Immunofluorescence and Image Analysis: Cells plated on coverslips were fixed with 3.7% ice-cold paraformaldehyde, permeabilized with 0.1% Triton X-100 for 10 min and incubated with primary antibody in 0.1% gelatin and incubated with the Alexa 488 goat anti-mouse IgG1 and 568 goat anti-rabbit IgG. Proteins were detected with a Nikon TE 2000S epifluorescence microscope equipped with a CCD camera by using a Nikon lamp shutter with a mercury lamp for excitation. In colocalization experiments, scanning was conducted with 25−30 optical series from the top to the bottom of the cell with a step size of 0.45 μm. A Z-stack was acquired using the MetaMorph software, and every two-color stack (red and green) acquired separately in black and white (B/W). Each stack is deconvolved using the AutoDeblur 9.1 function of AutoQuant and then merged by transforming the two channels corresponding to red (tetramethylrhodamine B isothiocyanate) and green (fluorescein isothiocyanate) into a single two color stack by using the "RGB merge" command of ImageJ software.
Results: Immunofluorescent staining using anti-EGFR and GFP-NHERF1 indicates that NHERF1 colocalized with EGFR upon EGF stimulation. Functional experiments with truncated and binding groove-mutated PDZ domain constructs demonstrated that NHERF1 regulates these interaction through its PDZ1 domain. Enhancing of the expression of NHERF1 by transfection of wild-type (wt) NHERF1 inhibited ligand-induced degradation of EGFR upon EGF stimulation, suggesting that NHERF1 plays an important role in regulation of EGFR degradation in cells.
Conclusions: Taken together, our studies suggest that NHERF1 senses signal of EGF and regulates ligand-induced degradation of EGFR

P64

The stability of matrix metalloproteinases and chemokines in blood stored under various conditions

D. Belobrajdic[1], A. Phatak[2], I. Priebe[1], A. Burgess[3], E. Nice[3], L. Cosgrove[1]. *[1]CSIRO Molecular and Health Technologies, Australia; [2]CSIRO Mathematical and Information Sciences, Australia; [3]Ludwig Institute of Cancer Research, Australia*

Background: It is highly important to understand the stability of proteins in blood if they are to be used as biomarkers of disease, particularly when the disease process may affect the stability of the protein of interest. In clinical trials biomarkers are measured using frozen blood samples, whereas clinical tests are conducted on fresh blood samples soon after collection. Rouy et al (2005), along with our observations (unpublished), have suggested that matrix metalloproteinases (MMP) and chemokines in blood may be particularly vulnerable to degradation when samples are frozen. The aim of this study was to evaluate the stability of MMPs and chemokines in serum from colorectal cancer patients and control subjects, by analysing fresh blood samples and aliquots stored at −80ºC or in liquid nitrogen.
Methods: Blood from 10 healthy controls was age and sex matched to pre-operative blood collected from 10 colorectal cancer patients. Immediately after processing the blood, plasma MMP-9 was quantified by ELISA, and MMP-1, -2, -3, -7, -8 was quantified in serum by multiplex assay (R&D Systems). Chemokines CXCL-2, -3, -4, -8 were analysed in serum by multiplex assay (R&D Systems). The serum and plasma was sub-aliquoted and stored at −80ºC and in liquid nitrogen. An aliquot was stored at 4ºC overnight and analysed on the following day. Stored samples were analysed at 1, 30 and 90 days following collection.
Results: Preliminary results show that only MMP-1 in normal and cancer samples significantly decreased in serum when stored at either −80ºC or in liquid nitrogen for up to 1 month ($p = 0.02$) in comparison to freshly analysed serum samples. No such trends are evident for any of the other biomarkers, although the data suggest that where such trends exist, they are more clearly visible in normal samples.
We intend on measuring biomarker levels up to a period of 18 months, at which time we will have sufficient data to be able to estimate the effect of time, storage condition, and whether these effects are different for normal or cancer samples.
Conclusions: Blood samples analysed fresh show similar levels of chemokines and MMPs (with the exception of MMP-1) as bloods that are analysed following storage at either −80ºC or in liquid nitrogen for up to 3 months.

