docetaxel, +/- trastuzumab, and investigate MammaPrint together with the molecular subtyping profile BluePrint as markers of pathological response in comparison to other biomarkers. **Methods:** This analysis was carried out on data from 122 patients enrolled in a multicenter study (XeNA) of neoadjuvant therapy for four 21-day cycles with capecitabine 825 mg/m$^2$ plus docetaxel 75 mg/m$^2$ if HER2-, and a standard trastuzumab dose if HER2+ (Glück, BCRT 2011). Clinical and pathological features, TP53 mutation analysis and PAM50 results were collected through GEO at NCBI (GSE22358). MammaPrint and BluePrint outcomes were determined from the available gene expression data and resulted in 4 distinct molecular groups: Luminal A (MammaPrint Low Risk/Luminal-type), Luminal B (MammaPrint High Risk/ Luminal-type), Basal-type and HER2-type. **Results:** In patients who completed 4 cycles of chemotherapy and surgery the overall pCR rate was 16%. Stratified by BluePrint pCR was observed in 1/15 (7%) of the Luminal A and 2/44 (5%) of Luminal B, in 10/22 (45%) of the HER2-type and in 7/41 (17%) of the Basal-type. The response rate among TP53 mutated patients was 6/61 (26%), which was significantly higher than TP53 wild-type patients (3/54 4%; p=0.012). Concordance of BluePrint/ MammaPrint with PAM50 molecular subtyping was 61%. **Conclusions:** Molecular Subtyping with BluePrint and MammaPrint can identify better outcomes of patients in the neo-adjuvant setting. Patients with Luminal A breast cancer have a good baseline prognosis with excellent survival and may not benefit from chemotherapy (Glück, SABCS 2013). MammaPrint and BluePrint provide predictive information for patients treated with treated with docetaxel-capecitabine +/- trastuzumab.
Tumoral MET/HGF expression and MET gene amplification in patients with ALK 2p23 fusion driven lung cancer.

Yan Feng, Eugen C. Minca, Wei Zhang, Lihong Yin, Nathan A. Pennell, Carol Farver, Angen Liu, Raymond R. Tubbs, Patrick C. Ma; Cleveland Clinic Taussig Cancer Institutie, Cleveland, OH; Cleveland Clinic Foundation, Cleveland, OH; Cleveland Clinic Taussig Cancer Institute, Cleveland, OH; Cleveland Clinic Pathology and Laboratory Medicine Institute, Cleveland, OH

Background: MET receptor and its ligand HGF are both promising targets in non-small cell lung cancer (NSCLC) therapy. Crizotinib, a recently approved ALK inhibitor for NSCLC harboring oncogenic ALK 2p23 fusion (ALK+), was initially developed as a bona fide MET inhibitor. The role of MET/HGF pathway in ALK+ NSCLC is still unknown. Methods: The study included 73 NSCLC patients tested for ALK rearrangements at Cleveland Clinic (2000-2012), including 21 ALK+ and 52 ALK-. Immunohistochemistry (IHC) on FFPE tumor tissue was performed for c-MET using a monoclonal CONFIRM antibody (SP44, Ventana) with Ventana Benchmark XT automated immunostainer and for HGF using a polyclonal antibody (R&D) following a manual protocol. IHC scoring was interpreted on a 4-tier system (0, 1+, 2+, 3+). MET gene amplification by MET/Chromosome 7 dual probe in-situ hybridization (DISH) (Ventana) was also performed. Statistical analysis was performed using Fisher exact test in JMP. Results: Of the tested tumors, 61 were adenocarcinoma (21 ALK+ and 40 ALK-), 6 squamous cell, 4 large cell and 2 NSCLC-NOS. None received any MET inhibitor prior to tissue collection. MET expression by IHC score 0-3 was: 35%, 33%, 15% and 17% in ALK-, and 5%, 37%, 42% and 16% in ALK+ tumor group, respectively. HGF IHC score 0-3 was 34%, 55%, 11% and 0% in ALK-, and 0%, 63%, 32% and 5% in ALK+ tumor group, respectively. The percentages of high MET or HGF expression (2+ or 3+) were higher in ALK+ group compared to ALK- (58% vs 32%, p=0.06, and 37% vs 11%, p=0.03). The correlation coefficient between MET and HGF expression was 0.48. MET gene amplification by DISH was detected in 15% (7/47) ALK- tumors but 0% (0/15) ALK+ tumors (difference not statistically significant, p=0.18). The correlation coefficient between MET IHC and MET gene amplification was 0.33. Conclusions: MET and HGF expression is commonly seen in NSCLC, with more frequent high expression levels in ALK+ than ALK- tumors. Using a newly developed DISH method, we show that MET gene amplification tend to be less frequent in ALK+ tumor. A prospective study with larger sample size is warranted to further define the role of MET/HGF as biomarkers in the biology of NSCLC with ALK rearrangements and their targeted therapy.
Application of next-generation sequencing (NGS) for evaluation of advanced epithelial cancers: A single institution experience.

Patrick McKay Boland, Alan P. Skarbnik, Massimo Cristofanilli, R. Katherine Alpaugh, Anthony J. Olszanski; Fox Chase Cancer Center, Philadelphia, PA

Background: The use of molecular targeted therapeutic agents may require the application of sophisticated diagnostic technologies for patients’ selection. Next generation DNA sequencing (NGS) has the ability to identify genetic alterations (GA) including mutations, copy number alterations, insertions/deletions, and rearrangements in tumor specimens. We sought to evaluate patients with advanced and refractory epithelial tumors to detect potentially actionable molecular abnormalities. Therapeutic intervention driven by GA findings was determined solely by the patient’s treating physician. Methods: Tumor samples from 77 patients ≥ 18 years old with any solid malignancy were sequenced. NGS of 186 genes was performed by FoundationOne through funding from Foundation Medicine using archival or newly acquired tumor tissue. Results: Seventy-four patients had specimens with adequate material for DNA extraction and analysis. Characteristics: 74% female, median age 55 years (19-82). Tumor sites included inflammatory breast (50%), colon (12%), unknown primary (5%) and other (33%). At least one genetic alteration was seen in 71 (96%) patients. The most common GA included mutations in 65 (60%) samples revealing TP53 (32%), KRAS (10%), PIK3CA (8%), and APC (6%) and amplifications in 38 (35%) samples which included MYC (18%), MCL1 (14%), CCND1 (12%), and ERBB2 (7%). Copy number loss (4%), fusion (1%) and deletions (2%) were also discovered. An actionable GA was seen in 46 of 74 patients successfully tested (62%), with 54% of GAs being amplifications and 43% mutations. Patients had a median of 3 GA (range 0-7). One patient with anal cancer had a concomitant PIK3CA mutation and amplification. NGS in association with immunohistochemistry helped identify site of origin for one patient with an occult primary. Conclusions: NGS identified GAs in the majority of patients with advanced epithelial cancers, including actionable abnormalities in a large fraction of this heterogeneous population. NGS shows promise in the diagnostic evaluation of advanced malignancies. Future studies should include the potential prognostic implications of genomic-driven personalized therapy.
Diffusion-weighted imaging using ADC mapping as an imaging biomarker for breast cancer invasiveness.

Hubert Bickel, Wolfgang Bogner, Peter Christian Dubsky, Rupert Bartsch, Margaretha Rudas, Thomas Helbich, Katja Pinker-Domenig; Medical University Vienna, Vienna, Austria; Medical University Vienna, Department of Radiology, Division of Molecular and Gender Imaging, Vienna, Austria; Department of Surgery, Medical University of Vienna, Vienna, Austria; Department of Medicine 1, Clinical Division of Oncology and Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria; Department of Pathology, Medical University of Vienna, Vienna, Austria

Background: Recently, functional imaging techniques such as diffusion weighted imaging (DWI) have been added to routine MR and have shown great potential for improving breast cancer diagnosis. DWI depicts cellular diffusivity on a molecular level and can be quantified using the apparent diffusion coefficient (ADC). In malignant tumors diffusivity is restricted, leading to lower ADC values than benign tumors. The aim of this study was to proof, that DWI can be used to differentiate benign from malignant tumors and to elucidate if ADC can serve as an imaging biomarker for breast cancer invasiveness. Methods: In this IRB-approved study 250 patients with 267 suspicious breast lesions (BI-RADS IV-V) were included. All patients underwent routine MR at 3T. A DWI-sequence was added to a standard imaging protocol, increasing measurement time by 2:30 min. The lesions were identified in routine MR and DWI sequences and ADC values of the lesions were calculated. Histopathology was used as the standard of reference for all lesions. Appropriate statistical tests were used to compare the ADC values of benign and malignant tumors (cut-off value $1.25 \times 10^{-3} \text{mm}^2/\text{s}$), of invasive and non-invasive disease and between different invasive tumor subtypes. Results: There were 91 benign (mean ADC $1.58 \times 10^{-3} \text{mm}^2/\text{s}$) and 176 malignant ($0.94 \times 10^{-3} \text{mm}^2/\text{s}$) lesions, sensitivity and specificity were 94.3% (PPV 95.4%, CI 0.91-0.98) and 91.2% (NPV 89.2%, CI 0.81-0.94). 155 lesions were invasive cancers (median ADC $0.90 \times 10^{-3} \text{mm}^2/\text{s}$), while 21 were non-invasive ductal carcinoma in situ ($1.22 \times 10^{-3} \text{mm}^2/\text{s}$). The invasive cancers were 130 invasive ductal (median ADC $0.91 \times 10^{-3} \text{mm}^2/\text{s}$) and 25 invasive lobular cancers ($0.83 \times 10^{-3} \text{mm}^2/\text{s}$). ADC was significantly different between benign and malignant lesions (p<.001) and between invasive and non-invasive cancers (p<.001), while no significant difference could be found between the invasive cancer subtypes (p=.163). Conclusions: Diffusion-weighted imaging reliably allows differentiation of benign and malignant breast tumors. The data suggest that ADC can be used as a non-invasive imaging biomarker for breast cancer invasiveness and may be of importance to treatment planning and outcome in breast cancer patients.
Utilizing a collaborative working model to optimize molecular analysis of solid tumors in the Cancer Research UK’s Stratified Medicine Programme.

Ian Walker, Fiona MacDonald, Helen Stuart, Rachel Butler, Rhianedd Ellwood-Thompson, Celia Brown, James Eden, David Gonzalez de Castro, Lisa Thompson, Sue Lillis, Debbie Mair, Vicky Cloke, Pauline Rehal, Jenny Bell, Matthew Smith, Brendan O’Sullivan, Philippe Taniere, Michael Griffiths, Peter W. M. Johnson, Stratified Medicine Programme Team; Cancer Research UK Institute for Cancer Studies, London, United Kingdom; Birmingham Women’s NHS Foundation Trust, Birmingham, United Kingdom; All Wales Genetics Laboratory, Cardiff, United Kingdom; Cardiff and Vale NHS Trust, Cardiff, United Kingdom; All Wales Medical Genetics Service, Cardiff, United Kingdom; The Institute of Cancer Research, The Royal Marsden NHS Foundation Trust, London, United Kingdom; The Royal Marsden NHS Foundation Trust, London, United Kingdom; Queen Elizabeth Hospital, Birmingham, United Kingdom; West Midlands Regional Genetics Laboratory, Birmingham, United Kingdom

Background: The Stratified Medicine Programme is demonstrating large scale molecular testing of solid tumours in the UK using a range of technologies. The collaborative model has allowed three laboratories to share and compare data on mutation frequencies including mutation exclusivity, test turnaround times and failure rates, to inform a future routine service for clinical care. Methods: Phase One is a two-year pilot study of molecular analysis of surplus diagnostic FFPE tumour tissue obtained from patients with cancer of the breast, colorectum, lung, ovary, prostate or malignant melanoma. Samples are tested for specified genes of clinical and research interest (for example KRAS, BRAF, NRAS, PIK3CA, TP53, PTEN, TMPRSS2-ERG, EGFR, EML4-ALK and KIT). The labs have developed and validated protocols with comparable sensitivity for the simultaneous molecular analysis of multiple genes. Results: By 31 December 2012, 4,734 sets of molecular results were completed with 60% of tumour-site specific reports issued within the target 15 days (from sample receipt). Failure rates vary with both sample quality and the type of analysis performed. The Table illustrates the link between turnaround times and failure rates, showing that repeat testing for specimens which initially fail may reduce overall failure rates but consequently increase average turnaround times. Conclusions: We will report comparative data across the three testing labs and identify multiple factors that affect mutation detection rates, failure rates, turnaround times and reporting procedures. The Stratified Medicine Programme acknowledges funding from Cancer Research UK, AstraZeneca, and Pfizer.

