

## Poster Sessions

### Poster session A: Cancer cell biology

#### A01

#### CO-EXPRESSION PATTERNS OF TUMOR-ASSOCIATED ANTIGEN GENES BY NON-SMALL CELL LUNG CARCINOMAS: IMPLICATIONS FOR IMMUNOTHERAPY

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**Background:** Polyvalent vaccination represents a recent attempt to improve the effectiveness of lung cancer immunotherapy. This study aimed to investigate whether a gene expression pattern of tumor-associated antigens (TAA) would exist indicating that their use will be most appropriate for the polyvalent vaccination of Caucasian non-small cell lung carcinoma (NSCLC) patients. We examined the concomitant expression of genes belonging to different TAA families for which expression frequencies either have never been detected in NSCLC or vary widely in the literature.

**Methods:** Tumor material from 23 patients with NSCLC (12 adenocarcinomas, 8 squamous cell carcinomas, 3 bronchoalveolar carcinomas) was examined. mRNA transcripts were detected for 5 genes of the survivin family, 5 MAGE-A genes as well as the genes of human telomerase reverse transcriptase (hTERT) and p53, by the use of quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) or semi-quantitative RT-PCR.

**Results:** 15/23 (65%) and 8/23 (35%) tumor samples were found expressing 6-11 and 2-5 out of the 12 examined TAAs, respectively, at levels >1% of the testis reference sample. The most prevalent TAA patterns observed were those of survivin standard (survivin-std)/survivin-2B expressed by 22/23 (95.5%) tumor samples and of survivin-std/survivin-2B/hTERT expressed by 19/23 (82.5%) tumor samples. The

expression levels of the survivin-std gene strongly positively correlated to those of the survivin-2B (p=0.001) and the hTERT genes (p=0.031). The number of concomitantly expressed genes was found to be positively correlated to the age of the patients (p=0.001) and the tumor size (p=0.048).

**Conclusion:** This study provides evidence that, in Caucasian patients with NSCLC, highly prevalent expression patterns of TAA genes, predominantly of overexpressed TAAs, do exist. This result implies that the combined use of these TAA could help in designing more effective NSCLC immunotherapeutic protocols.

#### A02

#### LOW-FREQUENCY CD8+ T CELL PRECURSORS SPECIFIC FOR SURVIVIN AND SURVIVIN-2B IN CANCER PATIENTS: A CAVEAT FOR IMMUNOTHERAPY?

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**Background and aims:** We measured, in patients with lung carcinomas expressing transcripts of Survivin and its variant survivin-2B, as well as in healthy individuals, the frequency of circulating pCTLs specific for two naturally processed and presented peptides of these proteins. Their magnitude has never been evaluated, despite being implicated in the effectiveness of immunotherapy.

**Methods:** Twenty patients with primary lung cancer and 5 aged matched apparently healthy male individuals, all expressing HLA-A2 and/or -A24, were studied. The frequency of peripheral blood peptide-specific pCTLs was estimated using the most sensitive amongst the available methods that combines HLA-multimer flow cytometric technology with a previous step of in vitro amplification under limiting dilution conditions. Peptides of Epstein-Barr virus and human telomerase reverse transcriptase were used as controls.

**Results:** Anti-survivin or anti-survivin-2B specific CTL clones were not detected in 10 out of the 13 tested patients and in none of the healthy individuals. In a number of peripheral blood mononuclear cell microcultures of the remaining 4 patients, we observed diffuse clusters stained weakly by the HLA-multimers.

These clusters were not amplified after further stimulation and, therefore, were finally considered as negative.

**Conclusion:** The absence of detectable naturally occurring CTL-responses, in both patients with lung cancer and normal subjects, at least against the peptides of survivin and survivin-2B currently utilized in anti-cancer vaccines, despite the expression of both antigens by the tumor samples, might indicate that their applicability in immunotherapeutic protocols should be reconsidered.

### A03

#### QUANTITATIVE AND QUALITATIVE ASSESSMENT OF ANTI-TUMOUR SPECIFIC T CELLS IN PATIENTS WITH LUNG CANCER AND CANCER FREE INDIVIDUALS

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**Background and aims:** We measured, in patients with lung carcinomas and healthy individuals, the frequency and qualitative characteristics of circulating precursor cytotoxic T lymphocytes (pCTLs) specific for naturally processed and presented peptides of human telomerase reverse transcriptase (hTERT) and MAGE-A3. Their magnitude as well as their functional properties, have not been thoroughly evaluated in lung cancer despite being used for the design of immunotherapeutic protocols.

**Methods:** Twenty patients with primary lung cancer and 5 aged matched apparently healthy male individuals, all expressing HLA-A2 and/or -A24, were studied. The frequency of peripheral blood peptide-specific pCTLs was estimated using the most sensitive amongst the available methods that combines HLA-multimer flow cytometric technology with a previous step of in vitro amplification under limiting dilution conditions. Peptides of Epstein-Barr virus were used as controls. Tetramer positive populations were sorted and clones generated. These were studied with respect to their TcR, cytokine profile and anti-tumour specific lytic ability.

**Results:** The estimated frequency of circulating pCTL against all tumour peptides studied was much higher in patients than cancer-free individuals. However, the isolated clones from both groups did not display any different characteristics with respect to secretion of cytokines, multiplication time, phenotype and lytic capacity. Conversely, when the phenotypic

characteristics of the in vitro amplified cell populations that contained tetramer positive cells was examined, a difference with respect to the differentiation profile according to staining with CD45RA and CCR7 was observed.

**Conclusion:** Patients with lung cancer and healthy individuals contain in their blood anti-tumour specific CTL clones. These do not appear to have any significant functional difference, questioning what their true role in vivo and how amplifying them by the application of various immunostimulating protocols could prove beneficial for patient outcome.

### A04

#### MODULATORY EFFECTS OF TRYPTANTHRIN ON THE MURINE MYELOID LEUKEMIA CELLS

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Conventional treatments against leukemia often cause severe adverse side effects, hence there has been an increasing interest on the development of novel tactics for leukemia treatments. Tryptanthrin (6,12-dihydro-6,12-dioxoindolo-(2,1-b)-quinazoline) is a naturally-occurring, weakly basic alkaloid isolated from a number of medicinal indigo plants, including *Polygonum tinctorium*, *Isatis tinctoria* and *Strobilanthes cusia*. It has been reported to have various biological and pharmacological activities, such as anti-microbial, anti-inflammatory, anti-allergic and anti-tumor effects. However, its modulatory effects and action mechanisms on leukemia cells remain poorly understood. In this study, tryptanthrin was examined for its modulatory effects on the proliferation, differentiation and apoptosis of the murine myeloid leukemia WEHI-3B JCS cells. Using the 3H-thymidine uptake assay, tryptanthrin was shown to suppress the proliferation of JCS cells in a dose- and time- dependent manner with an estimated IC<sub>50</sub> of 1.5  $\mu$ M at 48 h. However, it exhibited no significant direct cytotoxicity on the normal murine peritoneal macrophages and human hepatocyte-like WRL-68 cells. Flow cytometric analysis of the tryptanthrin-treated JCS cells showed an obvious cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase. The expression of cyclin A, cyclin D2, Cdk 4 and Cdk 6 genes in JCS cells was found to be down-regulated at 24 h as measured by RT-PCR. Moreover, tryptanthrin triggered apoptosis in the leukemia JCS cells, as evidenced by the induction of DNA fragmentation, phosphatidylserine externalization, and mitochondrial membrane depolarization after 18 h of tryptanthrin treatment. A time- and dose-dependent

