different classifiers (70 genes "prognosis profile" (supervised on clinical outcome), Wound Signature (WS) and Hypoxia Signature (HS), both unsupervised) that separate patients into relatively good and poor prognosis groups. The supervised approach has an excellent sensitivity, but somewhat lower specificity for metastasis free survival. The unsupervised approaches have a higher specificity to identify patients with poor prognosis, but a relatively low sensitivity. In order to optimize both sensitivity and specificity, we combined the unsupervised profiles.
Methods: In a previously described series of 295 stage I and II breast carcinomas treated at the Netherlands Cancer Institute, we have obtained gene expression data of 25,000 genes using micro-array analysis. We have categorized the patients according to previously established groups. The first group consists of patient with a quiescent WS and a non hypoxic signature, patients in the second group have either an activated WS or hypoxic signature and the third group consists of patients with both an activated WS and a hypoxic signature.
Results: At a median follow up of 12 years for patients alive, the metastasis free probability (MFP) at 12 years is 79% for group 1 (n = 110), compared to 64% for group 2 (n = 103) and 45% for group 3 (n = 82) (log rank: p < 1*10−6. HR: 2.1 (95%CI: 1.6−2.7)); these figures were 87%, 68% and 37%, respectively, for overall survival (OS) (p < 1*10−12. HR: 2.6 (95%CI: 2−3.4). In subgroups with a favorable clinico-pathological characteristics (pT1N0, ER+ and pN0) the predictive power remains highly significant, as in patients with unfavorable clinico-pathological characteristics (pT2N+, ER− and pN+). The true negative predictive value for OS for group 1 is 87% and the true positive predictive value for group 3 is 60%. In multivariate analysis the combining the WS and HS signatures resulted in the best prediction of MFP and OS, which was independent of clinico-pathological variables (ER, TN-stage, Grade, Angioinvasion, Chemotherapy and age).
Discussion: In this consecutively treated series of breast cancer patients, the combination of the Wound Signature and Hypoxia classification stratifies patients that differ with respect to prognosis in three risk categories. Combining different gene expression signatures may result in improved classification of breast carcinomas.

258 ORAL

Utilisation of microarray technology to refine molecular classes and improve clinical management of breast cancer

F. Hermitte[1], F. Bertucci[2], N. Borie[1], I. Treilleux[3], S. Deraco[1], A. Martinec[1], J. Jacquemier[2], T. Bachelot[3], P. Viens[2], D. Birnbaum[2]. *[1]Ipsogen, Marseille cedex 9, France; [2]Institut Paoli-Calmettes, Marseille, France; [3]Centre Léon Bérard, Lyon, France*

Background: The significant genetic heterogeneity among breast cancer patients is a primary obstacle to effective clinical diagnosis and management. Emerging technologies based on gene expression profiling (GEP) may provide clinically useful information to improve the management of breast cancer. GEP has been used to refine classification of previously undistinguishable tumour subgroups, and predict prognosis and response to anticancer agents. Here we report a multicentric GEP analysis to identify and validate predictors in order to improve tumour classification and predict patients most likely to respond to standard chemotherapy.
Material and methods: 323 patients with early breast cancer treated with adjuvant anthracycline-based chemotherapy were selected from Institut Paoli-Calmettes (IPC), Marseille and Centre Léon Bérard (CLB), Lyon. RNAs were analysed on 10 K nylon cDNA microarrays. Metagenes for tumour classification were identified based on adjusted t-test analysis and hierarchical clustering on an identification set. A Cox-based method was used to find predictors able to discriminate patients with favourable outcome (no metastasis) after chemotherapy, by combining validated metagenes with clinical factors on an identification set of 159 patients treated with anthracyclines (IPCa). The stability and robustness of these predictors were assessed on two different and independent validation sets (IPCb n = 54 & CLB n = 110). The best predictor was compared with the Nottingham Prognostic Index (NPI).
Results: A predictor was identified on patients treated with chemotherapy (anthracyclines). This predictor was based on a linear combination involving metagenes and clinical factors, i.e. A*(metagene 1)+B*(metagene 2)+C*(clinical factors). It classified patients in two groups with different outcome. The robustness of this predictor was then confirmed on the two validation sets of patients. Our predictor compared favourably with the NPI, improving the classification of the low-risk patients.
Conclusions: Our metagene-based predictor is highly efficient to discriminate patients with favourable outcome under adjuvant anthracycline-based chemotherapy. It uses a validated combination of genes known for their biological relevance, and is valid irrespective of the clinical centre. Additional clinical studies and technical developments are ongoing to translate this new tool into a decentralised test designed for routine clinical practice.