P67

Gene signature and lymph node metastasis in patients with early stage cervical cancer

P. Biewenga, M. Buist, P. Moerland, E. Ver Loren van Themaat, A. van Kampen, F. ten Kate, F. Baas. *Academic Medical Center, Amsterdam, The Netherlands*

Background: Pelvic lymph node metastases are the main prognostic factor for survival in early stage cervical cancer, yet accurate detection methods before surgery are lacking. In this study we examined whether gene expression profiling can predict the presence of lymph node metastasis in early stage squamous cell cervical cancer before treatment.
Methods: Tumour samples of 35 patients with early stage cervical cancer who underwent radical hysterectomy and pelvic lymph node dissection, 16 with and 19 without lymph node metastasis, were analyzed. We investigated differential expression and prediction of patient status for lymph node positive versus lymph node negative tumours. Classifiers were built by using a multiple validation strategy, enabling the assessment of both classifier accuracy and variability.
Results: Five genes (BANF1, LARP7, SCAMP1, CUEDC1, PEBP1) showed differential expression between tumour samples from patients with and without lymph node metastasis. However, the accuracy of class prediction is only 64.5% with a 95% confidence interval (CI) of 40−90%.
Conclusions: Expression profiling did not provide an accurate classification for lymph node status in early stage cervical cancer. Five genes were identified that may be attractive candidate markers for lymph node metastasis in early stage cervical cancer.

P71

Gene signature and early stage cervical cancer

M. Buist, P. Biewenga, P. Moerland, E. Ver Loren van Themaat, A. van Kampen, F. ten Kate, F. Baas. *Academic Medical Center, Amsterdam, The Netherlands*

Background: Cervical cancer is caused mainly by infection with a high-risk group of human papilloma viruses (HPV's). However, HPV infection alone is not enough for triggering cervical cancer. Few patients infected with high-risk HPV develop cervical cancer with a long incubation time, suggesting that additional factors or cellular events are required for progression to cervical cancer. In this study we identified genes involved in cervical carcinogenesis.
Methods: Tumour samples of 35 patients with early stage cervical cancer and samples of five normal cervical tissues were analyzed. We investigated differential expression and prediction of patient status for healthy versus cervical cancer tissue. Classifiers were built by using a multiple validation strategy, enabling the assessment of both classifier accuracy and variability.
Results: A total of 9313 probes representing human genes and transcripts were differentially expressed between healthy cervical tissue and early stage cervical cancer tissue with a q-value $\leqslant 0.005$. There is considerable overlap between previous studies and our study (top 200 genes upregulated) in terms of genes differentially expressed between normal cervical tissue and cervical cancer. Biological processes involved in cervical cancer oncogenesis are related to cell cycle, cell division, response to DNA damage stimulus and chromosome segregation. Highly accurate class prediction was obtained for healthy versus early stage cervical cancer tissue, mean accuracy of 99.5% (95% CI of 90–100%).
Conclusions: Expression profiling provides an accurate classification for early stage cervical cancer. A subset of genes involved in cervical cancer was identified.

P20

HER2-amplified breast carcinomas: molecular characteristic and response to trastuzumab treatment.

M. Campiglio, R. Orlandi, L. De Cecco, M. Gariboldi, L. Bertola, S. Ménard, E. Tagliabue. *Fondazione IRCSS – Istituto Nazionale Tumori, Milan, Italy*

Background: Therapy with trastuzumab of HER2-positive breast carcinomas has been shown to be active in 50% of patients in 4 clinical trials involving more than 10,000 cases. Forest plot of these studies did not identify any factors predictive of response and therefore all patients with HER2-positive breast carcinomas must be treated with trastuzumab even though it is known that halph of these patients will not benefit from this treatment. The identification of predictive factors of response is therefore mandatory. The mechanism underlying the antitumor activity of trastuzumab in vivo is still controversial. Different mechanisms have been proposed to account for its therapeutic effect including down-modulation of HER-2, activation of apoptotic signals, impairment of angiogenesis and interaction with the immune system. Analysis in animal models as well as neo-adiuvant clinical trials suggested that trastuzumab activity may depends on engagement of the Fc receptor, suggesting that Fc-dependent antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity are critical for trastuzumab efficacy.
Methods: To investigate the gene profiling of HER2-positive tumors, we selected 42 HER2-amplified breast carcinomas potentially targets of trastuzumab therapy that were profiled using cDNA microarray technology.
Results: The two groups obtained by unsupervised hierarchical clustering showed different modulation of genes belonging to ECM. One group presented the upmodulation of extracellular matrix (ECM) molecules (collagens, fibronectin, laminins) other ECM structural genes (i.e. fibulins,

SPARC) and peptidases (i.e. MMP2, cathepsins) suggesting a remodeling of tumor environments in these carcinomas. In the same group the upmodulation of genes specific of NK cells and genes involved in proliferation has been found by using the GSEA software that evaluates microarray data at the level of gene set. Markers emerged from this analysis are under evaluation on tumors section from patients who have received trastuzumab for metastatic disease and categorized as responder or not responder according to the Recist criteria with a medium follow-up of 31 months.
Conclusions: In conclusion, our reliminary results suggest that HER2-amplified breast carcinoma are a heterogenous group especially concerning extracellular matrix and infiltration composition.
Supported by AIRC.