A comparison of turnaround times and whole test failure rates for clinically relevant genes.

<table>
<thead>
<tr>
<th>Molecular analysis</th>
<th>Lab 1</th>
<th>Lab 2</th>
<th>Lab 3</th>
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<td>EGFR</td>
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<td>Complete fail rate</td>
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<td>EML4-ALK</td>
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<td>Complete fail rate</td>
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<td>Mutation rate (colorectal)</td>
<td>35%</td>
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<td>38%</td>
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MRI apparent diffusion coefficient in a murine orthotopic glioblastoma model as a clinically translatable early readout of efficacy for AMG 595, an antibody drug conjugate targeting EGFRvIII.

Brittany Yerby, Juan Estrada, Matthew D. Silva, Kevin J. Hamblett, Suzanne K. Coberly, John S. Hill, Sharon E. Uritersma, Pedro J. Beltran; Amgen, Inc., Thousand Oaks, CA; Amgen, Inc., Seattle, WA; Amgen, Inc., South San Francisco, CA; Amgen, Inc, Thousand Oaks, CA

Background: Epidermal growth factor receptor variant III (EGFRvIII) is a constitutively active mutant of EGFR present in thirty to fifty percent of glioblastoma (GBM) patients. AMG 595, currently in Phase I trials, is composed of a fully human anti-EGFRvIII-specific antibody conjugated to the maytansinoid DM1 via a non-cleavable linker. The MRI apparent diffusion coefficient (MRI ADC) has been shown to correlate with tissue cellularity, and changes in MRI ADC can be an early indicator of therapeutic efficacy. The aim of this work is to evaluate MRI ADC as a clinically translatable early readout of tissue changes due to AMG 595 treatment in a murine orthotopic GBM model. Methods: D317 human GBM cells were intracranially injected into female CB17 SCID mice at Day 0. Mice were randomized at Day 7, using tumor volumes calculated by MRI, and were treated with vehicle, 6.5, 11, or 22 mg/kg AMG 595 i.v. twice per week, or temozolomide 10 mg/kg p.o. daily five days per week (N=8/group). MRI was repeated at days 14 and 21. Tumor volumes were manually traced on multi-slice $T_2$-weighted RARE images covering the entire tumor volume. The mean MRI apparent diffusion coefficients for each tumor in the vehicle and 22 mg/kg AMG 595-treated groups were calculated from diffusion-weighted spin echo images ($b=100-1200$ s/mm$^2$).

Results: A dose-dependent effect of AMG 595 on tumor volume was observed at Day 21; growth was inhibited in both the temozolomide and AMG 595-treated groups (22 and 11 mg/kg) relative to vehicle ($p<0.0001$). At Day 14, this significant treatment effect on tumor volume was not yet detectable. However, mean MRI ADC values were already significantly higher in the AMG 595 (22 mg/kg) treated group than in the vehicle group (23% higher at Day 14, $p<0.01$ vs vehicle; 32% higher at Day 21, $p<0.0001$ vs vehicle). The increase in MRI ADC in the AMG 595-treated group preceded observable tumor growth inhibition in the AMG 595-treated animals. Conclusions: Increases in tumor MRI ADC in response to AMG 595 treatment precede measurable inhibition of tumor growth, supporting the use of MRI ADC as a clinically relevant early biomarker for therapeutic efficacy.
Liquid biopsy-based assays to monitor residual disease in cancer.

Gangwu Mei, Dragan Sebisanovic, Alain Mir, Zulfqar Gulzar, James D. Brooks, Stefanie S. Jeffrey, AmirAli Talasaz; Guardant Health Inc., Redwood City, CA; Department of Medicine at Stanford University, Stanford, CA; Stanford University, Stanford, CA

Background: One of the main challenges in cancer management is monitoring the non-organ-confined disease post metastasis. Recently analysis of circulating tumor nucleic acids has generated a new paradigm for monitoring the progression and molecular pathology of the residual disease through a non-invasive test.

Methods: We have developed a new sequencing workflow, which increases the sensitivity and specificity of detecting and quantifying rare tumor-derived nucleic acids among healthy fragments by at least ten-fold. Unlike conventional sequencing library preparation protocols, the majority of extracted circulating DNA fragments are hybridized to sequencing flow cells with minimal modifications. The sequencing data are processed using our proprietary bioinformatics pipelines to search for rare genetic abnormalities within the heterogeneous circulating fragments.

Results: To study the performance of our technology, we first evaluated its sensitivity in analytical samples. We spiked varying amounts of LNCaP cancer cell line gDNA into a background of normal gDNA and were able to successfully detect somatic mutations down to 0.1% sensitivity. Subsequently, we investigated the correlation of circulating DNA and tumor gDNA in human xenograft models in mice. In seven different mice models of two different human tumors, we found very strong correlation between somatic mutations detected in all pairs of tumor gDNA and mouse blood cfDNA. After preclinical studies, we initiated a pilot study on human samples across different cancer types. We found more than 90% correlation between tumor mutations in stage IIIB CRC cancer patients and the mutations detected in their circulation (n=7). In a pilot study in prostate cancer patients (n=3), we detected chromosomal abnormalities in multi locations in circulating DNA genome in both advanced and post-relapse stages of the disease.

Conclusions: The present work indicates the potential of using circulating nucleic acids to facilitate the integration of real-time molecular pathology into routine cancer care. Our assay will be used to identify residual disease after neoadjuvant chemotherapy and/or surgery, but may also identify a subset of patients who may benefit from alternative therapies.
Association of tumor perivascular desmin expression with survival in patients with sunitinib-treated renal carcinoma.

Magnus Frodin Bolling, Per Sandström, Artur Mezheyevski, Lars Egevad, Arne Ostman; Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden; Department of Oncology-Pathology, Karolinska University Hospital and Karolinska Institutet, Stockholm, Sweden; 1)Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden; 2)Department of Pathology, Belarusian State Medical University, Minsk, Belarus; Stockholm, Sweden

Background: The introduction of tyrosine kinase inhibitors has markedly improved the outcome for patients with metastatic renal carcinoma (mRCC), but lack of reliable response-predictive markers makes it difficult to individualize treatment. Pericytes surround endothelial cells of capillaries and regulate vessel function. Associations between pericyte marker expression and response to sunitinib have not been well characterized.

Methods: A tissue micro array with samples from 67 mRCC patients (34 males, 33 females, age 42 – 84) treated at Dept. of Oncology, Karolinska University Hospital where constructed from primary tumors. All patients included had received at least one course with the tyrosine kinase receptor inhibitor sunitinib. Data of treatment, progression free survival and overall survival was collected in a clinical registry. Tissues were double-stained with the endothelial cell marker CD 34 and either of three pericyte markers desmin, PDGF beta receptor and alpha smooth muscle actin (ASMA). By semi-quantitative visual scoring, the perivascular staining of these markers was evaluated and cases dichotomized into negative/low- or positive/high-expressing groups.

Results: For patients with desmin positive/high vessels (n = 12), median PFS where 5.0 months (2.9 – 7.1, 95% CI) vs 7.0 months for patients with desmin negative/low vessels (n = 55, 3.8 – 10.2, 95% CI, Mantel Cox log rank test p<0.02). The median OS in the desmin-positive/high group was 24 months (7 – 41, 95% CI) vs 46 months in the negative/low group (34 – 58, 95 % CI, Mantel Cox log rank test p<0.014). No significant associations were seen between PFS or OS and the expression of perivascular PDGF beta receptor or ASMA. Conclusions: Metastatic RCC displays a clinically relevant variation in perivascular desmin expression which is associated with survival in sunitinib-treated patients. Future studies are warranted which further explores the prognostic and/or response-predicative capacity of this novel candidate renal cell cancer biomarker.
Comparison of $^{99m}$Tc tilmanocept and $^{99m}$Tc sulfur colloid for identification of sentinel lymph nodes in clinically node-negative breast cancer patients.

Jennifer L. Baker, Minya Pu, Christopher A. Tokin, Karen Messer, Carl Hoh, David R. Vera, Anne M. Wallace; UC San Diego Moores Cancer Center, La Jolla, CA; University of California, San Diego, La Jolla, CA

**Background:** Receptor-targeted (CD206) $^{99m}$Tc tilmanocept is a radiopharmaceutical specifically engineered for sentinel lymph node (SLN) identification that has recently completed phase III clinical trials. The agent has been compared to vital blue dye in prior studies, but has not yet been compared to radio-labeled sulfur colloid in two cohorts of clinically node-negative breast cancer patients (BCP) who underwent SLN mapping at a single institution. Outcomes were degree of SLN localization and % positive nodes among those removed. **Methods:** The $^{99m}$Tc tilmanocept cohort was composed of UCSD-specific patients pooled from two phase III clinical trials (Jun 2008-Jun 2009, Jul 2010-Apr 2011); the fTcSC cohort was composed of consecutive BCP undergoing SLN mapping at UCSD (Mar 2011-Feb 2012). Demographic, lymph node-specific, and cancer characteristics were compared between groups. A zero-inflated binomial model compared % positive nodes among nodes removed. **Results:** There were 85 vs. 120 patients in the $^{99m}$Tc tilmanocept and fTcSC cohorts, respectively. The groups did not differ in demographic or clinicopathologic factors predictive of axillary metastatic disease (age, race, cancer stage, histologic subtype and grade, hormone and HER2-Neu status or presence of lymphovascular invasion). The $^{99m}$Tc tilmanocept group had significantly fewer SLNs removed (mean 1.9 vs. 3.9, p<0.001), achieved higher gamma counts/node (28 vs. 1.6 kcps, p<0.001), and detected a significantly higher percent of tumor-positive SLNs (73% vs. 49%, p=0.016) while identifying the same rate of node-positive patients (24% vs. 18%, p=0.4). **Conclusions:** $^{99m}$Tc tilmanocept identified the same rate of node positive patients and removed fewer SLNs compared to fTcSC among BCP with similar risk of axillary metastatic disease. These data suggest that $^{99m}$Tc tilmanocept more precisely targets true SLNs and may minimize morbidity while maintaining or improving the accuracy of axillary staging in clinically node-negative breast cancer patients.
Performance of next-generation sequencing for detection of clinically actionable genetic variants in cancer.