activation of caspases 3, 8, 9 activities was also observed in tryptanthrin-treated JCS cells. Further mechanistic studies showed that tryptanthrin treatment up-regulated the expression of pro-apoptotic Bax and Bad genes but down-regulated the anti-apoptotic Bcl-2 and Bcl-xL genes in JCS cells. Interestingly, morphological and flow cytometric studies revealed that tryptanthrin induced the differentiation in JCS cells, as indicated by the increases in vacuolization, cell size and granularity. Collectively, our results suggest that tryptanthrin may exert its anti-leukemic activities through inhibition of cell proliferation, induction of leukemic cell differentiation and triggering of apoptotic cell death.

#### A05

### THE ALCAM/CD166 ECTODOMAIN CLEAVAGE BY THE METALLOPROTEASE ADAM17/TACE IS A TYROSINE KINASE-INDUCIBLE PROCESS INVOLVED IN INVASIVENESS BY OVARIAN CARCINOMA CELLS

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The activated leukocyte cell adhesion molecule (ALCAM) is expressed by tumors and plays a relevant role, since the loss of surface ALCAM expression may result in reduced cell aggregation and in metastatic growth. Here we show that ALCAM is shed from epithelial ovarian cancer (EOC) cells in vitro, leading to the generation of a soluble ALCAM (sALCAM), consisting of most of the extracellular domain. A similar sALCAM molecule was also found in the ascites and sera from EOC patients. sALCAM is constitutively produced by EOC cells and this process can be enhanced by cell treatment with pervanadate, or EGF, a growth factor for EOC. Pharmacological inhibitors of matrix metalloproteases (MMPs) and of a disintegrin and metalloproteases (ADAMs), and the tissue inhibitor of metalloproteases TIMP-3, significantly inhibited sALCAM release by EOC cells. The ADAM17/TACE (TNF-alpha converting enzyme) molecule was expressed in all EOC cell lines tested and ADAM17/TACE silencing by specific siRNA reduced ALCAM shedding. To visualize the involvement of MMP/ADAM in EOC cell motility, we utilized a wound-healing assay in the presence of EGF, which

showed that the ADAM inhibitor CGS yielded 40±10% (mean±SD of four experiments) inhibition of EOC cell migration. Conversely, an antibody blocking ALCAM adhesive functions and inducing ALCAM internalization enhanced EOC cell motility. Altogether our data indicate that ADAM17/TACE is responsible for the shedding of ALCAM in EOC, a process that may be relevant to its invasive potential. Indeed, decreased/lost ALCAM membrane expression can be a marker of poorer outcome in EOC patients and is associated with loss of cell-anchorage both in vitro and in vivo. Indeed, preliminary data indicate that ALCAM may also be involved in interaction with the tumor matrix. These data indicate that ADAM17/TACE may represent a suitable target molecule to inhibit ovarian cancer invasiveness.

#### A06

### ESTABLISHMENT OF NSCLC XENOGRAPTS WITH DIFFERENT VASCULOGENESIS

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**Background:** Vascular endothelial growth factor is a key mediator of new blood vessels formation (vasculogenesis) and tumor growth promotion by invitation of endothelial progenitor cells (EPC). Both tumor growth and vessels development are correlated with number of EPC. In non-small cell lung cancer (NSCLC), the majority of studies support correlation between VEGF expression, microvessel density, and poor survival prognosis.

**Aim:** Establishment of NSCLC tumor xenografts in Nude rat model suitable for in vivo PET/CT and MRI investigations of neoangiogenesis and new target therapy applications.

**Material and methods:** The relevant literature in the field of transplantation and own data about tumor Xenografts established in Nude rats model with H1299 and A549 lung cancer cell lines expressing different amount of VEGF are reviewed.

**Results:** The source of EPC of xenografts and clinical tumors is different. Irradiation, applied for immunosuppression is well known to diminish the number of bone-marrow stem cells. Resulting xenografts are not comparable to the clinical data due to significant necrosis. Bone marrow transplantation (BMT) or EPC injection could reduce radiation effects. However, radiation may be substituted by certain chemical compounds traditionally used in organ transplantation eliminating specific groups of cells. In

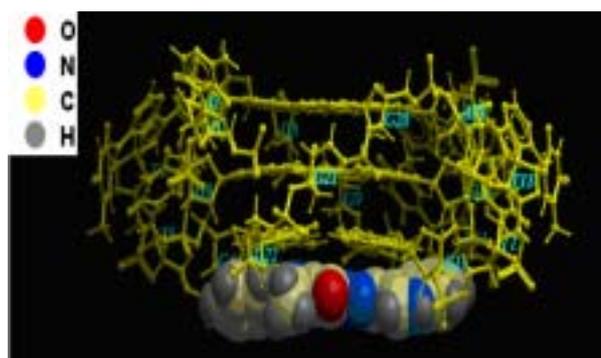
Nude rats (T-cell deficient) xenoantibody formation (B-cells) may be blocked by Leflunomide and Natural killer cells (NK) may be depleted by addition of an anti-NK cell serum without changing of EPC number. Established NSCLC Xenografts in rat model are suitable for PET/CT or MRI studies using clinical equipment.

**Conclusion:** Combination of different immunosuppression methods together with VEGF and EPC administration allows establish tumor Xenograft models with wide range of vasculogenesis. Tumors with different vasculogenesis reflecting to angiogenic stimuli or antiangiogenic therapy are suitable for PET/CT or MRI and translation of these examinations into the clinic.

**A07**  
**DISCOVERY OF DRUG-LIKE HIT OF G-QUADRUPLEX BINDING LIGAND BY HIGH-THROUGHPUT DOCKING**

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There has been considerable interest in the study of G-quadruplex DNA due to its involvement in the regulation of telomerase activities. Human telomeric DNA is composed of a repeated double-stranded [TTAGGG/CCCTAA]<sub>n</sub> sequence except in the 3'-terminal region, which consists of a single-stranded tandem [TTAGGG] repeated sequence over several hundred bases. In normal somatic cells, approximately 100 bases will be lost during every cell division, and after reaching a critical shortening of the telomere, the cell undergoes apoptosis. In cancer cells, telomeric length is maintained by telomerase, and telomerase activity is expressed in over 90% of tumor cell lines but in relatively few normal cell types. Thus, the inhibition of telomerase activity by ligand-induced stabilization of G-quadruplex has therefore become an attractive strategy for developing new anti-cancer drugs. In order to develop a high-throughput platform for G-quadruplex DNA stabilizing ligands, a computer model has been constructed by using the X-ray crystal structure of the intramolecular human telomeric G-quadruplex DNA. Preliminary results indicated that some of the small molecules found through in silico screening are potential stabilizers for G-quadruplex DNA with telEC50 (effective concentration that inhibited 50% of the telomerase activity vs a drug-free control) in the micro-molar concentration range.