259 ORAL

Standardisation of HER2 testing: results of an international proficiency testing ring study

M. Dowsett[1], W. Hanna[2], M. Kockx[3], F. Penault-Llorca[4], J. Rueschoff[5], T. Gutjahr[6], K. Habben[7], M. van de Vijver[8]. *[1]Royal Marsden Hospital, London, Academic department of Biochemistry, London, United Kingdom; [2]University of Toronto, Division of Pathology, Toronto, Canada; [3]Histogenex, Dept of Pathology, Antwerp, Belgium; [4]Centre Jean Perrin, Département de Pathologie, Clermont-Ferrand, France; [5]Klinikum Kassel, Institut für Pathologie, Kassel, Germany; [6]F. Hoffmann-La Roche Ltd, Basel, Switzerland; [7]Roche Diagnostics GmbH, Penzberg, Germany; [8]The Netherlands Cancer Institute, Amsterdam, The Netherlands*

Background: HER2-positive breast cancer indicates aggressive tumour growth, poor prognosis and treatment response to trastuzumab. Early and accurate determination of HER2 status is essential for optimal management of breast cancer. Because current HER2 tests (immunohistochemistry [IHC], fluorescence in-situ hybridisation [FISH], and chromogenic in-situ hybridisation [CISH]) are reader-dependent, validation by laboratory proficiency testing is important to improve standardisation. The study compared IHC and FISH testing between five international pathology reference centres.
Methods: A total of 20 IHC and 20 FISH breast cancer specimens were evaluated separately in five testing rounds (8-week intervals). In each round, a designated laboratory selected two sets of four different invasive tumour specimens (set A for IHC [HercepTest™]; set B for FISH [PathVysion]). The lab sent samples from each set to the other four testing centres in a blinded fashion, while retaining samples for its own evaluation. IHC scores were stated as negative (0, 1+), ambiguous (equivocal, 2+) or positive (3+). FISH scores were based on the ratio of HER2 signals to chromosome 17 centromere signals: negative (<2.0) or positive (⩾2.0). At the end of each round, an independent co-ordinator analyzed and discussed the results among the centres.
Results: All centres reported the same findings for nine out of 20 IHC specimens (45%). Although reports differed in the remaining 11 specimens, there were no cases wherein a laboratory reported a specimen as HER2 positive and another reported it as negative. At least one laboratory reported an ambiguous HER2 status in each of the 11 specimens. Sixteen out of 20 (80%) FISH specimens had similar scores from all the centres. The four cases in which the centres did not agree had mean amplification levels of 1.95, 1.48, 1.72 and 1.82. In the second of these cases, the difference in the report was due to one centre reporting a value of 2.0, while the others reported <2.0.
Conclusions: Equivocal IHC and borderline FISH cases are difficult to interpret, even for highly experienced and validated laboratories. To help determine the treatment, FISH retesting of IHC 2+ samples and retesting of FISH borderline cases with FISH, IHC, or CISH is recommended. As a follow-up of this study, equivocal IHC samples will be retested by FISH. Each testing laboratory should regularly validate their HER2 testing to ensure proper reporting of test results.

260 ORAL

Comparison of her2/neu expression on the primary tumor and on isolated tumor cells in the bone marrow of breast cancer patients

B. Rack, A. Schoberth, C. Schindlbeck, S. Schulze, W. Janni, M. Heinrigs, B. Strobl, U. Jeschke, H. Sommer, K. Friese. *University of Munich, Department of Gynecology and Obstetrics, Munich, Germany*

Background: There is growing evidence that the presence of isolated tumor cells in the bone marrow (ITC) of breast cancer patients, both at primary diagnosis and during follow-up, indicates an increased risk for subsequent recurrence (Braun, NEJM 2000; Janni, Cancer 2005). Therefore, ITC might be a potential target for tailored treatment options in these patients. Aim of this study was to establish a new method to analyse cytokeratin-positive (CK+) cells for her2/neu gene amplification.
Methods: ITC were detected using a standardized immunoassay with monoclonal antibody A45-B/B3, directed against cytokeratin 8, 18, 19 (CK) and stained according to the APAAP-technique. 2×10^6 cells per patient were screened by bright field microscopy. Subsequently, cytospins with CK-positive cells were further characterized by fluorescence in situ hybridisation (FISH) using a her2/neu DNA probe (Zymed, Germany) or a multi-colour probe (Vysis, Il., USA) for hybridisation of centromer 17 (polyploidy) and the her2/neu growth factor gene. A ratio of her2/neu and centromer chr. 17 signals of at least two was regarded as amplification.
Results: 232 bone marrow aspirates of 156 patients with breast cancer were analyzed at the time of primary diagnosis and during follow-up. ITC were detected in 68 samples (29%) in this patient group. The median number of detected cells was 2 (range 1−58). In 45 randomly assigned aspirates with ITC, the her2/neu status on these cells was evaluated and