Mohammed Omar Hussaini, Ian S. Hagemann, Teresa Mary Cox, Christina Lockwood, Karen Seibert, Shashikant Kulkarni, John Pfeifer, Eric James Duncavage; Washington University in St. Louis, St. Louis, MO

Background: Next-generation sequencing (NGS) allows for simultaneous detection of numerous actionable somatic variants in cancer. We have implemented a clinical NGS panel to detect genetic alterations in 25 genes with established roles in cancer and report here the frequency of clinically actionable genetic variants in a variety of cancer types. Methods: NGS testing was performed in a CAP-certified, CLIA-licensed environment on DNA extracted from FFPE tissue in 209 cases spanning 41 histologic tumor types. DNA was enriched by hybrid capture and sequenced to >1,000x average coverage on Illumina sequencers with 2x101bp or 2x150bp reads. Variants were called using clinically validated parameters using the Genome Analysis Toolkit, Pindel, and the custom-written Clinical Genomicist Workstation. Results: Non-small cell lung cancer (45%), pancreatic cancer (10%), and colorectal cancer (8%) were the most common tumors sent for NGS analysis. An average of 3 (range 1-16) non-synonymous, non-SNP sequence variants per case (SNVs and indels) were detected in the 130kb exonic target. Variants were most commonly seen in TP53, KRAS, and EGFR. 27% of cases (56/209) had one or more variants with therapeutic implications for the tumor type tested (e.g., EGFR mutation in NSCLC). 15% of cases (32/209) showed actionable variants not generally associated with the malignancy tested (e.g., detection of an activating KIT variant in thymic carcinoma). 10% of cases (21/209) had variants that were prognostically significant but not directly targetable. Some cases (9%) had variants that were prognostic/diagnostic and targetable. In 117 cases (56% of total), no therapeutically or prognostically significant variants were identified. Overall, in 92 cases (44%), NGS testing yielded information with therapeutic (majority), prognostic, or diagnostic ramifications. Conclusions: We found that 44% of unselected cancer cases have clinically relevant sequence variants in a set of 25 commonly mutated cancer genes. Our data suggest that clinical NGS testing may serve as an integral tool in realizing the potential of precision medicine in oncology.
Clinical next generation sequencing (NGS) of fine needle aspiration (FNA) biopsies in non-small cell lung (NSCLC) and pancreatic cancers.

Matthew J. Hawryluk, Jeffrey S. Ross, Christine E. Sheehan, Jie He, Geneva Young, Geoff Otto, Roman Yelensky, Doron Lipson, Gary A. Palmer, Phil Stephens, Vincent A. Miller; Foundation Medicine, Inc., Cambridge, MA; Albany Medical College, Albany, NY

Background: FNA is a common diagnostic procedure for the evaluation of pulmonary and pancreatic masses. We sought to determine whether NGS could be performed on these small specimens and to characterize heterogeneity across classes of genomic alterations (GA) in a subset of paired FNA and matched resected primary tumors. Methods: DNA was isolated from formalin fixed paraffin embedded (FFPE) sections of FNA cell blocks using 40μm total sections for NSCLC and 20μm total sections for pancreatic cancers in a CLIA-certified lab (Foundation Medicine). DNA sequencing was performed for 3,320 exons of 182 cancer-related genes and 37 introns of 14 genes frequently rearranged in cancer on indexed, adaptor ligated, hybridization-captured libraries to a median depth of 931x for the NSCLC and 416x for the pancreatic FNAs. Results: Genomic profiles were successfully generated from 19/19 of the NSCLC and 22/23 of the pancreatic FNA cases. We found 97 GA in the 19 NSCLC FNAs (range 2-9, average 5.1 GA per patient). 68% of the patients had GA in TP53 and 21% in KRAS. Only 16% (3/19) patients did not exhibit an actionable alteration. We found 99 GA in the 23 pancreatic cancer FNAs (range 0-12, average 4.3 per patient). 74% of the patients had GA in KRAS. There was 94% concordance of GA found in 4 matched FNA and primary tumor pairs for the pancreatic cases. For the single discordance, manual inspection of the reads of the discordant allele indicated evidence of loss of heterozygosity. Conclusions: NGS can be reliably performed on small FNA samples processed into cell blocks, and the GA discovered significantly correlates with the GA found in matched primary tumors. This study demonstrates the feasibility of NGS in analyzing FNA samples and further broadens the spectrum of commonly encountered specimen types to which this approach can be successfully applied.
The role of the glucocorticoid receptor (GR) in inhibiting chemotherapy-induced apoptosis in high-grade serous ovarian carcinoma (HGS-OvCa).

Erica Michelle Stringer, Maxwell N. Skor, Gini F. Fleming, Suzanne D. Conzen; University of Chicago Medical Center, Chicago, IL; University of Chicago, Chicago, IL; Alliance for Clinical Trials in Oncology, Chicago, IL; The University of Chicago, Chicago, IL

Background: Ovarian cancer is the leading cause of death from gynecologic malignancies. High-grade serous ovarian cancer (HGS-OvCa) is often initially sensitive to platinum-based therapy, but relapse rates remain high. The TCGA recently found that HGS-OvCas have a gene expression and mutational profile similar to that of triple negative breast cancer (TNBC). Previously, our group demonstrated that dexamethasone treatment decreased chemotherapy-induced tumor cell apoptosis in TNBC and HGS-OvCa cell lines. We have also shown that glucocorticoid receptor (GR) activation induces expression of anti-apoptotic genes SGK1 and MKP1/DUSP1 in both HGS-OvCa and TNBC cell lines and in primary human ovarian and TNBC tumors. 

Methods: We examined glucocorticoid receptor (GR), estrogen receptor (ER), and progesterone receptor (PR) expression in a panel of HGS-OvCa cell lines by Western analysis and qRT-PCR. We also performed apoptosis assays with and without mifepristone, glucocorticoid and/or chemotherapy treatment using IncuCyte live-cell imaging technology in order to measure the effect of GR modulation of chemotherapy sensitivity. 

Results: HGS-OvCa cell lines (including CAOV3, HeyA8, SKOV3, Monty-1) all had detectable GR expression; HeyA8, SKOV3, and Monty-1 cell lines expressed very low levels of ER-alpha while all other HGS-OvCa cell lines did not express any detectable ER-alpha. Furthermore, none of the HGS-OvCa cell lines tested expressed PR. Apoptosis assays revealed that GR activation significantly inhibited gemcitabine/carboplatin-induced apoptosis in HGS-OvCa cell lines and that mifepristone could reverse this cell survival effect, presumably through GR antagonism. 

Conclusions: These results suggest that treatment with mifepristone, a GR antagonist, reverses GR-mediated cell survival signaling in HGS-OvCa and increases chemotherapy-induced tumor cell death. To further investigate the role of GR activity in HGS-OvCa, we are currently performing xenograft experiments with chemotherapy +/- mifepristone treatment.
A community-based program for personalized cancer care using next-generation sequencing (NGS).

Shile Liang, Pranil Chandra, Zeqiang Ma, Debbie Haynes, James Prescott, Lisa H. Morrissey, Suzanne Fields Jones, David R. Spigel, Michael R. Savona, John D. Hainsworth, Jeffrey R. Infante, Howard A. Burris; Sarah Cannon Research Institute, Nashville, TN; PathGroup, Nashville, TN; Sarah Cannon Research Institute; Tennessee Oncology, Nashville, TN

Background: Despite growing interest and need, molecular profiling of tumor samples is largely unavailable in community cancer centers, where nearly 80% of cancer patients (pts) are treated. In 10/12, Sarah Cannon Research Institute (SCRI) launched a community-based molecular profiling program to: 1) better understand the molecular constituency of cancer patients, 2) identify appropriate pts for phase I and II clinical trials of targeted agents, and 3) identify pts with molecular abnormalities responsive to FDA-approved agents. Methods: Eligible pts consented to testing of available biospecimens, which were interrogated for alterations in 35 cancer-related genes using NGS (1000X average coverage) in a CLIA/CAP laboratory. Results were reported to the treating physician within 14 days and stored in a database to enable correlation with clinical outcomes. Results: As of 1/13, 209 pts had been enrolled with 84% having sufficient material for assay. At least 1 mutation was detected in 46% of tumors. Results in the 3 most commonly assayed tumor types are summarized (Table). Mutations for which there are FDA-approved targeted agents were found in 14 off-label tumors (EGFR 4, KIT 3, SMO 3, BRAF 2, HER2 2). 40 pts (27%) were subsequently enrolled in clinical trials; in 19 of these, assay results influenced clinical trial selection.

Conclusions: This program provides molecular profiling data to community oncologists for clinical decision making. Experience to date indicates this information can be provided in a timely manner for incorporation into clinical practice. Profiling results will enable: 1) selection of pts with appropriate tumor targets for investigational targeted agents, 2) enhanced study enrollment, 3) evaluation of FDA approved targeted agents in off-label tumor types, and 4) correlation of treatment outcomes with patterns of tumor molecular abnormalities.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th># of patients</th>
<th>Mutation frequency</th>
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<tbody>
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<td>Lung</td>
<td>32</td>
<td>50%</td>
<td>KRAS(22%), EGFR(19%), STK11(6%), FGFR3(3%), GNA11(3%), KIT(3%), MAP2K1(3%), SMO(3%), WT1(3%)</td>
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<td>Colorectal</td>
<td>25</td>
<td>72%</td>
<td>KRAS(56%), PIK3CA(24%), ABL1 (4%), BRAF (4%), ERBB2(4%), GNAS(4%), IDH1(4%), IDH2(4%), NRAS(4%), PIK3R1(4%), RUNX1(4%), SMO(4%)</td>
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<td>Breast</td>
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</tbody>
</table>

Background: Several MET inhibitors are currently being developed that block aberrant HGF/MET signaling in different cancers. We utilized validated MET pharmacodynamic (PD) assays to compare time course, magnitude, and reversal of MET suppression by 5 MET inhibitors in preclinical models. Methods: Immunoassays (total MET, pY1234/35MET, and pY1356MET) were developed and validated to measure modulation of MET by 5 MET inhibitors (crizotinib, tivantinib, cabozantinib, foretinib, and EMD1214063). The comparison was implemented in 3 sequential stages: 1) establish time course and magnitude of MET inhibition after single drug administration of 4 different doses; 2) determine dose(s) and schedule for sustained MET inhibition and downstream signaling at optimal levels; and 3) compare efficacy of MET inhibitors at MTD and equal MET inhibition. The preclinical models include an autophosphorylation gastric tumor (SNU5) model and a paracrine MET activation model in hHGF knock-in mice. Plasma and tumor exposures were measured using LC-MS/MS to correlate with PD effects. Results: We completed phase one in the SNU5 model and determined inhibition of pY1234/35MET and total MET in tumor tissues after single administration of MET inhibitors. Time course and magnitude of pY1234/35MET inhibition varied considerably among MET inhibitors, with the most rapid (>80% suppression in 30 min) and sustained inhibition (up to 48 h) observed with EMD1214063 at a dose of 30 mg/kg. The maximal inhibition of pY1234/35MET and time taken for biomarker recovery were wide-ranging among MET inhibitors. Tumor drug exposures were concomitantly higher than plasma for all drugs and correlated inversely with pY1234/35MET, except for tivantinib which, unlike other drugs, is not ATP competitive inhibitor. Conclusions: We applied validated PD assays to directly compare similarities and differences in extent and duration of MET inhibition by 5 MET inhibitors. Our results provide important foundation for head-to-head comparison of efficacies of MET inhibitors at MTD and equal MET inhibition. Funded by NCI Contract No HHSN261200800001E.
Characterization of a lung cancer growth factor, LASEP1, as a serologic and prognostic biomarker and a therapeutic target.