*Diagram of G-quadruplex DNA stabilizing ligand*

**A08**  
**SOCS-2 EXPRESSION IN MCF-7 AND MDA-MB-231 CELLS**

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We used two breast carcinoma cell lines having different genetic and biologic characteristics to investigate the relation between SOCS-2 expression, EGFR and cell proliferation. SOCS-2 is a member of the Suppressors of Cytokine Signalling (SOCS) protein family, a group of proteins induced by interleukins (ILs) and various peptide hormones that may prevent sustained activation of JAK-STAT and other signalling pathways. In a previous study on 50 breast carcinoma samples, we found that SOCS-2 expression was inversely correlated with EGFR presence and high proliferation indicators. To better define the role of SOCS-2 in breast cancer, we stably transfected MCF-7 and MDA-MB-231 breast carcinoma cells with SOCS-2 cDNA. We compared EGFR presence in control (CTRL) and SOCS-2 transfected clones (trSOCS-2) by immunofluorescence and flow cytometry. Cell growth and S-phase were studied by MTT and [3H]thymidine incorporation assay. trSOCS-2 MCF-7 only showed that high SOCS-2 expression was related to loss of EGFR on the plasma membrane and reduced cell growth (MTT assay  $p < 0.01$ ). In contrast, MDA-MB-231 clones did not show any difference in EGFR presence, distribution and cell growth. We suggest that the reduced MCF-7 cell growth in presence of high SOCS-2 expression may be related with EGFR redistribution, possibly attenuating EGFR signalling pathway. These findings suggest a role of SOCS-2 in reducing cell growth in breast carcinoma cells. SOCS-2 function might be differentially regulated in breast carcinoma cells.

**A09**  
**TOPOTECAN ANTAGONIZES HYPOXIA EFFECTS ON GENE EXPRESSION OF NEUROBLASTOMA TUMORS**

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Angiogenesis is essential for neuroblastoma tumors development and correlates with their aggressive behavior, metastatic spread, and poor clinical outcome. One of the major drives to tumor angiogenesis is hypoxia, a local decrease in oxygen tension that characterizes solid tumors. Transcriptional activation by hypoxia is mediated primarily by the hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor that transactivates the hypoxia responsive elements (HRE) in the promoter sequence of many genes. We investigated the effects of topoisomerase-1 inhibitor, topotecan, on neuroblastoma cell lines exposed to hypoxia. Using Affymetrix GeneChip we studied the changes in gene expression profile of neuroblastoma cell line LAN-5 caused by exposure to hypoxia and by topotecan treatment. Cells were exposed to hypoxia (1% oxygen) or normoxia (20% oxygen) and treated with 500 nM topotecan for 6 hours. The gene expression data and the presence of the HRE in the promoter sequences were analyzed using GeneSpring GX 7.3 software. We found that 60% of the genes upregulated by hypoxia are not regulated when the cells are treated with topotecan in hypoxic condition and 39% of the genes are even downregulated. This means that 99% of the genes generally induced by hypoxia are affected by topotecan treatment. A gene ontology analysis reveals that most of the genes upregulated by hypoxia but downregulated by topotecan are belonging to the pathways of apoptosis, angiogenesis, cell cycle regulation, and signal transduction. Furthermore, we found a statistical significant enrichment of the presence of HRE in the promoter region of the genes downregulated by topotecan. In fact, 80% of the genes show the consensus sequence of HRE in their promoter region confirming the regulation of HIF-1 function by topotecan. Our data indicate that topotecan prevents the hypoxia effects on gene expression and that pharmacological inhibition of HIF-1 is a promising therapeutic approach for the reduction of neuroblastoma angiogenic potential.

**A10**  
**THE N-TERMINAL BH4 DOMAIN OF BCL-2 IS REQUIRED FOR THE PROANGIOGENIC PROPERTIES OF BCL-2**

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The classic function of bcl-2 is that of a prosurvival protein. Recent studies have demonstrated that bcl-2 not only functions as a survival molecule but it can also promote tumor progression and angiogenesis. However, the molecular mechanism by which bcl-2 induces angiogenesis is poorly understood. In this context, we have previously demonstrated that forced overexpression of bcl-2 in melanoma cells exposed to hypoxia enhances angiogenesis through Hypoxic Inducible Factor (HIF)-1-mediated-Vascular Endothelial Growth Factor (VEGF) expression. Herein, using conditioned medium from cells overexpressing bcl-2 wild-type or lacking the BH4 domain, we showed that the BH4 domain of bcl-2 protein is required for the proangiogenic activity of bcl-2. In fact, while bcl-2 wild-type overexpressing cells exposed to hypoxia increased *in vitro* and *in vivo* angiogenesis, cells overexpressing bcl-2 lacking the BH4 domain showed angiogenic potential similar to that observed in control cells. We also observed that the deletion of the BH4 domain abrogated the ability of bcl-2 to synergize with hypoxia to induce VEGF and HIF-1 $\alpha$  expression and HIF-1 transcriptional activity in melanoma cells, suggesting that the BH4 domain of bcl-2 is a requirement for the enhancing effect on bcl-2 induced angiogenesis through VEGF and HIF-1.

Furthermore, we also showed that the BH4 domain fused to the protein transduction domain of HIV-TAT protein had a significant effect on VEGF production in human melanoma cells exposed to hypoxia. Finally, we evaluated the relevance of other BH domains in bcl-2-induced HIF-1 $\alpha$  expression under hypoxic conditions. In particular, we analysed the effect of mutations of the highly conserved residues into BH1 (G145E) and BH2 (W188A) domains, which abrogate the ability of bcl-2 to dimerize with BH3 domain of proapoptotic proteins. We found that bcl-2 protein carrying one of the two mutations did not synergize with hypoxia to increase HIF-1 $\alpha$ , as observed after overexpression of wild type bcl-2 protein.

In conclusion, our results provided evidence for the regulation of angiogenesis by bcl-2 through a mechanism that requires the BH4 domain of bcl-2, and they may be useful to permit the design of new agents to

block tumor angiogenesis by interrupting the function of the BH4 domain.