13 samples were diagnosed with her2/neu amplification on ITC (29%). In 41 cases, both the her2/neu status of the primary tumor and on CK+ cells was available. In comparison, in 31 cases (76%) her2/neu status on the primary tumor corresponded to the her2/neu status on ITC. 7 patients (17%), however, with her2/neu overexpression or amplification on the primary tumor showed her2/neu negative ITC, whereas in 3 patients (7%) with her2/neu negative tumors we found ITC with her2/neu amplification.
Conclusion: Heterogeneity of antigen expression on the primary tumor results in a discrepancy of her2/neu status between the primary tumor and ITC in the bone marrow in a relevant subgroup of patients. Therefore, the amplification of her2/neu on persisting ITC may proof useful to stratify patients for a tailored treatment approach. New targeted agents such as her2/neu antibodies might be considered as an individualized treatment option in these patients.

261 ORAL

CCR7 receptor expression correlate with node involvement and survival in primary breast carcinoma

J. Blay[1], F. Andre[2], J. Guastalla[3], T. Bachelot[3], M. Mathieu[2], I. Ray-Coquard[3], A. Bremond[1], C. Caux[1], L. Zitvogel[2], I. Treilleux[3].
[1]INSERM U590 Ctr L Berard & Hop Ed. Herriot, Medecine, Lyon, France; [2]Institut Gustav Roussy, Medecine & Pathology, Villejuif, France; [3]Centre Leon Berard, Medecine & Pathology, Lyon, France

Rationale: The role for chemokine receptors in primary breast carcinoma dissemination in regional lymph node is unclear. The expression of chemokine receptors CCR6 and CCR7 was investigated in primary breast carcinoma and tested for possible correlations with lymph node invasion and distant relapse.
Methods: CCR6, CCR7 and CCR7 ligand CCL19 expression was investigated in a prospectively collected series of 256 patients with invasive non metastatic breast cancer (NMBC) treated in the Centre Leon Berard in 1996 and 1997 (Clin Cancer Res 2004). Correlations between these markers and the characteristics of the tumors, relapse free and overall survival were analyzed in univariate and multivariate analysis. These observations were analyzed on a retrospective series of the Institute Gustave Roussy analysing the phenotype of a series of long term (>20 years) NMBC as compared to a controlled matched series.
Results: CCR7 expression was observed in 75% of tumor samples, only in non tumoral cells and mostly in fibrocytes. In univariate analysis, CCR7 expression on fibrocytes correlated to tumor size ($p = 0.001$), SBR ($p = 0.001$), node involvement ($p = 0.003$), and HER2+++ ($p = 0.01$). CCR7+ fibrocytes were also detected at the contact of tumor cells in invaded axillary lymph nodes: 16 of 32 N+ patients had detectable fibrocytes in the axillary lymp node vs 0/15 of N− patients ($p < 0.0001$). CCR7 fibrocytes were observed in the axillary lymph nodes only in patients whose primary tumors contained CCR7 fibrocytes ($p < 0.001$).
Conversely, CCR6 expression was observed in 40% of primary breast carcinoma cells. CCR6 expression on tumor cells correlated to tumor size ($p = 0.001$) and node involvement ($p = 0.02$).
CCL19 was detectable in tumor cells (57%) and in infiltrating DC (48%). CCL19 expression correlated to low SBR ($p < 0.01$), no HER2 overexpression ($p = 0.01$), and lack of ER expression ($p < 0.001$). In multivariate analysis using a logistic regression model, tumor size, CCR7 expression on fibrocytes, and CCR6 expression on tumor cells were all found to be independent predictors of nodal involvement. CCR7 expression on fibrocytes correlated to a poor relapse free (RFS) (5 year RFS: 94 vs 81%, $p = 0.01$) and overall survival (OS) (5 year OS: 97 vs 86%, $p = 0.06$) in univariate analysis, but not CCR6 expression on tumor cells.
In the IGR series, CCR7 expression also correlated to SBR grade, HER2 expression ($p < 0.01$). The proportion of long term disease free survivors was significantly higher in patients with CCR− tumors as compared to CCR7+ tumors ($p = 0.01$).
Conclusion: CCR7+ expression in primary breast carcinoma tumor is associated with a high risk of distant relapse in primary breast carcinoma.