Atsushi Takano, Yusuke Nakamura, Yataro Daigo; Department of Medical Oncology, Shiga University of Medical Science, Otsu, Japan; Human Genome Center, Institute of Medical Sciences, University of Tokyo, Tokyo, Japan

Background: Identification and evaluation of oncoproteins are an effective approaches to develop novel diagnostic/prognostic biomarkers or therapeutic targets. Methods: We established a strategy as follows. i) To identify up-regulated genes in non-small cell lung cancers (NSCLCs) using the cDNA microarray, ii) To verify the candidate genes for their no or low expression in 23 normal tissues by northern-blot, iii) To validate clinicopathological significance of their protein expression by tissue microarray, iv) To verify whether they are essential for the growth of cancer cells by siRNA, and v) To measure their serum protein levels by ELISA. Results: We identified LASEP1 (Lung cancer Associated Serum Protein 1) as a candidate target molecule. Immunohistochemical staining using tumor tissue microarrays consisting of 374 NSCLC confirmed positive staining of LASEP1 was observed in 210 (56.1%) of 374 NSCLC. In addition, a high level of LASEP1 expression was associated with poor prognosis of NSCLC patients. Serum LASEP1 levels were higher in NSCLC than in healthy volunteers. The proportion of serum LASEP1-positive cases was 127 (38.6%) of 329 lung cancers, while 4 (3.9%) of 102 healthy volunteers were falsely diagnosed. Furthermore, treatment of lung cancer cells with siRNAs against LASEP1 suppressed its expression and resulted in growth suppression of the lung cancer cells; on the other hand, induction of exogenous expression of LASEP1 conferred growth-promoting activity in vitro. We found its 50-kDa receptor (LASEPR) which interacts with LASEP1 on lung cancer cell surface. Suppression of LASEPR expression by siRNAs inhibited the growth of cancer cells. The LASEP1-LASEPR interaction promoted the cell growth in an autocrine manner. In addition, the growth activity of the LASEP1-positive cells was neutralized by the addition of originally developed anti-LASEP1 monoclonal antibodies into their culture media. The systemic administration of the anti-LASEP1 antibody to tumor-implanted mice significantly suppressed tumor growth without any adverse events. Conclusions: We have identified LASEP1 as potential targets for development of biomarkers and therapeutic target for lung cancer.
Cross-species synthetic lethal interaction screening as a strategy for the identification of novel therapeutic targets in cancer.

John P. Shen, Rohith Srivas, Ana Bojorquez-Gomez, Katherine Licon, Jian Feng Li, Robert W. Sobol, Trey Ideker; University of California, San Diego, La Jolla, CA; University of Pittsburgh Cancer Institute, Pittsburgh, PA

**Background:** Mutation, deletion, or epigenetic silencing of tumor suppressor genes is a near universal feature of malignant cells. However, therapeutic strategies for restoring the function of mutated or deleted genes have proven difficult. Synthetic lethality, an event in which the simultaneous perturbation of two genes results in cellular death, has been proposed as a method to selectively target cancer cells. Identifying and pharmacologically inhibiting proteins encoded by genes that are synthetic lethal with known tumor suppressor mutations should result in selective toxicity to tumor cells. **Methods:** To identify candidate target proteins we measured all pair-wise genetic interactions between all known orthologs of human tumor suppressor genes (162 genes) and all orthologs of druggable human proteins (~400 genes) in the model organism *S. Cerevisiae*. Analysis of the data uncovered 2,087 distinct synthetic lethal interactions between a tumor suppressor and druggable gene. A computational algorithm was then developed to identify those interactions which were likely to be conserved in humans based on conservation of the synthetic lethal relationship in the distant fission yeast *S. pombe*. **Results:** Our bioinformatic analysis suggested a high probability of conservation of the synthetic lethal interactions between the yeast *RAD51* (ortholog of *BRCA1*) and *RAD57* (ortholog of *XRCC3*) with HDA1 (a histone deacetylase; HDAC). We confirmed this by treating LN428 cells with stable lentiviral knockdown of BRCA1 or XRCC3 with the HDAC inhibitors vorinostat (SAHA) and entinostat (MS-275). Both the BRCA1 and XRCC3 knockdown cell lines were significantly more sensitive to HDAC inhibition relative to wild-type (non-silencing lentiviral control) cell line (Table). **Conclusions:** These results demonstrate that high-throughput approaches for screening synthetic lethal interactions in model organisms such as *S. cerevisiae* and *S. pombe* can serve as a valuable resource in helping to identify novel therapeutic targets in human cancer.

<table>
<thead>
<tr>
<th></th>
<th>IC50 (μM)</th>
<th>(95% CI)</th>
</tr>
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<tr>
<td></td>
<td>SAHA</td>
<td>MS-275</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.57 (.52 - .62)</td>
<td>0.90 (.78 - 1.0)</td>
</tr>
<tr>
<td>BRCA1-kd</td>
<td>0.39 (.35 - .45)</td>
<td>0.48 (.41 - .56)</td>
</tr>
<tr>
<td>XRCC3-kd</td>
<td>0.34 (.32 - .37)</td>
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Hyperactivated mTOR and JAK2/STAT3 pathways: Crucial molecular drivers and potential therapeutic targets of inflammatory breast cancer (IBC).

Komal L. Jhaveri, Eleonora Teplinsky, Rezina Arju, Shah Giashuddin, Yasmeen Sarfraz, Melissa Alexander, Farbod Darvishian, Deborah Silvera, Paul H Levine, Salman Hashmi, Heather J. Hoffman, Baljit Singh, Judith D Goldberg, Tsivia Hochman, Amanda Valeta, Robert Schneider; New York University School of Medicine, New York, NY; The Brooklyn Hospital Center, Brooklyn, NY; New York University Langone Medical Center, New York, NY; Department of Pathology, New York University School of Medicine, New York, NY; Department of Epidemiology and Biostatistics, George Washington University, Washington, DC; The George Washington University, School of Public Health and Health Services, Washington, DC; George Washington University, Washington, DC; New York University, New York, NY

Background: IBC is an aggressive form of breast cancer with poor prognosis. Combined multimodality Rx results in 5 year median OS of 30-50%, underscoring the unmet need for novel targeted strategies. Our preclinical research in cell lines and xenografts suggests a role for activated PI3K/AKT/mTOR pathway in IBC. IBC cells not only express high levels of IL-6 and IL-8 but can recruit tumor activated macrophages (TAMs), which can further induce IL-6, IL-8 and activate JAK2/STAT3 pathway. We therefore investigated independent and combined activity of these pathways.

Methods: Archived tissue specimens of 42 IBC pts (1999 - 2009) and 13 controls (normal breast) were analyzed using IHC and scored by 3 independent pathologists. Results were defined as: 0, 1+ neg; 2+ pos for activated mTOR (phosphorylatedS6) and 0 = neg; 1+, 2+ = pos for activated nuclear JAK2/STAT3 (pJAK2; pSTAT3), cytokine (IL-6), macrophage infiltration (CD68) and TAMs (CD163). Proportion of IBC cases with pos expression were compared to proportion among controls (Fishers exact test). Clinical and survival data were obtained.

Results: Median age at diagnosis - 44.5 yrs (29-64). 22 had HER2 overexpression (8 also ER+ ) and 9 were ER-/HER2--; ER & HER2 unknown for 1 and 2 pts respectively. Majority were Rxed with neoadjuvant anthracycline and/taxane without adjuvant trastuzumab. There were 24 deaths. Median OS: 67 mths (95% CI: lower 41). Proportions of IBC cases with pos expression when compared to controls are listed in the table (Fishers p value: <0.0001). Of the 31 pts with complete biomarker data who were PS6 pos, 97% had activated JAK2 & 58% had activated STAT3 (McNemar’s chi square, p <0.001). 24/31 (80%) showed strong infiltration of macrophages and TAMs. All cases had widespread IL6 staining. Conclusions: This study validates our preclinical findings and shows hyperactivation of mTOR and JAK2 signaling in vast majority of IBC specimens, with close association between mTOR, TAMs, cytokines and JAK2/STAT3 pathways. These findings support a role for dual blockade of mTOR and JAK/STAT pathways in clinical trials.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>N = 42</th>
<th>Pos N (%)</th>
<th>Neg N (%)</th>
<th>No tumor</th>
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<tbody>
<tr>
<td>PS6</td>
<td>35 (90)</td>
<td>4 (10)</td>
<td>3</td>
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</tr>
<tr>
<td>pJAK2</td>
<td>37 (97)</td>
<td>1 (3)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>pSTAT3</td>
<td>22 (59)</td>
<td>15 (41)</td>
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<td>CD68</td>
<td>32 (86)</td>
<td>5 (14)</td>
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<tr>
<td>CD163</td>
<td>36 (90)</td>
<td>4 (10)</td>
<td>2</td>
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</tbody>
</table>

Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human AML.

Kapil N. Bhatta, Warren Fiskus, Karissa Peth; University of Kansas Medical Center, Kansas City, KS; Kansas University Medical Center, Kansas City, KS

**Background:** The bromodomain-containing protein 4 (BRD4) is a member of the BET (bromodomain and extraterminal) protein family, which regulate the assembly of chromatin complexes and transcriptional regulators at the target gene promoters, e.g., of Myc and BCL2. JQ1 competitively binds and inhibits the bromodomains of the BET proteins, including BRD4. Treatment with JQ1 attenuates Myc and BCL2 levels and promotes growth arrest, differentiation and apoptosis of AML cells. **Methods:** Here, we determined the growth inhibitory, differentiation and apoptotic effects of JQ1 (100 to 500 nM) alone and in combination with low concentrations of the deacetylase inhibitor panobinostat (PS) (5 to 20 nM) (gift from Novartis) in cultured (OCI-AML3, MV4-11 and MOLM13) and primary AML blast progenitor cells. We also determined the in vivo anti-AML activity of JQ1 (50 mg/kg/day IP) and/or PS (5 mg/kg/MWF IP) for 3 weeks against the OCI-AML3 xenograft in the NOD/SCID mice. **Results:** Treatment with JQ1 decreased the binding of BRD4 at the chromatin associated with Myc and BCL2 promoters (by ChIP-qPCR) and attenuated the mRNA and protein levels of Myc, BCL2 and cyclin D1, while increasing the levels of p21, CEBPa, BIM and cleaved PARP in the cultured and primary AML blasts. This was associated with an increase in the % of G1 phase cells, attenuation of the colony growth, and induction of apoptosis of the cultured (OCI-AML3 ->MV4-11 ->MOLM13) and primary AML cells. JQ1 treatment also dose-dependently inhibited the viability of CD34+ primary AML progenitor cells (n=10). Gene expression microarray analysis revealed a JQ1 treatment-associated signature of the most up- and down-regulated mRNAs that correlated with its anti-AML effects. Co-treatment with JQ1 and PS synergistically induced apoptosis of AML cells (by isobologram analysis; CI < 1.0). As compared to the mice treated with vehicle alone, or with each agent alone, co-treatment with JQ1 and PS, without inducing toxicity, significantly improved the survival of the OCI-AML3 engrafted NOD/SCID mice (p < 0.01). **Conclusions:** These pre-clinical findings warrant further in vivo evaluation of the efficacy of the combination of a BRD4 antagonist and PS, and its predictive biomarkers, against human AML.
NGS-based targeted RNA sequencing for expression profiling and relative quantitation of specific gene isoforms and fusions in tumor-specific panels.