**A11  
HPV-INFECTION IN ORAL SQUAMOUS CELL  
CARCINOMA IN RELATION WITH S-PHASE  
KINASE-INTERACTING PROTEIN 2 AND  
P27KIP1 PROTEIN EXPRESSION**

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High risk (HR) human papillomavirus (HPV) infection could play an important role in the pathogenesis of a proportion of oral squamous cell carcinomas (OSCCs), leading to consider HPV-positive OSCCs as a distinct clinicopathological entity with different outcome. The carcinogenic role of HR-HPV in this group of tumours could be related to specific mechanisms of cell-cycle deregulation promoted by the viral oncoproteins, respectively HPV-E6, -E7 and -E5. Reduced expression levels of p27Kip1, a cyclin-dependent kinase inhibitor, is associated with high aggressiveness and poor prognosis of various malignant tumors, including squamous carcinoma. It has been demonstrated that S-phase kinase-associated protein 2 (Skp2), a member of the F-box family proteins, is responsible for p27Kip1 ubiquitination and degradation. We examined the expression of Skp2 in OSCC histological samples grouped by HPV infection and its correlation with HPV infection and p27Kip1 expression.

Methods: 63 paraffin-embedded biopsies of OSCC were previously tested for HPV-DNA by nPCR (MY09/MY11 and GP5+/GP6+ primers) and then undergone immunohistochemical evaluation. Results: 24/63 (38,1%) of samples shown the presence of HPV DNA: HR HPV-18 and 16 were the most frequently found genotypes (respectively 9/24 and 8/24 cases), other genotypes were HPV-6 (4/24) and HPV-53 (3/24). Based on immunohistochemical evaluation, 30/63 (48%) OSCC samples presented significantly high expression of Skp2 with a reduced histological differentiation state and no significant difference with respect to HPV infection, whereas p27Kip1 expression was high in 14/63 (22%), moderate in 17/63 (27%) and low in 32/63 (51%) and significantly related to HPV infection. Moreover, in HPV-negative OSCC tumors we found an inverse correlation between Skp2 and p27Kip1 protein expression, behaviour not revealed in HPV-positive cases. Our data suggest that Skp2 may contribute to the progression of OSCC, however

p27Kip1 is unlikely to be the major target protein contributing to malignant progression in HR-HPV-positive OSCCs.

**A12  
TRANSKETOLASE-LIKE 1 EXPRESSION IN  
PAPILLARY THYROID CARCINOMAS  
SMALLER THAN 1.5 CM**

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Malignant tumours are able to ferment glucose to lactate even in the presence of oxygen (Warburg effect). In the non-oxidative part of the pentose phosphate pathway (PPP), controlled by transketolase enzyme, glucose is degraded into ribose for nucleic acid synthesis. Transketolase-like-1 (TKTL1) is a transketolase-related gene which encode a transketolase with unusual enzymatic properties. TKTL1 has been found overexpressed in several epithelial cancers and it has been associated with aggressiveness and poor patient survival. Patients with small papillary thyroid carcinoma (PTC) have a high incidence of regional lymph node (LN) metastases at presentation which are considered an independent risk factor for tumor recurrence. The aim of our study was to investigate the expression of TKTL1 protein in a series of PTCs  $\leq$  1.5 cm and its relationship with the presence of LN metastases. We have taken into consideration 256 consecutive cases of PTCs  $\leq$  1.5 cm and we have analyzed TKTL1 protein expression by immunohistochemistry using a specific anti-TKTL1 antibody. Immunoreactivity for TKTL1 was seen in the majority of primary tumors, and in lymph nodal metastatic tumor tissues. In the adjacent non-neoplastic tissues, as well as in control healthy samples expression of TKTL1 was not detected. Examination of the TKTL1 protein expression in primary tumors did not show any significant differences for sex, age, tumor diameter, encapsulation and clinical signs. Statistical analysis demonstrated a significant association between TKTL1 protein expression and the presence of LN metastases. Differences were not found in TKTL1 protein expression in relation to primary tumors and their paired LN metastases or to compartment involved, whereas a positive correlation was found between the TKTL1 protein expression in primary tumors and the number of metastatic LNs, as well as between the TKTL1 protein expression and the diameter of the largest metastatic area of LNs. Our results suggest that TKTL1 is involved in papillary thyroid cancer metabolism and that TKTL1

overexpression may be considered as factor facilitating tumor growth and progression in PTCs  $\geq 1.5$  cm.

**A13**  
**USE OF SALIVA AS A BIOLOGICAL MEDIUM**  
**IN NON-INVASIVE SCREENING FOR ORAL**  
**CANCER**

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**Background:** An early detection is critical to reduce the mortality from oral squamous cell carcinoma (OSCC), but there is insufficient evidence to support the effectiveness of current screening methods (visual examination, toluidine blue, brush biopsy, fluorescence imaging). Molecular markers could be useful as adjunctive screening tool, and proteomics could allow the discovery of new OSCC biomarkers. One advancement in proteomic analysis is the surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), that allows the generation of an accurate protein profile from minimal amounts of biological samples. As a non-invasive method, salivary diagnostics is highly attractive. We aimed to compare the proteomic profile, obtained using the SELDI-TOF-MS technology, of saliva from patients with OSCC and healthy subjects.

**Methods:** Whole saliva was collected from 43 patients with OSCC and 30 healthy subjects. Specimens were centrifuged (10 min, 13000 g); the Q10 ProteinChips were prepared according to the manufacturer's instructions and were loaded with the supernatants. A saturated solution of sinapinic acid was used as energy-absorbing matrix. The analysis was performed in a m/z range from 2500 to 25000 Da, and the proteomic profiles was compared by a specific data analysis software.

**Results:** The average intensities of peaks at m/z 8048, 6243, 5260, 8007, 19667, 5324, 6108, 7316, 6320, 8090, 10650, 23497 Da were significantly higher in OSCC patients than healthy controls, while peaks at m/z 3483 and 6919 Da showed a higher intensity in healthy controls.

**Conclusion:** Qualitative differences were noted between patients with OSCC and healthy controls with regard to the salivary proteomic profile obtained using the SELDI-TOF-MS technology. These data suggest that the proteomic analysis of saliva is a promising new tool for a non-invasive screening for OSCC.

**A14**  
**DMXAA [ASA404], A NOVEL TUMOUR**  
**VASCULAR DISRUPTING AGENT UNDER**  
**CLINICAL INVESTIGATION IN LUNG AND**  
**PROSTATE CANCERS**

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DMXAA (ASA404) belongs to the class of small-molecule tumour vascular disrupting agents (VDAs), which target established tumour blood vessels. DMXAA has a distinctive mechanism of action involving disruption of the actin cytoskeleton of tumour vascular endothelial cells and localised release of cytokines within the tumour. It induces apoptosis of tumour vascular endothelial cells, inhibits tumour blood flow, and ultimately causes haemorrhagic necrosis of the tumour core. In animal studies, DMXAA demonstrated synergistic anti-tumour effects in combination with cytotoxics, in particular with taxanes. Phase II clinical trials have therefore combined DMXAA with taxane-based regimens.