262 ORAL

Prognostic effect of estrogen receptor status across age in primary breast cancer

N. Bentzon[1], M. Düring[2], B.B. Rasmussen[3], H. Mouridsen[2], N. Kroman[2,4]. *[1]Herlev University Hospital, Department of Breast Surgery, Herlev, Denmark; [2]Rigshospitalet, Danish Breast Cancer Cooperative Group, Copenhagen, Denmark; [3]Roskilde County Hospital, Department of Pathology, Roskilde, Denmark; [4]Rigshospitalet, Department of Breast and Endocrine Surgery, Copenhagen, Denmark*

Background: Estrogen receptor (ER) status is the most important combined predictive and prognostic factor in breast cancer. It is well known that the chance of contracting an endocrine responsive tumor is age dependant. It is less well investigated whether the prognostic effect of ER status varies across age.
Material and methods: We used a well-established population-based registry with detailed information regarding clinical and histopathological presentation, postoperative therapy and follow-up status on Danish women with breast cancer.
Results: Overall, 26,944 patients with primary breast cancer diagnosed from 1989 to 2004 were included in the study. The chance of being ER positive increases from 51% in women < 35 yrs. to 82% in women 70–74 yrs. In a multivariate analysis ER status was found to be a significantly positive prognostic factor in women $\geqslant$ 40 yrs., but in women < 40 yrs. the survival was unaffected of ER status. The positive effect in relation to survival of being ER+ was limited to the first 5 yrs. after diagnosis, while survival after 5 yrs. was superior for women with ER− tumors. Same results were found when restricting the analysis to patients in low risk group (not receiving adjuvant therapy), n = 6,272.
Conclusion: In contrast to other studies we cannot confirm that ER+ status confer a negative impact on survival in young women. We find that the prognosis is generally worse in patients with ER− breast cancer but this applies only during the first 5 years whereafter the prognosis becomes worse in ER+ patients in the entire study group where ER status serves as a combined predictive and prognostic factor. This result stays unchanged when analyzing the untreated group separately where ER status is a prognostic factor only. This observation may be of clinical importance for future designing of adjuvant therapy beyond five years.

Oral presentations (Mon, 31 Oct, 13.45–15.45)
Large adjuvant breast cancer clinical trials relevant to clinical practice

263 ORAL

Doxorubicin and cyclophosphamide (AC) followed by paclitaxel (T) with or without trastuzumab (H) as adjuvant therapy for patients with HER2-positive operable breast cancer (BC): combined analysis of NSABP B-31 and NCCTG N9831

E.H. Romond[1], E.A. Perez[2], J. Bryant[3], V. Suman[4], C.E. Geyer[5], N. Davidson[6], S. Paik[7], S. Martino[8], P. Kaufman[9], N. Wolmark[10]. *[1]National Surgical Adjuvant Breast & Bowel Project, Pittsburgh, Philadelphia, USA; [2]North Central Cancer Treatment Group, US Breast Intergroup, Rochester, Minnesota, USA*

Introduction: B-31 and N9831 are two parallel phase III randomized clinical trials evaluating the addition of H to T following AC in women with HER2+BC.
Methods: In B-31 following AC $\times$ 4, T (175 mg/m^2 q 3 w $\times$ 4) was given alone (Arm 1) or concurrently with weekly H (Arm 2). In N9831 following AC $\times$ 4, T (80mg/m^2 q w $\times$ 12) was given alone (Arm A) or concurrently with weekly H (Arm C). Both trials required normal left ventricular ejection fraction (LVEF) and negative cardiac history. All B-31 and 88% of N9831 patients (pts) had positive axillary nodes. HER2 status had to be 3+ by IHC or positive by FISH. Cardiac function was monitored by LVEF measurement at 3, 6, 9, and 18 months from randomization with strict criteria for discontinuing H due to substantial asymptomatic declines. A proposal for combined efficacy analysis (control arms 1+A vs. investigational arms 2+C) was approved in Jan. 2005.
Results: First planned interim combined data analysis in 3351 patients with follow-up showed risk reduction in disease free survival (DFS) at 3 years (yrs) of 52% (HR = 0.48, $p = 3 \times 10{-}12$) and absolute improvement in DFS of 12% at 3 yrs and 18% at 4 yrs. All subsets of pts showed strong relative benefit from addition of H. Improvement in DFS was similar across protocols. First distant recurrence was reduced by 53% with addition of H (HR = 0.47, $p = 8 \times 10{-}10$) and absolute reduction in distant recurrence was 9% at 3 yrs and 16% at 4 yrs. Distant recurrences were markedly decreased after the second year with addition of H. Relative risk reduction of death was 33% (HR = 0.67, $p = 0.015$) and absolute improvement in overall survival is 2.5% at 3 yrs and 4.8% at 4 yrs. NYHA Class III/IV CHF was 3–4% and generally responded to therapy. In the B-31 cohort 19% of pts stopped H due to symptomatic or asymptomatic cardiac dysfunction and risk of CHF correlated with post-AC LVEF and age. Review of similar data from the N9831 cohort is ongoing.
Conclusion: AC followed by TH should be considered for adjuvant therapy of women free of cardiac disease with high-risk HER2-positive BC but careful cardiac monitoring is essential when using the therapy._