Gordon Vansant, Mark Landers, Lien Vo, Kahuku Oades, Hyunsuu Kim, Jerry Lee, Rhonda Meredith, Byung-In Lee, Joseph Monforte; Althea Technologies, San Diego, CA; AltheaDx, San Diego, CA

**Background:** Gene expression signatures have become a useful tool for the identification of tumor subtypes and response to specific therapies. Expression of tumor, metastatic and macrophage specific transcripts utilizing alternative promoters and transcriptional start sites can further characterize these tumors. NGS is a powerful tool for gene expression analysis, however larger sample input requirements (>100ng) and excessive sequencing depth requirements (30-40M tags/sample) to detect the expression of rare isoforms or fusions in tumor samples are prohibitive for clinical assay development. We describe the development of a targeted RNA sequencing assay for the relative quantitation of specific gene expression signatures, known splice variants and gene fusions from less than 100 ng of starting material in a single tube universal amplification format. **Methods:** Primers for 52 genes, isoforms and gene fusion products were designed using the universal amplification strategy. 10 ng of RNA from 5 matched tumor/adjacent normal breast cancer tumor pairs were assayed. Libraries were prepared for sequencing by emPCR and sequenced on Ion Torrent PGM. Data were aligned via TMAP. Relative expression was determined vs. housekeeping genes or wild type transcripts. **Results:** All gene targets were detected at significant levels in at least one tumor sample. Robust expression profiling (5 log dynamic range) was obtained from FFPE macrodissected tumor and normal samples with as little as 200K reads/sample. Immune specific transcripts demonstrated differential expression (CCL3, AIF, FCGR3A and CSF1) across patients and matched pairs as well as an upregulation of CXCL12, indicative of tumor associated macrophages. **Conclusions:** Targeted RNAseq demonstrates detection and quantitation of relative expression levels of not only tumor subclass specific gene expression signatures, but immune cell specific transcripts from 10ng of FFPE derived total RNA derived from macrodissected tumor samples. The lower input requirements, quicker turnaround time and incredible sensitivity of targeted RNAseq make this assay a useful tool for clinical assay development.
Plasma osteopontin and the prognosis of pleural mesothelioma.

Harvey I. Pass, Jessica S. Donington, Shirish M. Gadgeel, Abraham Chachoua, Antoinette J. Wozniak, Geoffrey Liu, Ming Sound Tsao, Marc de Perrot, Chandra Goparaju; New York University School of Medicine, New York, NY; Karmanos Cancer Institute, Wayne State University, Detroit, MI; New York University Medical Center, New York, NY; Princess Margaret Hospital, Ontario Cancer Institute, Toronto, ON, Canada; Princess Margaret Hospital, University Health Network, University of Toronto, Toronto, ON, Canada; Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada

Background: Cytoreductive surgery for malignant pleural mesothelioma (MPM) should be reserved for patients with favorable tumor biology. Osteopontin (OPN) and the ratio of absolute neutrophil to absolute lymphocyte counts (NLR) have been reported as possible prognostic biomarkers. These were studied with other clinical/laboratory variables in a mixed surgical/non-surgical MPM population to define independent predictors of survival (OS) and progression (TTP).

Methods: Forty-four MPM patients (12 F, 32 M; 26 cytoreduction, 18 no cytoreduction; 31 epithelial, 13 non-epithelial; 15 Stage I/II, 29 Stage III/IV) were examined with regard to pretreatment plasma OPN (ELISA, R&D, Minneapolis, MN), NLR age, gender, therapy, histology, stage, platelet count and WBC count. Cut points for age, OPN, NLR, platelets, and WBC were determined by X-tile Software (Yale, New Haven, CT) and univariate/multivariate Cox analyses performed.

Results: Median OS were 11 m, 21m, and 8m for all 44 MPMs, cytoreduced and non-cytoreduced MPMs, respectively. Of platelet count, WBC, NLR, and OPN, only OPN was statistically significant between Stage I/II and Stage III/IV (80.3 ng/ml vs 148 ng/ml, p<0.018). The only independent covariate predictive of OS was plasma OPN. For TTP in cytoreduced patients, only age, stage, platelet count, and OPN were significant in univariate analysis, and multivariate modeling retained stage (p=0.04, HR=2.75, 95% CI=1.0517 to 7.1879) and OPN (p=0.0008, HR=17.471, 95% CI=3.3054 to 92.3461).

Conclusions: Plasma OPN is promising for the stratification of tumors into good or bad risk categories and to help select potential candidates for cytoreduction and further postoperative therapy.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
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<tbody>
<tr>
<td>Age &gt; 61</td>
<td>0.0008</td>
<td>2.95</td>
</tr>
<tr>
<td>Male vs female</td>
<td>0.8141</td>
<td>1.09</td>
</tr>
<tr>
<td>Other vs epithelial</td>
<td>0.2129</td>
<td>1.54</td>
</tr>
<tr>
<td>Cytoreduced yes vs no</td>
<td>0.0018</td>
<td>0.3885</td>
</tr>
<tr>
<td>Platelet count &gt; 376</td>
<td>0.0026</td>
<td>2.96</td>
</tr>
<tr>
<td>WBC &gt; 8.0</td>
<td>0.0227</td>
<td>1.93</td>
</tr>
<tr>
<td>NLR &gt; 7.2</td>
<td>0.0009</td>
<td>4.11</td>
</tr>
<tr>
<td>Plasma OPN &gt; 99.8</td>
<td>&lt; 0.0001</td>
<td>5.56</td>
</tr>
</tbody>
</table>

*NR: not retained in the final multivariate model.
The role of Pirh2 C-terminal residues and ubiquitin lysine chains in ubiquitination mechanism.

Rami Mahmoud Abou Zeinab, Roger Leng; University of Alberta, Edmonton, AB, Canada

Background: Pirh2, a p53 inducible gene, is proposed to be a main regulator of p53 family proteins fine tuning the DNA damage response by acting as an E3 ligase. A negative feedback loop between Pirh2 and p53 exists where under unstressed conditions, Pirh2 induces p53 ubiquitination followed by proteosomal degradation. In case of cellular stress when the activity of tumor suppressors is essential, Pirh2 is self-ubiquitinated releasing p53 continuous repression. Interestingly among all E3 ligases, Pirh2 is the only one to be over-expressed in a wide range of human tumors. This over-expression indicates a disruption in the self-ubiquitination mechanism or a defect in the degradation mechanism post ubiquitination. Based on that, we aimed to analyze Pirh2 ubiquitination mechanism through mapping Pirh2 domains to reveal the essential residues for this process and the ubiquitin chains utilized. Methods: Pirh2 constructs deleting major residues in the three domains were designed, and also ubiquitin mutant constructs were designed through single/multiple mutations at specific positions where lysine residues are mutated to arginine. Using these constructs and in comparison to WT proteins, we tested Pirh2 in-vitro self and p53 ubiquitination activity. Results: Regarding Pirh2, we were able to reveal that residues 240-250 of the c-terminal along with the ring domain are essential for self-ubiquitination. K63R and K48R ubiquitin did not affect Pirh2 self-ubiquitination minimizing the impact of ubiquitin mutations on Pirh2 self-ubiquitination. However, KO, which had all lysine residues mutated to arginine, showed total inhibition of Pirh2 self-ubiquitination confirming the importance of lysine residues. Interestingly, Pirh2 self-ubiquitination reaction showed no difference in the presence or absence of p53 proteins. Concerning Pirh2-p53 ubiquitination, K48 was found to be critical for E3 ubiquitin ligase activity because K48R and not K36R showed defective ubiquitination. All results were confirmed by quantifying ubiquitin. Conclusions: Our data added knowledge to the Pirh2 self-ubiquitination mechanism that can resolve the constant overexpression of Pirh2 proteins hence maximizing p53 response to DNA damage.
Prognostic significance of the maximal value of the baseline standardized uptake value on fluorine-18 fluorodeoxyglucose positron emission tomography/computed tomography for predicting pathologic malignancy of operable breast cancer.

Takayuki Kadoya, Kenjiro Aogi, Sachiko Kiyoto, Emiko Kanno, Etsushi Akimoto, Akiko Emi, Hideo Shigematsu, Norio Masumoto, Yoshifumi Sugawara, Morihito Okada; Hiroshima University, Hiroshima, Japan; National Hospital Organization Shikoku Cancer Center, Ehime, Japan; Department of Surgical Oncology, Hiroshima University, Hiroshima, Japan; National Hospital Organization Shikoku Cancer Center, National Hospital Organization Shikoku Cancer Center, Department of Diagnostic Radiology, Ehime, Japan

Background: [18F]-fluorodeoxyglucose (FDG) positron emission tomography (PET)/computed tomography (CT) is potentially useful in predicting prognosis of breast cancer patients. Methods: 344 breast cancer patients (mean age: 58.0 ± 12.5) with clinical stage IxIII between January 2006 and December 2011, were prospectively evaluated (median follow-up period: 52.0 months). Patients underwent a whole-body FDG PET/CT before operation. The maximal value of the baseline standardized uptake values (SUVmax) were assessed for predicting disease free survival (DFS). For the evaluation of relationship between SUVmax values and prognostic factors such as hormone receptors, human epidermal growth factor receptor 2 (HER2), nuclear grade, lymph node metastasis and tumor size, statistical analyses were performed using Student t test and log-rank test, and p values of less than 0.05 were considered to indicate statistically significant differences. Results: Clinical stage included were I (n = 194), II (n = 134) and III (n = 16). Tumors with estrogen receptor (ER) positive were 292 (84.9%) and negative were 52 (15.1%). Patients were divided into two groups according to cut-off SUVmax established on the basis of receiver operating characteristic analysis (≥3.0 vs >3.0, AUC=0.713). There was a significant difference in DFS between two groups (p = 0.001) and, hormone receptor, HER2, nuclear grade, lymph node metastasis were found strong relation to SUVmax values. SUVmax and ER status were predictive factors with multivariable analysis using cox proportional hazard regression model (p = 0.033 and p = 0.004, respectively). Conclusions: SUVmax on FDG PET/CT before operation has a predictive value for high-grade malignancy and prognosis in operable breast cancer.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revised maxSUV</td>
<td>4.350 (1.127-16.786)</td>
<td>0.033</td>
</tr>
<tr>
<td>Age</td>
<td>1.338 (0.496-3.607)</td>
<td>0.565</td>
</tr>
<tr>
<td>Clinical T stage</td>
<td>1.668 (0.597-4.656)</td>
<td>0.329</td>
</tr>
<tr>
<td>Clinical N stage</td>
<td>1.183 (0.418-3.344)</td>
<td>0.751</td>
</tr>
<tr>
<td>Nuclear grade</td>
<td>0.955 (0.332-2.745)</td>
<td>0.932</td>
</tr>
<tr>
<td>Estrogen receptor status</td>
<td>4.624 (1.635-13.082)</td>
<td>0.004</td>
</tr>
<tr>
<td>HER2 status</td>
<td>1.247 (0.407-3.824)</td>
<td>0.699</td>
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</table>

Hormone receptor-dependent regulation of ABAT and beta-alanine metabolism in breast cancer.

Jan Budczies, Scarlet F. Brockmoeller, Berit Mueller, Manfred Dietel, Oliver Fiehn, Carsten Denkert; Charité-Universitätsmedizin Berlin, Berlin, Germany; University of Cambridge, Cambridge, United Kingdom; Institute of Pathology, Charité Universitätsmedizin, Berlin, Germany; University of California, Davis, Davis, CA; Charité-Universitätsmedizin Berlin, Institute of Pathology, Berlin, Germany

Background: Recently, we identified beta-alanine as biomarker for breast cancer using GC-MS based metabolomics. Beta-alanine is increased in breast cancer compared to normal tissues and in the more aggressive ER- subtype compared to ER+ breast cancer. Beta-alanine is a substrate of 4-aminobutyrate aminotransferase (ABAT), can be catabolised to malonate semialdehyde and used for reduction of NAD and acetylation of coenzyme A. The aim of the current study is to analyze ABAT protein and RNA expression in a large cohort of breast cancers.