Development of DMXAA is currently ongoing in non-small cell lung cancer and hormone-refractory prostate cancer.

A randomised phase II trial in non-small cell lung cancer enrolled chemotherapy-naïve patients with stage IIIb or IV disease of any histology. 73 patients were treated with carboplatin and paclitaxel with or without 1200 mg/m<sup>2</sup> DMXAA. Patients receiving DMXAA had a higher RECIST response rate (31.2 vs 22.2%), longer time to tumour progression (5.4 vs 4.4 months) and longer median survival (14.0 vs 8.8 months) than patients receiving chemotherapy alone. Most adverse events were those typically associated with chemotherapy and occurred with similar frequency in the DMXAA and chemotherapy alone arms. There was no discernable difference in safety findings between patients with squamous and non-squamous histologies. These findings were corroborated by a single arm study in which 30 patients with similar characteristics received carboplatin, paclitaxel and 1800 mg/m<sup>2</sup> DMXAA. The response rate was 38% and median survival was 14.9 months. A phase III trial is now planned.

In a randomised phase II trial in hormone-refractory prostate cancer, 71 chemotherapy-naïve patients received docetaxel with or without 1200 mg/m<sup>2</sup>

DMXAA. Prostate specific antigen (PSA) response rates were 59% in patients receiving DMXAA and 37% in those receiving docetaxel alone. Fewer patients receiving DMXAA experienced an increase in PSA than those receiving docetaxel alone (16% versus 37%, respectively). Addition of DMXAA to docetaxel was generally well-tolerated. Follow-up for survival is ongoing. Findings to date suggest that DMXAA has the potential to improve outcomes without adding unacceptable toxicity in patients receiving chemotherapy for various solid tumours.

### **A15 N-MYC GENE EXPRESSION: IMPACT ON LEUKOCYTE INFILTRATION IN 3D NEUROBLASTOMA SPHEROIDS**

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**Background:** High N-myc expression is associated with advanced neuroblastoma stage and poor prognosis, but the relationship between N-myc and immunity has remained obscure. Multicellular neuroblastoma spheroids are a 3D in vitro model system that can reflect the pathophysiological in vivo situation of avascular neuroblastoma microregions and micrometastatic sites; in particular the core region of spheroids well mimics hypoxic conditions of neuroblastoma.

**Methods:** To investigate whether N-myc gene expression together with hypoxia affects leukocyte infiltration we used 72 hours cocultures of peripheral blood mononuclear cells (PBMCs) and preformed 4 days old SHEP21N spheroids. Both the starting spheroids culture and the following spheroids-PBMCs coculture was grown with (N-myc<sup>-</sup>, bare SHEP21N N-myc expression) or without (N-myc<sup>+</sup>, high SHEP21N N-myc expression) tetracycline. The distribution of hypoxia and leukocyte infiltration was determined from 5- $\mu$ m-thick paraffin-embedded spheroids sections using respectively monoclonal antibodies to pimonidazole (Hypoxiprobe kit) together with antibodies to hypoxic markers (e.g. HIF1a, HIF2a, VEGF, CaIX) and

appropriate leukocyte-specific antibody (CD3, CD20 or CD68). Moreover using Affymetrix GeneChip we studied the differences in gene expression profile of N-myc<sup>+</sup> and N-myc-SHEP21N spheroids. The gene expression data were analyzed using GeneSpring GX 7.3 software.

**Results:** We found that distribution of hypoxic regions and hypoxic markers was similar both in N-myc<sup>+</sup> and N-myc<sup>-</sup> SHEP21N spheroids while infiltration of leukocytes, especially macrophages, was detectable only into N-myc<sup>-</sup> SHEP21N spheroids. Regarding gene expression experiments we found that some chemokines (e.g. CXCL12 and CXCL14) were upregulated in SHEP21N spheroids that barely express N-myc gene relative to SHEP21N spheroids with high N-myc expression.

**Conclusions:** Our data suggest a negative correlation between overexpression of a prognostically relevant oncogene, N-myc, and leukocyte infiltrate into neuroblastoma tumors. Therefore N-myc mediated tumorigenesis may be coupled with mechanisms of immune escape that are dependent on N-myc involvement in the regulation of immunologically relevant genes.

### **A16 ISOLATION, CHARACTERIZATION AND COMPARISON OF CANCER STEM CELLS OBTAINED FROM LOW-GRADE ASTROCYTOMAS AND THEIR RECURRENCES**

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Tamara Ius<sup>2</sup>, Marco Vindigni<sup>2</sup>, Antonio Paolo Beltrami<sup>1</sup>,  
Daniela Cesselli<sup>1</sup>, Miran Skrap<sup>2</sup>, Carlo Alberto Beltrami<sup>1</sup>  
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**Background:** At the present, pathological and clinical criteria for the prediction of relapse expectation after surgery, of low-grade astrocytomas, are suboptimal.

**Aims:** The purpose of our study was to obtain and compare cancer stem cells (CSCs) from primitive, low grade astrocytomas (n=2) and their respective recurrences (n=2), in order to identify markers associated with tumor progression.

**Methods and Results:** Primitive grade II astrocytomas and their respective relapses were obtained by surgical resection at the Neurosurgical Unit of the Azienda Ospedaliero-Universitaria of Udine (Italy). Neoplasms were mechanically-enzymatically dissociated and cells cultured in adhesion following an optimized method for

the expansion of multipotent adult stem cells. Each obtained cell line was characterized, *in vitro*, by tumorigenicity, as assessed by loss of contact inhibition (soft agar assay, transformation foci assay, growth curve analysis). Moreover, their stemness was confirmed by the analysis of their surface immunophenotype (CD13<sup>high</sup>/CD59<sup>high</sup>/CD49b<sup>high</sup>/CD90<sup>high</sup>/CD73<sup>low</sup>/CD44<sup>low</sup>/HLA-

ABC<sup>low</sup>/CD29<sup>low</sup>/CD105<sup>low</sup>/KDR<sup>low</sup>/CD49a<sup>low</sup>/CD49d<sup>low</sup>/CD14<sup>neg</sup>/CD45<sup>neg</sup>/CD38<sup>neg</sup>/HLA-DR<sup>neg</sup>/CD133<sup>neg</sup>/CD117<sup>neg</sup>/CD34<sup>neg</sup>), stem cells transcriptional markers expression (Oct-4, Nanog and Sox-2) and multilineage differentiation capacity.

Cell lines obtained from relapsed tumours showed, with respect to primitive ones, a significant increase, both in CD133 (12,05 vs 0,3%), and in CD117 expression (3,54 vs 0,16%).

**Conclusions:** Low grade glioma recurrence is characterized by an increase in the cancer stem cell population expressing CD133 and CD117. This support the notion that cancer stem cells are responsible for self-maintenance, regrowth and cancer progression.