P29
aCGH analysis of male breast cancers (MBC)

P. Chiarappa[1], A. Mangia[1], S. Tommasi[1], E. Rossi[2], F. Menolascina[1], L. Ottini[3], M. Mottolese[4], Z. Zuffardi[2], A. Paradiso[1]. *[1]National Cancer Institute "Giovanni Paolo II", Bari, Italy; [2]University of Pavia, Pavia, Italy; [3]University La Sapienza Policlinico Umberto I, Roma, Italy; [4]Instituto Regina Elena, Roma, Italy*

Background: Male breast cancer (MBC) is a rare disease whose causes are poorly understood. Information on genome alterations by CGH in MBC showed similar pattern of imbalances with female breast cancer (FBC), suggesting a common aetiology. To elucidate the somatic genetic changes of MBC we analysed a series of 30 MBC using array Comparative Genomic Hybridisation (aCGH). aCGH has been successfully used in post-genomic cancer research studies because the screening of gene copy number covers a key role in the understanding of biological pathways involved in the complex tumorigenic process.
Methods: 30 male patients who had received a primary diagnosis of breast cancer and had been analyzed for familial characteristics in Genetic Outpatients Clinics were investigated and compared with aCGH analysis from sporadic and familial FBC.
Genomic DNA was extracted from 20 mg of frozen tumor tissues using the DNeasy tissue kit (Qiagen). The reactions were checked on 0.8% agarose gel and the DNA obtained was quantified by spectrophotometry (Nanodrop, Celbio). aCGH analysis was performed using the Microarray Kit 44B (Agilent). We identified the most significant sequences altered as ones with $p < 0.0001$ and log2(ratio) value +0.5 and −0.5 for gains and losses, respectively. The data set was analysed with MATLAB software to extrapolate profiles for the 30 MBC in study.
Results: Preliminary results on 20 patients showed the presence of a wide range of chromosome alterations spanning all the genome. The most frequent chromosomes involved in gains were 8q21−24, 17q12, 20q13 in which some interesting genes map, such as PLEC1, a protein involved in cytoskeleton-membrane attachment, DOK5, an adapter protein involved in signal transduction and BCAS1, a candidate oncogene for breast cancers. The most frequent losses were on chromosomes 2p23.2, 19p13, 22q13 and Y, in which the mapped genes were: GSTT1, a Glutathione S-transferase and APOBECC3A, which has a role in growth or cell cycle control. The deletions on Y chromosome encompass TBL1Y gene and PRKY gene, whose function is important in cytotipic differentiation in males.
Conclusions: Our approach allows to identify somatic genetic changes that are specific in MBC. We think it is important to report these alterations because our data could show some possible association functions in tumour development.

P47
BRCA1, ER-alpha expression and molecular BRCA1 alterations in familial and sporadic breast cancer

A. Chiriatti, A. Mangia, S. Tommasi, F. Menolascina, S. Petroni, G. Simone, C. Salvatore, A.F. Zito, F. Schittulli, A. Paradiso. *National Cancer Institute "Giovanni Paolo II", Bari, Italy*