Methods: The specificity of a polyclonal antibody against ABAT was validated using siRNA in MFC7 cells. A cohort of 164 paraffin-embedded breast cancer tissues from the METAcancer biobank was investigated by immunohistochemistry. Tumor cells were evaluated separately for staining intensity (low, moderate or high) and percentage of stained cells. A cohort of 156 METAcancer samples was investigated for gene expression using whole genome DASL.

Results: ABAT protein intensity correlated strongly with estrogen receptor (ER) status (p < 0.001), but not with HER2 status. In particular, the ABAT protein was highly expressed in 41% of the ER+ tissues, but only in 2% of the ER- tissues. Further, ABAT intensity correlated strongly with tumor grade (p < 0.001). ABAT intensity did not correlate with tumor stage or nodal status. Explorative evaluation of ABAT protein in normal cells revealed weak expression in some of the ducts and negative expression in adipose cells. ABAT protein and RNA expression strongly correlated in the subcohort investigated by DASL. Analysis of a large external data set (publicly available at www.kmplot.com) showed that low ABAT expression is associated with an unfavorable prognosis of breast cancer. Both, ABAT protein and RNA expression, showed a strong negative correlation with beta-alanine abundance.

Conclusions: We reported on changes in beta-alanine metabolism that occur between the molecular subtypes of breast cancer and normal breast tissue. High expression of ABAT in ER+ breast cancer is compatible with catabolic use of beta-alanine. Differently, beta-alanine might be preferable used anabolic in ER- breast cancer, possibly for the synthesis of carnosine.
Roles of cMET/ErbB3 activation and overexpression in the development of resistance to EGFR inhibitors in NSCLC patients.

Myung-Ju Ahn, Silvia Park, Jong-Mu Sun, Jin Seok Ahn, Keunchil Park, Emma J. Langley, Phillip Sangwook Kim, Sharat Singh, Steve Lockton; Samsung Medical Center, Seoul, South Korea; Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea; Department of Medicine, Division of Hematology-Oncology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea; Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea; Prometheus Laboratories Inc., San Diego, CA; Prometheus Therapeutics & Diagnostics, San Diego, CA

Background: Multifaceted efforts in the molecular and cellular research of lung cancer revealed many critical pathway dysregulations due genetic and epigenetic alterations to alter balances in signal transduction leading to carcinogenesis. Methods: US guided FNAs or pleural fluid were collected from 44 NSCLC patients enrolled in on-going prospective study involving EGFR inhibitors (gefitinib, erlotinib, or afatinib) and evaluated for EGFR as well as other various receptor tyrosine kinases (RTKs, i.e. ErbB2, ErbB3, cMET, IGF1R, ALK, etc) and downstream AKT and MAPK pathway proteins for their level of expression and activation in order to 1) Compare objective response rate according to the expression/activation of RTK and pathway proteins; 2) Evaluate the modulation of the RTK and pathway proteins during the treatment of EGFRi; 3) Correlate between activating EGFR gene mutations and RTK activation in patients treated with EGFRi. Results: Majority of patients over-expressed EGFR. While not overexpressed, varying and significant levels of cMET and ErbB3 were found and quantitated in each patient. A striking difference in PFS was observed with respect to relative levels of cMET to EGFR in pretreatment FNA. Regardless of EGFR mutation status, patients with higher levels of EGFR to cMET (or higher E/M-Index) showed superior PFS. With an increase in cMET involvement (or decrease in E/M-Index; continuous variable), NSCLC patients exhibited worse clinical outcome with reduction in PFS (as shown in the Table). Furthermore, patients with shorter PFS (<8 months) had significantly higher levels of total and phosphorylated ErbB3 (67% positive) than patients with longer PFS (>8 months, 27% positive). Conclusions: This study clearly demonstrates the critical roles of cMET and ErbB3 in resistance to EGFR inhibitors in NSCLC patients. E/M-Index and activation may serve as predictive markers for treatment selection with EGFRi and cMETi, and should be validated in prospective clinical studies.

<table>
<thead>
<tr>
<th>E/M-Index</th>
<th>&gt; E/M-Index (N)</th>
<th>&lt; E/M-Index (N)</th>
<th>Median PFS (M)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>10</td>
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<td>6.1</td>
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Two lipids based on lipidomics as novel biomarkers for early detection of squamous cell lung cancer.

Youping Deng, Junmei Ai, Jeffrey Allen Borgia, Hankui Chen, Brett Mahon, Michael J. Liptay, Philip Bonomi, James L. Mulshine; Rush University Medical Center, Chicago, IL

Background: Lipids play roles in membrane structure, energy storage, and signal transduction as well as lung cancer. Lipidomics, a new technology aims to measure all the lipids in a cell, has not been applied to diagnostic test development for a variety of cancer types. Here, we adopt lipidomics as a means to identify plasma lipid markers for the early detection of lung cancer and complement CT-based methods for lung cancer screening. Methods: Using mass spectrometry, we profiled 390 individual lipids in a training discovery cohort comprised of cohorts that were either at “high-risk” for lung cancer (n=22) and squamous cell carcinoma at early stages (n=22). Cases had a minimum of two years clinical follow-up and were matched in terms of race, sex, age and smoking status. Gain ratio feature selection and local weighted classification model were employed to find the best training classifier, which was further validated against an additional cohort, including high-risk individuals (n=20) and squamous cell carcinoma patients (n=17).

Results: In the training discovery stage, we found 20 distinct lipids that were significantly distributed between high-risk and cases of squamous cell carcinoma. We further defined a two lipid marker panel had a training accuracy at 95.5% sensitivity, 90.9% specificity and 95.2% AUC (Area under ROC curve). The validation accuracy against the additional cohort is 100.0% sensitivity, 90.0% specificity and 99.0% AUC (Table). The power for sample size we used in both discovery training and validation stages were over 90%.

Conclusions: Using lipidomics we identified two lipid markers capable of discerning cases of squamous cell carcinoma from individuals at high risk for lung cancer, with a high sensitivity, specificity and accuracy. The markers maybe further developed as a quick, safe blood test for early diagnosis of squamous cell lung cancer and reduce unnecessary follow-up imaging or invasive procedures.

The training and validation results using a two lipid marker panel.

<table>
<thead>
<tr>
<th>Stages</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Odds ratio</th>
<th>AUC</th>
<th>Sample size</th>
<th>power</th>
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<tbody>
<tr>
<td>Training</td>
<td>95.45</td>
<td>0.909</td>
<td>0.913</td>
<td>0.952</td>
<td>+ inf.</td>
<td>0.952</td>
<td>inf.</td>
<td>0.99</td>
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<tr>
<td>Validation</td>
<td>1</td>
<td>0.900</td>
<td>0.895</td>
<td>1.000</td>
<td>+ inf.</td>
<td>0.99</td>
<td>inf.</td>
<td>0.96</td>
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</tbody>
</table>
Targeting radiation resistance in p53 mutant tumors with AKT inhibitors.

Aswin George Abraham, Garth Hamilton, Eric O’Neill; Gray Institute for Radiation Oncology and Biology, Department of Oncology, Oxford, United Kingdom

Background: Increasing our understanding of how resistance to radiation occurs, helps us develop more efficient and personalised radiotherapy treatments for cancer patients. Through molecular dissection of events that drive tumour initiation and progression, we have uncovered a functional connection between the most frequent oncogenic mutations that are likely to contribute to therapeutic resistance in 30% of all human tumours. Mutations activating the "AKT" signalling pathway and inactivation of the "TP53" tumour suppressor gene are common mechanisms that cancer cells require to proliferate and escape pre-programmed cell death. We find that in combination these events lead to therapeutic resistance that is reversible by the AKT clinical candidate, MK-2206 and the PI3K inhibitor PI-103. Methods: Using a combination of in vivo and in vitro techniques we have tracked the molecular mechanism for AKT mediated resistance to treatment in solid tumours. This has helped us to simultaneously derive potential biomarkers that could highlight where the greatest efficacy may be achieved in clinical practise. Results: Tumours employ many strategies to inactivate p53; however sequence mutations that result in mutant p53 protein (p53mut) are most often observed. p53mut tumours not only fail to respond to DNA damaging therapy, but are also described to promote therapeutic resistance by dominant negative suppression of p53 dependent promoter activity. We demonstrate that AKT inhibitors- and as proof of concept, the clinical candidate AKT inhibitor, MK-2206, and PI3K inhibitor, PI-103, are effective in treatment of mice with therapeutically resistant tumours with elevated AKT and carrying p53mut. AKT inhibition promotes reduced cellular levels of p53mut via a novel mechanism and promoted re-engagement of cell cycle arrest, senescence and increased sensitivity to ionising radiation in both in vivo and in vitro systems. Conclusions: We show that AKT inhibitors sensitisise xenografts carrying p53 mutations to DNA damaging therapy. We have also been identifying potential molecular markers to select the cohort of patient most likely to benefit from this treatment.
Comparison of in vivo skin and in vitro blood lymphocyte models for the prediction of late normal tissue responses in breast radiotherapy (RT) patients.

Melvin Chua, Navita Somaiah, Sue Davies, Lone Gothard, Kai Rothkamm, John Yarnold; University College London Cancer Institute, London, United Kingdom; Institute of Cancer Research, Sutton, United Kingdom; Institute of Cancer Research, Radiotherapy and Imaging, Sutton, United Kingdom; Health Protection Agency, Chilton, United Kingdom; The Royal Marsden NHS Foundation Trust, Sutton, United Kingdom

Background: Critical opinions for the lack of success of DNA double-strand break (DSB) repair as a predictive marker of normal tissue radiosensitivity include the argument that in vitro cellular responses correlate poorly with in vivo responses due to the modifying influence of tissue environment. In this study, we test the hypothesis that a DNA damage assay based on in vivo irradiated skin tissues better predicts clinical responses in human skin, as opposed to the same assay performed in ex vivo irradiated lymphocytes.

Methods: DSB levels (24 h post-4 Gy) were quantified using γH2AX/53BP1 immunostaining in irradiated skin tissues and G0 lymphocytes of 35 breast RT patients. Patients were selected on the basis of late RT effects in their breast and individuals with marked or minimal effects were classified as cases and controls, respectively. Risk factors of late effects established from multivariate analyses of outcomes of two breast RT trials were also considered in patient selection. They were 1) total RT dose, 2) RT dosimetry, 3) tumour bed boost, 4) breast size, 5) surgical cavity, and 6) axillary treatment. Results: Clinical parameters were balanced in both patient groups. Residual foci levels in skin epidermis and dermis were comparable between cases (n = 20) and controls (n = 15). Mean foci per cell were 3.29 in cases, 2.80 in controls for dermal fibroblasts (p = 0.07); 3.28 in cases, 2.60 in controls for endothelial cells (p = 0.08); 2.87 in cases, 2.41 in controls for superficial keratinocytes (p = 0.45); 2.32 in cases, 2.35 in controls for basal keratinocytes (p = 0.27). Residual foci levels in lymphocytes were however significantly higher among cases (foci per cell = 12.1) compared to controls (foci per cell = 10.3, p = 0.01). Of the different cell types, only residual foci levels of dermal fibroblasts and lymphocytes correlated with clinical severity (R = 0.722, p < 0.001; 0.593, p = 0.01, respectively). Interestingly, foci levels were not correlated between skin cells and lymphocytes of the same patients. Conclusions: DSB repair of ex vivo irradiated lymphocytes appears to be a better predictive marker of late effects to breast RT than DSB repair of in vivo irradiated skin.
Can BCL2L12 be a promising favorable prognostic biomarker in breast cancer?