#### A17

### ISOLATION AND CHARACTERIZATION OF CANCER STEM CELLS PRESENTS IN HUMAN LIVER HEPATOCELLULAR CARCINOMA

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**Background:** Hepatocellular carcinoma (HCC) represents the ultimate event of viral- or metabolic-related chronic liver processes, characterized by a gradual accumulation of genetic mutations. Recently, these alterations have been shown to occur not only in the mature parenchymal cells, but also in the resident stem cell compartment.

Histologically, the multistep process of hepatocarcinogenesis is a continuum of lesions, in which dysplastic nodules have been suggested to behave as pre-neoplastic lesions.

**Aims:** Purpose of this project was to isolate, expand *in vitro*, characterize, and compare stem cells obtained from normal livers (n=17) and from three different regions of neoplastic cirrhotic livers (n=12): the neoplastic area (N), the region bordering the neoplasia (C1) and a remote, macroscopically non-neoplastic region (C2).

**Methods and Results:** Utilizing a method optimized in our lab to grow multipotent-adult-human stem cells, we obtained cell lines from every normal and pathological specimen. Human liver stem cells (hLSCs) and human liver cancer stem cells (hLCSCs) shared a similar mesenchymal immunophenotype, expressing high levels of CD90 and CD105 and being mainly negative for CD34, CD45 and CD117. Interestingly, FACS analysis showed an increased expression of CD29, CD49a and CD49b in hLCSCs than in hLSCs. Regarding pluripotent state-specific transcription factor expression, every evaluated cell line showed high levels of OCT-4 (97,9±1,6%), SOX-2 (97,28±2,1%) and Nanog (99,28±0,9%). In contrast they were almost negative for hepato-specific markers such as cytokeratins (0,5±0,4%). hLCSCs were characterized, with respect to hLSCs, by a more restricted multipotency, failing to differentiate into glial or neural derivatives. Importantly, every tested hLCSC line possessed *in vitro* tumorigenicity, being able to grow in soft agar. Moreover, even cell lines obtained from regions distant from the neoplasia were able to grow embedded in soft agar. These areas were morphologically characterized by the presence of dysplastic nodules in the absence of overt neoplasia.

**In conclusion:** We have isolated hLCSCs cells from livers affected by HCC; they possess both stemness and *in vitro* tumorigenic characteristics. The tumorigenicity of C1/C2-derived cell lines could be envisioned as supporting evidence for the presence, within dysplastic lesions, of cells already showing properties typical of cancer stem cells.

#### A18

### MORPHOLOGICAL AND QUANTITATIVE CHARACTERIZATION OF ENDOCYTOSIS ABILITY AND MICROCELLS DEVELOPMENT IN HELA CANCER CELLS

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Exploration of endocytosis in cancer cells could improve tumor treatment results. The aim of this study was to investigate morphological aspects of HeLa (Henrietta Lacks cervical cancer cells) microcells development and to estimate endocytosis capability of these cells. The HeLa cell line was maintained at 37°C in DMEM medium 3-4 days. The cell samples were supravitaly stained with different markers (acridine orange, water soluble CdSe/ZnS nanoparticles, indian ink, carmin red)

and microscope pictures were taken using Leica DM6000B microscope connected with DFC 490 digital camera. Microscope images were analyzed and measured with image analyzing program IPP 5.0. Geometrical and photometrical measurements were gained in order to distinguish particular cell classes in HeLa cell line subpopulations. We found that different markers accumulate more in specific morphological states of cancer cells. Increasing fluorescence intensity testifies about more intense staining and higher endocytosis capability in early microcell development stages. We observed water soluble CdSe/ZnS nanoparticle interaction and binding with cell surface in all cell classes but enhancement of fluorescence intensity was observed in microcells. Although it is known that different sized nanoparticles have different fluorescence emission spectra in our experiments red and green nanoparticle emission spectra were similar suggested that nanoparticle spectral properties are determined by mutual binding with cell structures. All used supravital staining (Indian ink, carmin red, acridine orange, red and green CdSe/ZnS nanoparticles) methods allows to determinate different endocytosis ability in various HeLa cancer cell classes. Measured fluorescence intensities and endocytosis degree are significant for characterizing different cancer cell classes' endocytosis ability.

**A19**  
**DIFFERENTIAL EXPRESSION OF HOX GENES**  
**IN ORAL KERATINOCYTES AND ORAL**  
**CANCER CELL LINES**

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 Terence Lappin, Jacqueline James  
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HOX genes are a subset of a large family of transcription factor encoding genes, characterized by a highly conserved 183-base pair sequence, known as the homeobox. They control a variety of processes including, proliferation, differentiation and apoptosis during embryonic morphogenesis. HOX genes are expressed in adult tissues but often in distinct patterns from those found in their embryonic precursors. Aberrant expression of HOX genes has been reported during malignant transformation of many solid cancer types but the role of these genes in cancer of the oral cavity has not been investigated. The present study aims to compare the HOX gene expression profiles of normal oral keratinocytes with oral cancer cell lines. The HOX gene expression profiles of normal oral keratinocytes and four oral carcinoma cell lines were

determined by real-time quantitative-PCR. Comparison of these profiles identified 22 HOX genes expressed in all four carcinoma cell lines, but absent in all of the normal keratinocytes studied. Comparison of the results obtained from the oral cancer cell lines with a subset of leukaemia cell lines resulted in selection of a subset of HOXD genes for further investigation. Preliminary western blots and immunocytochemistry for the HOXD8-D11 proteins showed good correlation with the real-time quantitative-PCR results.

It is concluded that the HOXD genes may have an important role in oral cancer. The functional significance of these genes on cellular functions such as adhesion, migration, invasion and proliferation are currently under laboratory investigation using siRNA knockdown and over-expression vectors to modulate their expression in various cell lines.

The degree of DNA methylation in the promoter regions of the selected HOX genes in oral cancer cell lines, normal oral keratinocytes and patient tissue samples is currently under investigation using pyrosequencing assays.

The expression and potential sequence mutations of microRNAs predicted to target these HOX genes are also under investigation using real-time quantitative-PCR and other bioinformatic techniques.

**A20**  
**VESSEL REMODELLING DURING TUMOUR**  
**PROGRESSION OF CARCINOMA EX**  
**PLEOMORPHIC ADENOMA**

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**Introduction:** Tumor progression from adenoma to carcinoma induces remodeling of the vessel architecture due to changes in the tumor-host interaction, particularly the tumor stroma. The aim of our investigation was to describe the remodelling of the vessels during tumor progression of carcinoma ex-pleomorphic adenoma applying computerized texture analysis.