Background: There are major discrepancies concerning the usefulness of various antibodies in detecting BRCA1 protein expression and its subcellular localization. The aim of the present study was to evaluate the performance of immunohistochemical MS110 expression with respect to molecular BRCA1 alterations in a series of familial and sporadic breast cancer patients.
Methods: An immunohistochemical study was performed on TMA samples from 93 sporadic and 94 familial breast cancer patients with (7/94) and without BRCA1 germline mutations. In all 94 patients, BRCA1 alterations gene have been studied by dHPLC and direct sequencing. BRCA1 protein expression level has been evaluated by using the monoclonal MS110 antibody, suitable for immunohistochemical analysis of paraffin-embedded tissue sections.
Results: Immunohistochemistry cattied out using the MS110 antibody, showed positive nuclear-staining for BRCA1 protein in 34 (41%) sporadic and 37 familial (44%) breast tumours respectively. All the tumours from patients carrying BRCA1 mutations showed complete loss of both BRCA1 and ERá expression, regardeless of the type of mutation ($p = 0.02$). BRCA1 wild type expression resulted similar both in sporadic and in familial tumours, while ERá expression was higher in sporadic tumour patients (62% vs 40% in sporadic and familial cases respectively; $p = 0.012$). Interestingly, the presence of the E1038G polymorphism in BRCA1 exon 11 was significantly associated with protein expression ($p = 0.029$). Furthermore, confirming what found in previous studies, loss or reduction of both BRCA1 and ER-alpha expression in familial patients were correlated both with higher histological grade ($p < 10^{-6}$, $p = 0.004$ respectively) and lower PgR positive rate ($p = 0.001$, $p = 0.022$, respectively). No significant correlation between BRCA1 and ER-alpha expression was found in both familial and sporadic patients.
Conclusions: Lack of MS110-immunostaining is significantly associated with molecular alterations. However, the frequency of MS110 negative cases also detected in BRCA1-wild type tumours, points to the unability of the IHC-BRCA1 expression in discriminating between familial and sporadic breast cancer, further suggesting that ER-alpha more than BRCA1 could be associated with sporadic breast cancer.

P42
Treatment of locally advanced head-and-neck squamous carcinomas with chemotherapy alternated to radiation and Cetuximab (ALTERCC Phase II study)

I. Colantonio, G. Numico, N. Crosetto, C. Lo Nigro, M. Merlano. *Ospedale "S. Croce e Carle", Cuneo, Italy*

Background: The gold-standard in the treatment of locally advanced squamocellular carcinomas is the combination of cisplatin-based chemotherapy with radiation therapy. To improve the outcome of this approach, the anti-EGF receptor (EGFr) monoclonal antibody C225 (Cetuximab) has been used with radiation alone or chemo-radiotherapy. We have initiated a phase II trial to evaluate the feasibility and activity of a regimen containing Cetuximab and radiation alternated to cisplatin-based chemotherapy (ALTErnating Radiotherapy and Chemotherapy plus Cetuximab, ALTERCC).
Methods: Chemotherapy (Cisplatin 20 mg/mq/day for 5 days plus 5-FU 200 mg/mq/day for 5 days) was given in weeks 1, 4 and 7, while radiation (10 Gy over 5 fractions, 1 fraction per day) in weeks 2, 3, 5, 6, 8, 9. Cetuximab was given at 400 mg/mq loading dose followed by 250 mg/mq weekly, concomitantly with the radiotherapy. Most patients (82%) had a disease stage IV and 92% had nodal involvement. All cases had primary tumours of either the oral cavity, oro-pharynx, hypo-pharynx or larynx, that were immune-positive for EGF receptor when analysed by immune-histochemistry.
Results: To date, we have treated 35 patients. Out of 23 measurable responses, on an intent-to-treat analysis, 15 (65%) were complete and 5 (22%) were partial. Two patients included in the study died, one patient refused treatment. No patient underwent progression during the treatment. Overall, the regimen was tolerable and characterized by the same spectrum of toxicities as in conventional regimens of combined chemo- and radiotherapy. Grade 3−4 neutropenia and mucositis were the most frequent adverse effects and were seen in 13 and 16 out of 24 cases, respectively. Interestingly, two thirds of the patients (18/24 = 75%) developed a benign, humid epidermo-lysis of the neck, which spontaneously regressed after one week of topic nursing procedures. This toxic effect is probably radiation-related and never appeared before an administered cumulative dose of 45 Gy. However, a cutaneous toxic interaction between Cetuximab and fluorouracile is also possible.
Conclusions: All together, our data demonstrate the feasibility and activity of Cetuximab plus radiation alternated to chemotherapy in patients with locally advanced head-and-neck squamocellular carcinomas. Our results also prompt the implementation of phase III studies to compare the ALTERCC protocol with conventional regimens of cisplatin-based chemotherapy combined with radiation.

P43
Molecular features in locally advanced head-and-neck squamous carcinomas might predict response to treatment with chemotherapy alternated to radiation and Cetuximab

N. Crosetto, R. Cusano, P. Catarsi, R. Lantermo, A. Comino, M. Merlano, C. Lo Nigro. *Ospedale "S. Croce e Carle", Cuneo, Italy*

Background: The anti-EGF receptor (EGFr) monoclonal antibody C225/Cetuximab has shown a promising activity in patients with locally advanced head-and-neck squamocellular carcinoma (HNSCC), when