Alexandros Tzovaras, Athina Kladi, Kleita Michaelidou, Nikolaos Tsoukalas, Gregory Tsoukalas, Pantelis Kountourakis, Sofia Stamatopoulou, Georgios Oikonomopoulos, Ioannis Misitizis, Alexandros Ardavanis, Andreas Scorilas; First Medical Oncology Department of Α“Saint Savvas” Anticancer Hospital, Athens, Greece; 1Department of Biochemistry and Molecular Biology, University of Athens, Athens, Greece; Department of Biochemistry and Molecular Biology, University of Athens, Panepistimiopolis, Athens, Greece; 401 General Army Hospital, Athens, Greece; AGIOS SAVVAS, Athens, Greece; Saint Savvas Anticancer Hospital, Athens, Greece; First Department of Medical Oncology, Saint Savvas Hospital, Athens, Greece; 1st Department of Medical Oncology, Agios Savvas Hospital, Athens, Greece; The Breast Clinic, Saint Savvas, Anticancer Hospital, Athens, Greece; First Medical Oncology Department of Α“Saint Savvas” Anticancer Hospital, Athens, Greece

Background: Many genes of the BCL2 family were found to be implicated in breast carcinogenesis and to serve as possible prognostic markers. BCL2-like 12 (BCL2L12) is a new member of BCL2 gene family which was discovered and cloned by members of our group and found to be expressed in the mammary gland. The aim of the study was the quantification and the evaluation of the prognostic value of BCL2L12 in breast cancer. Methods: BCL2L12 mRNA levels were determined in 140 samples (81 cancerous and 59 adjacent non-cancerous) of breast tissue using a highly sensitive quantitative real-time polymerase chain reaction (qRT-PCR) method. Relative quantification analysis was conducted using the comparative Ct (2-ΔΔCt) method, whereas the association between BCL2L12 expression and clinopathological data, disease free survival (DFS) and overall survival (OS) were defined by statistical analysis. Results: Significant relationships between BCL2L12 expression and early TNM stages (P<0.01), absence of metastasis (P<0.001), Estrogen Receptor (ER) positivity (P=0.031) and breast tumors of 2-5cm diameter (P=0.032) were observed. Moreover univariate analysis indicated that BCL2L12 expression is a significant prognostic factor of disease-free survival (DFS) and overall survival (OS), whereas multivariate analysis revealed strong association between BCL2L12 expression and DFS. Conclusions: With regard to breast cancer, patients bearing BCL2L12-positive tumors have significantly longer DFS and OS. These results denote a promising, independent favorable prognostic marker and a significant association of BCL2L12 expression with good outcome.
Analysis of prognostic factors after 16 years of follow-up in a randomized phase II trial of neoadjuvant FAC compared with CMF in stage III breast cancer.

Julieta Leone, Jose Pablo Leone, Carlos Teodoro Vallejo, Juan Eduardo Perez, Alberto Omar Romero, Mario Raul Machiavelli, Luis Romero Acuna, Maria Ester Dominguez, Mario Langui, Hebe Margot Fasce, Bernardo Amadeo Leone, Eduardo Ortiz, Julian Iturbe, Ariel Osvaldo Zwenger; Grupo Oncologico Cooperativo del Sur, Neuquen, Argentina; University of Pittsburgh Cancer Institute, Pittsburgh, PA; Grupo Onc Cooperativo del Sur, Bahia Blanca, Argentina; Grupo Oncologico Cooperativo Del Sur, Santa Fe, Argentina; Grupo Oncologico Cooperativo Del Sur, Tres Arroyos, Argentina; Grupo Oncologico Cooperativo Del Sur, La Plata, Argentina; Grupo Oncologico Cooperativo Del Sur, La Pampa, Argentina; Hospital Regional Neuquen, Neuquen, Argentina

Background: Neoadjuvant chemotherapy is a standard treatment in stage III breast cancer. Prognostic factors can help to identify patients (pts) with high risk of recurrence. The aim of this study was to assess several prognostic factors after a long follow-up, in stage III breast cancer pts, treated with neoadjuvant chemotherapy. Methods: We evaluated 126 pts with stage III breast cancer that participated in a phase-II randomized trial of neoadjuvant 5-fluorouracil, doxorubicin and cyclophosphamide (FAC every 21 days) compared with cyclophosphamide, methotrexate and 5-fluorouracil (CMF days 1 and 8 every 28). Chemotherapy was administered for three cycles prior to definitive surgery and radiotherapy, and then for six cycles as adjuvant. Response was assessed by WHO criteria. Results: The median age was 52 years (range 24-75). Median follow-up was 4.5 years (range 0.2-16.4), disease free survival (DFS) 4.8 years and overall survival (OS) 6.4 years. Results of the phase-II study showed no difference in efficacy between groups. Univariate analysis showed that the number of pathologically involved lymph nodes (pLN), pathologic response and estrogen and progesterone receptor status correlated with DFS and OS. Number of pLN was the only prognostic factor with statistical significance in Cox regression test for both, DFS and OS (P=0.0004 and P=0.0006, respectively). In a subgroup analysis of pts with pLN, we found no difference in survival when we compared FAC with CMF. Conclusions: The prolonged follow-up of this study provides a unique opportunity to evaluate factors that predict long-term outcomes. After 16 years of follow-up, the number of pLN remains the most powerful predictor of survival. The subset of pts with pLN had similar survival regardless of the regimen used. Clinical trial information: NCT00002696.
A Ki-67 proliferation index cutoff value of 1% to predict 5-year RFS and OS in patients with pulmonary carcinoid tumors.

Thanyanan Reungwetwattana, Sumithra J. Mandrekar, Trynda Kroneman, Nathan R. Foster, Marie-Christine Aubry, Eunhee S. Yi, Sarah E Kerr, Ping Yang, Axel Grothey, Vijayalakshmi Shridhar, Jesse S Voss, Benjamin Kipp, Julian R. Molina; Mayo Clinic, Rochester, MN; Mayo Clinic and NCCTG, Rochester, MN; Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN

Background: Evaluation of prognostic factors in carcinoid tumors of the lung is limited due to the rarity of disease. This study assessed Ki-67 expression and other clinical variables as prognostic factors in 262 patients. Methods: A systematic search of Mayo Clinic lung cancer epidemiology and tumor registry databases from 1997 to 2009 identified 449 consecutive patients, with 262 having available tissue blocks [40 atypical carcinoids (AC) and 222 typical carcinoids (TC)]. Clinical data were collected by chart review. Tissue blocks were reviewed by 1/3 pathologists using WHO criteria. Tumors were tested for the Ki-67 index using digital image analysis (tumor tracing) by two operators. The association and predictive value of the factors with recurrence-free and overall survival (RFS and OS) were explored using univariable Cox proportional Hazards model and concordance (c) index. Results: Age, stage, smoking history, lymph node (LN) involvement and Ki-67 index were significant prognostic factors for RFS and OS. Median follow-up on alive-patients is 5 years (range: 0.006-5). Median percentage of Ki-67 index of AC and TC were 1.61% and 0.56% (P<0.0001), respectively. Patients with Ki-67 ≥ 1% had significantly worse RFS (HR=3.69, P=0.0001) and OS (HR=3.69, P=0.0007) compared with Ki-67 < 1% group. The c-index of Ki-67 (0.65) was comparable to the pathologic distinction between AC and TC (0.62 for original diagnosis and 0.63 for central-reviewed diagnosis). Conclusions: Ki-67 index cutoff value of 1% is a valuable prognostic biomarker for pulmonary carcinoids based on this large cohort. Our data also provide strong evidence for clinical variables such as age, stage, smoking history, and LN involvement as clinical prognostic factors in pulmonary carcinoids. A prognostic calculator incorporating Ki-67 and clinical variables is under development.

<table>
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<th>Outcomes</th>
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<th>OS</th>
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<td>HR; 95% CI</td>
<td>C-index</td>
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<td>HR; 95% CI</td>
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<td></td>
<td>(p value)</td>
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<td>Ki-67 ≥ 1% vs. &lt; 1%</td>
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<tr>
<td>(original diagnosis)</td>
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<td>AC vs. TC</td>
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<td>(original diagnosis)</td>
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<td>AC vs. TC (central-reviewed diagnosis)</td>
<td>4.24; 2.40-7.48</td>
<td>0.63</td>
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<td></td>
<td>(0.0001)</td>
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<td>AC vs. TC (central-reviewed diagnosis)</td>
<td>4.02; 1.90-8.50</td>
<td>0.64</td>
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Identification and validation of an assay predictive of response and prognosis following anthracycline-based chemotherapy for early breast cancer.

Jude M. Mulligan, Laura A. Hill, Steve Deharo, Katherine E. Keating, Olaide Raji, Fionnuala A. McDyer, Jacqueline James, Gareth Irwin, David Boyle, Jennifer E. Quinn, Paul B. Mullan, Colin R. James, Manuel Salto-Tellez, Timothy S. Davison, Patrick Johnston, Fergus J. Couch, D. Paul Harkin, Richard D. Kennedy; Almac Diagnostics, Craigavon, United Kingdom; Almac Diagnostics, Craigavon, Northern Ireland; Almac Diagnostics Ltd., Craigavon, Northern Ireland; Queen's University Belfast, Belfast, United Kingdom; Centre for Cancer Research and Cell Biology, Queen's University of Belfast, Belfast, Northern Ireland; Centre for Cancer Research and Cell Biology, Queen’s University of Belfast, Belfast, Northern Ireland; Mayo Clinic, Rochester, MN

Background: Currently there is no biomarker to predict specific benefit from DNA-damaging anthracycline and cyclophosphamide-based chemotherapy in the clinic. Loss of the Fanconi anemia/BRCA (FA/BRCA) DNA-damage response pathway occurs in approximately 25% of breast cancer and results in sensitivity to DNA-damaging agents. We therefore developed an assay to detect loss of the FA/BRCA pathway, for the purpose of predicting benefit from chemotherapy. Methods: 21 FA patient samples were analyzed to identify genetic processes associated with loss of the FA/BRCA pathway. Unsupervised hierarchical clustering was then performed using 60 BRCA1/2 mutant and 47 sporadic tumor samples and a molecular subgroup was identified that was defined by the molecular processes representing loss of the FA/BRCA pathway. A 44-gene DNA Damage response deficient (DDRD) assay was developed that could identify this subgroup from formalin fixed, paraffin embedded (FFPE) samples in the clinic. Results: In a publicly available independent cohort of 204 patients, the assay predicted response to neoadjuvant DNA-damaging chemotherapy (5-fluorouracil, anthracycline and cyclophosphamide) with an odds ratio of 4.01, (95% CI:1.69-9.54). We also analysed samples from an independent cohort of 114 node-negative breast cancer patients treated with adjuvant 5-fluorouracil, epirubicin and cyclophosphamide treatment at the Northern Ireland Cancer Centre. The DDRD assay significantly predicted 5-year relapse free survival with a hazard ratio of 0.27 (95% CI:0.10-0.83). The assay was not predictive of survival in patients who did not receive chemotherapy. Conclusions: An FFPE tissue-based assay that detects loss of the FA/BRCA pathway has been developed and independently validated as a predictor of response and prognosis following DNA damaging anthracycline/cyclophosphamide-based chemotherapy in the neoadjuvant and adjuvant settings.
Correlation of $^{64}$Cu-DOTA-trastuzumab positron emission tomography (PET) imaging with HER2 status by immunohistochemistry (IHC).