**Material and Methods:** The study comprised 10 cases of pleomorphic adenoma, 8 cases of early phase carcinomas ex pleomorphic adenomas ( intracapsular and minimally invasive ) and 8 cases of advanced phase carcinomas ex pleomorphic adenoma (frankly invasive ). Tumor vascularization was assessed in gray-level

transformed digitalized images of CD34 stained paraffin sections after filtration. We calculated basic morphometric features, Shannon's entropies of the first and second order, fractal dimensions applying different algorithms (Blanket, Minkowski, and Sarkar ) and variables derived from the gray-level co-occurrence matrix. The data obtained by texture analysis were correlated with the three categories characterizing tumor progression ( adenoma, early phase and advanced phase carcinomas ; Spearman rank order correlations)

**Results:** Whereas the number of vessels and the area occupied by CD34 stained endothelial cells did not change, the intensity of CD34 antigen expression was lower in carcinomas (  $p= 0.022$ ; t-test ). During tumor progression entropy values of the first (  $r = 0.784$  ;  $p< 0.0001$  ) and second order (  $r = 0.838$  ;  $p<0.0001$  ) increased, as well as the fractal dimensions (  $r$  Blanket =  $0.536$ ;  $p = 0.006$ ;  $r$  Minkowski =  $0.521$  ;  $p = 0.009$ ;  $r$  Sarkar =  $0.492$ ;  $p = 0.0146$ ;) In contrast to that, values of the second angular moment (  $r = - 0.871$  ;  $p<0.0001$ ), local homogeneity (  $r = - 0.879$  ;  $p<0.0001$  ), and peak prominence (  $r = -0.872$ ;  $p<0.0001$ ) derived from the gray-level co-occurrence matrix were reduced during transition from adenoma to advanced invasive carcinoma.

**Conclusion:** Although the total vessel number did not change during tumor progression there was an important remodelling of the vessel architecture suggesting a gain in complexity. The loss of endothelial CD34 expression in carcinomas might indicate alterations of the vessel metabolism.

*Supported by: FAPESP, CNPq*

## **A21 INTERGRIN LINKED KINASE (ILK) AND E- CADHERIN EXPRESSION IN LARYNGEAL CANCER**

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**Background:** It is evidenced in cases of many epithelial cancers that the ILK is a central molecule in many pathways that are being activated during the cancer formation which endow the cancerous cells with molecular and phenotypic traits that potentates the cell to metastasize.

Moreover it is documented in cases of many carcinomas that the abolishment of the adhesion molecules such as E-cadherin represents a hallmark of more aggressive behavior of the cancer. Experimental data in specific

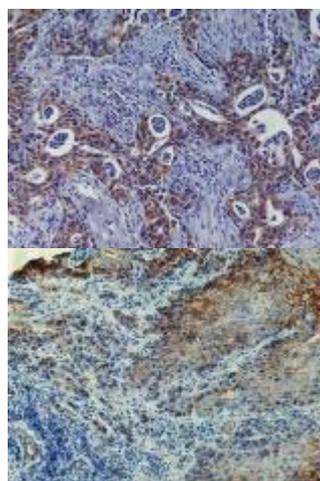
carcinomas implicate ILK in the molecular mechanism that leads to the loss of E-cadherin.

**Methods:** In the current study the expression of ILK and its potential downstream target E-cadherin was investigated by immunohistochemistry in human laryngeal cancer. Paraffin embedded tissue samples from 97 (n=97) squamous laryngeal carcinomas and adjacent non neoplastic laryngeal tissue was evaluated for ILK and E-cadherin expression. Statistical analysis was performed by SPSS for windows. The correlation between the expressions of the two proteins was evaluated by Spearman statistical test.

**Results:** It was detected an increased expression of ILK in 81% of tumor specimens and a decreased expression of E-cadherin in 71,1%, comparing to the normal controls. However the correlation of expression pattern between ILK and E-cadherin was not statistical significant ( $p= 0,067$ ).

**Conclusion:** The crucial role of ILK for the transition of a cancerous epithelial cell to a cell with all the proper molecular mechanisms that potentiates it to invade is been under extensive investigation in different types of cancers. This was the first time, to the best of our knowledge, the enhanced expression of ILK in laryngeal cancer to be described.

In addition our results confirmed the observation that laryngeal cancer is characterized by reduced expression of E-cadherin. However ILK does not seem to be involved in the loss of E-cadherin from the membrane of laryngeal cancer cells.



*Enhanced ILK expression in laryngeal cancer E-cadherin abolishment from cancer cell membranes*

## Poster session B: Cancer genomics

B01

### LOW-RISK HPV-11E6 CAN TRAP P53 PROTEIN IN CYTOPLASM, STUDIED IN VITRO WITH 293T CELLS

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Mucosal high-risk HPVs have been proved to be the major cause of cervical cancer, whereas low-risk HPVs induce only benign genital wart lesions. The mechanism underlining HPVs causing cancer has been studied extensively, and concluded that their E6,E7 proteins are the oncoproteins which interact with tumor suppressors p53 and pRB respectively, leading to the infected cell transformation and de-regulatory proliferation. However, why the low-risk HPVs only cause proliferative but non-invasive lesions of infected epithelia remains elusive. Given that p53 interacting with HR-HPVs E6 plays a very important role in the carcinogenesis, it is assumed that LR-HPVs E6 might interact with p53 in a different pattern.

In the present study, we used a mammalian EGFP expression system to express HPV-16E6 and HPV-11E6 fusion protein respectively in wtp53 cell lines, 293T, HCT116 (human colon carcinoma cell line), SMMC-7721(human hepatoma cell line), to trace the traffic and interaction between the E6s and p53, and here we show the major findings. 1. after transfecting, EGFP-HPV16 E6 mainly expressed in the nuclei of the three cells used, but EGFP-HPV11 E6 exclusively in the cytoplasm of all the cells used (Fig1,293T).

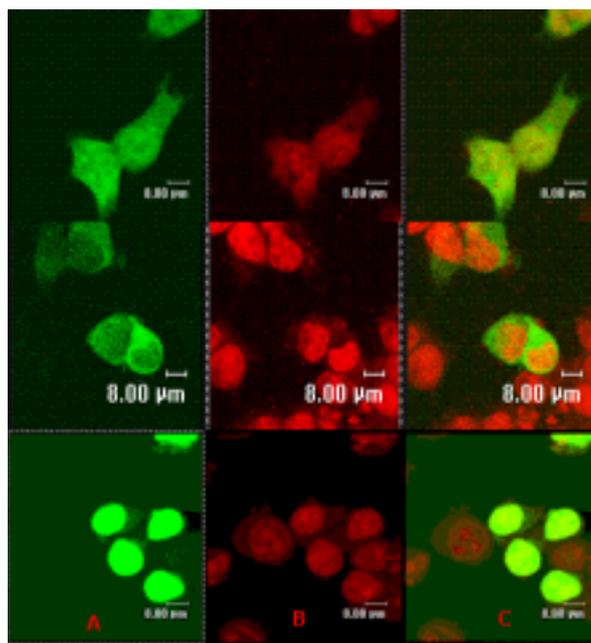


Fig. 1. A. EGFP,EGFP-11E6 and EGFP-16E; B. PI; C.Merge.