Joanne E. Mortimer, Jinha Mark Park, Mary I. Carroll, Kofi Poku, Joshua Miles, David Colcher, Andrew Antony Raubitschek, Tri Tran, James R. Bading; City of Hope, Duarte, CA; City of Hope Cancer Center/Beckman Research Institute, Duarte, CA; City of Hope Medical Center/Beckman Research Institute, Duarte, CA

**Background:** We have developed $^{64}$Cu-DOTA-trastuzumab for PET imaging of HER2-positive breast cancer. We have determined that administering trastuzumab (45 mg) prior to $^{64}$Cu-DOTA-trastuzumab sharply reduces liver uptake of the radiotracer. We are now testing whether tumor uptake of $^{64}$Cu-DOTA-trastuzumab correlates with variable IHC staining in women with advanced breast cancer. **Methods:** Eligibility criteria included biopsy confirmation of metastatic disease that was HER2 1+, 2+, or 3+ by IHC, no anti-HER therapy within the prior 4 mo, and at least 1 non-hepatic site of metastasis > 20 mm outside the biopsy site. Staging workup included $^{18}$F-FDG PET-CT. Patients received 45 mg of cold trastuzumab prior to $^{64}$Cu-DOTA-trastuzumab. PET-CT scans were obtained at 21-25 h (Day 1) and 47-48 h (Day 2) over axial fields of view chosen in reference to $^{18}$F-FDG. Uptake in prominent lesions was measured in terms of maximum single-voxel SUV (SUV$_{\text{max}}$). Lesions identified on CT and judged to have image intensity > adjacent tissue by an expert radiologist were considered positive on PET. Results: Fourteen women (median age 56, range 35-75 y) have undergone imaging. HER2 status by IHC was 3+ in 9 pts., 2+ in 5 and 1+ in 1. Two women with IHC 2+ disease were FISH+. In the patients considered clinically HER2 positive (IHC 3+ or 2+, FISH+), $^{64}$Cu-DOTA-trastuzumab sensitivity was 75 and 90%, respectively, on Days 1 and 2, compared with 94% for $^{18}$F-FDG. Tumor uptake of $^{64}$Cu-DOTA-trastuzumab was also readily visualized in HER2-negative patients (measured detection sensitivity 90%). There were no false positive findings with $^{64}$Cu-DOTA-trastuzumab. Lesion uptake of $^{64}$Cu-DOTA-trastuzumab was higher in HER2+ than in HER2- patients (SUV$_{\text{max}}$mean ± sem: Day 1 8.9±0.6 vs 4.3±0.2; Day 2 9.9±0.8 vs 5.4±0.3, p < 0.001). **Conclusions:** $^{64}$Cu-DOTA-trastuzumab PET visualizes HER2 1+, 2+ and 3+ metastatic breast cancer with high sensitivity and specificity. Tumor uptake of $^{64}$Cu-DOTA-trastuzumab-PET in IHC 1+ and 2+ patients implies possible benefit from anti-HER2 therapies for individuals whose cancers are currently considered HER2 negative. Research Support: DOD1024511. Clinical trial information: NCT01093612.
The prospective MammaPrint MINT (Multi-Institutional Neo-adjuvant Therapy) study.

Charles E. Cox, Peter William Blumencranz, Ruben A. Saez, Robert Wesolowski, Lisette Stork, Femke De Snoo, Jessica Gibson, Nicole Howard, Eli Avisar; USF Health, Tampa, FL; Morton Plant Hospital, Clearwater, FL; Plano Cancer Institute, Plano, TX; The Ohio State University, Columbus, OH; Agendia NV, Amsterdam, Netherlands; Agendia, Inc, Irvine, CA; University of Miami School of Medicine, Miami, FL

Background: Patients with locally advanced breast cancer (LABC) are often treated with neo-adjuvant chemotherapy to reduce the size of the tumor before definitive surgery. Complete pathologic Response (pCR) predicts better long term outcome. Genomics assays that measure specific gene expression patterns in a patient’s primary tumor have become important prognostic and predictive tools for early breast cancer. This study is designed to test the ability of molecular profiling, as well as traditional pathologic and clinical prognostic factors to predict responsiveness to neo-adjuvant chemotherapy in patients with LABC.

Methods: Women ≥ 18 yrs with histologically-proven invasive breast cancer T2(≥3.5cm)-T4,N0M0 or T2-T4N1M0, with measurable disease, adequate bone marrow reserves and normal renal and hepatic function who signed informed consent are enrolled. Axillary lymph nodes will be staged according to protocol. MammaPrint risk profile, BluePrint molecular subtyping profile, TargetPrint ER, PR and HER2 single gene readout, and the 56-gene TheraPrint Research Gene Panel will be analysed using the whole genome expression array. Patients will receive neo-adjuvant chemotherapy treatment according to protocol. Response will be measured by centrally assessed Residual Cancer Burden (RCB). Objectives are: (1) To determine the predictive power of MammaPrint and BluePrint for sensitivity to neo-adjuvant chemotherapy as measured by pCR. (2) To identify and/or validate predictive gene expression profiles of clinical response or resistance to neo-adjuvant chemotherapy. (3) To compare TargetPrint ER, PR and HER2 with local and centralized IHC and/or CISH/FISH assessment. (4) To identify correlations between TheraPrint and response to neo-adjuvant chemotherapy. (5) To compare BluePrint molecular subtype with IHC-based subtype classification. To achieve a difference of 20% in chemotherapy sensitivity for patients stratified by MammaPrint, a total of 226 samples is needed (significance level 0.05 and power of 0.90). So far 45 patients have been enrolled from multiple institutions. Clinical trial information: NCT01501487.
A pilot study utilizing molecular profiling to find potential targets and select individualized treatments for patients with metastatic breast cancer.

Gayle S. Jameson, Emanuel Petricoin, Jasjit C. Sachdev, Lance A. Liotta, David Loesch, Stephen Patrick Anthony, Manpreet Chadha, Mariaelena Pierobon, Alex Reeder, Monica Fulk, Linda Vocila, Nina Cantafio, Bryant Dunetz, Nicholas J. Robert; Virginia G. Piper Cancer Center at Scottsdale Healthcare, Scottsdale, AZ; George Mason University, Manassas, VA; Caris Life Sciences, Phoenix, AZ; Evergreen Hematology and Oncology / US Oncology Research Affiliate, Spokane, WA; Translational Drug Development (TD2), Scottsdale, AZ; The Side-Out Foundation, Fairfax, VA; Virginia Cancer Specialists/US Oncology, Fairfax, VA

Background: The objective of this prospective pilot study was to determine if treatment selected by molecular profiling (MP) of metastatic breast cancer (MBC) tumors at time of disease progression provides greater clinical benefit than empiric treatment selection. Methods: Eligible pts had MBC, ≥ 3 prior lines of therapy, and > 6 wks - < 6 months time to progression on last treatment prior to study entry. Fresh core biopsy samples were taken from the metastatic site for MP. Analysis by IHC, FISH, DNA microarray and reverse phase protein microarray (RPMA) a quantitative protein pathway activation mapping technique using laser capture micro dissected tumor cells was done, with results in ≤14 days reviewed by a Treatment Selection Committee. The primary objective was to compare the progression-free survival (PFS) using a MP selected treatment regimen to the preceding PFS. Clinical benefit for the pt is defined by PFS ratio (PFS on MP selected therapy/PFS on prior therapy) is ≥ 1.3, (JCO,28:4877-83:2010). N= 25 was determined (exact single-stage design for phase II studies, type I error rate of 5% [one-sided], power of 90%). MP selected therapy would warrant further investigation if ≥ 35% of the pts demonstrate a PFS ratio of ≥ 1.3. Results: Three U.S. sites enrolled 25 evaluable pts who were treated based on MP results. Pts were heavily pretreated with 4 -11 prior therapies (median 7.24). Ten pts (40%) met or exceeded the PFS ratio of 1.3. The most common targets used in drug treatment selection were TOPO1, TS, ER/PR, TOP2A, HER2. Sixty percent of pts’ samples had activation of drug targets determined by RPMA in 3 major clusters: pan-HER-AKT; EGFR/SRC/ERK/mTOR; IGFR/RAF/MEK/PLK1. Days on MP selected treatments range from 9 - 816+. 3 pts continue on treatment for 199, 254 and 816 days. Conclusions: This study demonstrates the feasibility of a highly multiplexed genomic and proteomic MP study for treatment selection in a timely fashion. Patient-specific target driven treatment selection based on MP of a metastatic lesion provided clinical benefit for 10 of 25 heavily pretreated MBC pts. Thus, this approach merits further investigation in future studies. Funded by The Side-Out Foundation. Clinical trial information: NCT01074814.
A randomized, phase II trial of AEZS-108 in chemotherapy refractory triple-negative (ER/PR/HER2-negative) LHRH-R positive metastatic breast cancer.

Stefan Buchholz, Joerg Engel, Andrew V. Schally, Stephan Seitz, Olaf Ortmann, Guenter Emons, Reshma L. Mahtani, Stefan Gluck, Charles L. Vogel, Alberto J. Montero; University Medical Center Regensburg, Regensburg, Germany; VA Medical Center; University of Miami School of Medicine, Miami, FL; AGO, Goettingen, Germany; Division of Hematology/Oncology Sylvester Comprehensive Cancer Center University of Miami Health System, Deerfield Beach, FL; Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL; University of Miami School of Medicine, Comprehensive Cancer Research Group, Inc., Miami, FL

Background: Triple-negative breast cancer (TNBC) is a subtype of breast cancer that is clinically negative for expression of estrogen and progesterone receptors (ER/PR) and human epidermal growth factor receptor-2 (HER2). It is characterized by its unique molecular profile, aggressive behavior, distinct patterns of metastasis, and lack of commercially available targeted therapies. Chemotherapy has been the mainstay of treatment for women with TNBC, but this current standard-of-care is suboptimal. AEZS-108 is an LHRH-cytotoxic hybrid drug whose rational design covalently couples the carrier D-Lys$^6$-LHRH (an LHRH agonist) to the cytotoxic doxorubicin radical. Because LHRH receptors are expressed in a majority of TNBC, AEZS-108 represents a novel way to selectively deliver cytotoxic chemotherapy in patients with TNBC via a new therapeutic target. Methods: In this open label randomized two-arm multicenter phase II study, patients will be randomized in a 1:1 ratio into one of the two treatment arms: AEZS-108 (267 mg/m$^2$ every 21 calendar days) [Arm A] or SSC (standard single agent cytotoxic chemotherapy [Arm B]) at discretion of treating oncologist cycled every 21 calendar days. Stratified randomization will be used with number of prior lines of therapies (1-2 vs. >2), ECOG performance status 0-1 vs. 2, and liver metastases (absent vs. present). Analysis of the main study endpoint, TTP, will follow a group sequential design with two interim analyses, including the final analysis. O’Brien Fleming stopping boundaries will be used. The primary endpoint is to evaluate the median time to progression (TTP) of AEZS-108 in patients with chemotherapy resistant advanced TNBC treated with AEZS-108 in relation to patients receiving standard single agent cytotoxic chemotherapy. Secondary endpoints include: overall response, clinical benefit, duration of response, overall survival, toxicity profile and quality of life (QoL). Clinical trial information: NCT01698281.