2. wtP53 labeled with immunocytochemistry was found to distributed in the nuclei of 293T cells transfected with EGFP-HPV16 E6, but distributed in the cytoplasm of the cells transfected with EGFP-HPV11 E6, in which wtp53 was trapped in the cytoplasm and never translocated into cell nuclei. 3. furthermore, we observed an obvious increase of apoptosis of HPV-E6 transfected 293T cells by double staining with Annexin V and PI from 24h to 72h posttransfection. In short, our finding of wtp53 being trapped in the cytoplasm by LR-HPV E6 may be one of the reasons why LR-HPV is not able to induce malignant transformation.

## B02 METHYLATION PATTERN OF HIGH RISK FLAT ADENOMAS IN CRC

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**Introduction:** Flat colorectal adenomas have been found to have a different molecular pathogenesis than regular polypoid-shaped lesions, and are associated with more aggressive clinical behaviour. Although genome wide chromosomal aberrations, mutations in the K-RAS and BRAF, as well as promoter methylation in the RASSF1 have been studied in flat tumours, substantial parts of the genomics of these tumours remains to be elucidated. We have previously shown that promoter methylation of hMLH1, O6MGMT, APC, p14ARF, p16INK4A, RASSF1A, GATA-4, GATA-5 and CHFR frequently occurs in colorectal cancer. The aim of the present study is to analyze the methylation status of these genes and 5 other genes reported to be methylated in colorectal cancer, in a large well characterized series of flat colorectal adenomas and compare these with established methylation patterns of polypoid adenomas.

**Method:** A series of 39 FFPE flat adenomas, that were classified as type IIa according to the Paris classification were used. Promoter methylation status of 14 genes (O6MGMT, hMLH1, APC, p14ARF, p16INK4A, CHFR, HMTF, RASSF1A, RASSF2A, ADAM23, JPH3, GATA-4, GATA-5, and NEURO1G) was studied by methylation-specific PCR (MSP) after bisulfite treatment.

**Results:** In the panel of flat adenomas analyzed, we observed methylation in the promoter region of all genes with frequencies ranging from 19 to 83 percent. p14ARF and GATA4 were most frequently methylated, with frequencies of 83% and 82%, respectively. The lowest frequency of methylation was observed in RASSF1A (19%). The methylation frequency of these genes was comparable to that observed in polypoid adenomas, except for p16, which showed a significantly

higher methylation in flat adenomas ( $P = 0.02$ ) and for GATA-5, where the methylation frequency was lower in flat adenomas ( $P = 0.001$ ).

**Conclusion:** We show that O6MGMT, hMLH1, APC, p14ARF, CHFR, HMTF, RASSF1A, RASSF2A, ADAM23, JPH3, GATA-4, and NEURO1G are methylated in flat adenomas in similar frequencies as have been described for polypoid adenomas, while there is a tendency towards higher promoter methylation of p16 and lower promoter methylation for GATA-5 in flat adenomas.

## B03 INTEGRATION OF DNA AND EXPRESSION MICROARRAY DATA UNRAVELS SEVEN PUTATIVE ONCOGENES ON 20Q AMPLICON INVOLVED IN COLORECTAL ADENOMA TO CARCINOMA PROGRESSION

Beatriz Carvalho<sup>1</sup>, Cindy Postma<sup>1</sup>, Sandra Mongera<sup>1</sup>, Erik Hopmans<sup>1</sup>, Sharon Diskin<sup>2</sup>, Mark van de Wiel<sup>1</sup>, Wim van Criekinge<sup>3</sup>, Olivier Thas<sup>3</sup>, Anja Matthäi<sup>4</sup>, Miguel Cuesta<sup>1</sup>, Jochim Terhaar<sup>1</sup>, Mike Craanen<sup>1</sup>, Evelin Schröck<sup>4</sup>, Bauke Ylstra<sup>1</sup>, Gerrit Meijer<sup>1</sup>

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**Introduction:** Chromosomal instability (CIN) is a hallmark of colorectal cancer progression, occurring in 85% of colorectal cancers. Gain of the long arm of chromosome 20 is one of the most prominent features of adenoma to carcinoma progression in CIN colorectal cancers, although the oncogene(s) underlying this chromosomal gain are still unknown. In the present study we investigate the effects of chromosomal instability on gene expression in colorectal adenoma to carcinoma progression, focusing on gain of chromosome 20, with the aim of identifying the oncogenes in this amplicon.

**Methods:** We have analysed two independent series of colorectal tumours, containing 34 non-progressed adenomas, 41 progressed adenomas (i.e. adenomas that harbour already a focus of cancer, also called malignant polyps) and 33 adenocarcinomas, and studied DNA copy number alterations by array CGH and mRNA expression by microarray analysis. Data analysis was done focusing on putative oncogenes whose expression was correlated with DNA copy number gain of the genomic region involved.

**Results:** Three small regions of overlap (SRO's) of copy number gain were defined and seven genes within these regions showed overexpression in progressed adenomas and carcinomas when compared to non-progressed adenomas.

**Conclusion:** With this approach seven genes were identified as having a putative oncogenic role in CIN related adenoma to carcinoma progression.

#### B04

### PROMOTER HYPERMETHYLATION IN STOOL-BASED DNA TESTS FOR COLORECTAL CANCER SCREENING

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Promoter hypermethylation of tumor suppressor genes is one of the common molecular alterations in the carcinogenesis of colorectal carcinoma (CRC). Since CRC has a long asymptomatic preclinical phase, and survival is better when treated in an early-stage disease, a screening test would be beneficial. The detection of methylated DNA by MSP is relatively simple, economic, and fast. Therefore, this technique has potential to be used in a stool-based DNA test for CRC screening. Sensitivity of these tests usually is measured in percentage of cases with a colorectal cancer that have detectable DNA alterations in their feces. Yet, the actual amount of tumor DNA present in stool of a given patient is unknown, and consequently also the analytical sensitivity of stool DNA based tests. Moreover, the loss of DNA in stool over time (stability) is not well defined. The aim of this study is to test the sensitivity of detecting aberrant methylated DNA in a stool-based DNA test for CRC screening, and determine the stability of DNA in stool.

A range of HCT116 cells was spiked into 80 milligram taken from homogenized stool of a colonoscopy negative individual, and three replicates were incubated at room temperature for different time periods. Whole genomic DNA was isolated using the QIAamp DNA stool mini kit. The analytic sensitivity of the assay, and the stability of DNA in stool was determined by detecting human DNA and HCT116-derived DNA in the different stool samples. Human DNA was detected using a human specific beta globin PCR and HCT116-derived DNA was detected by a nested MSP for promoter methylation of CHFR. Human DNA was detectable even in stool samples without cells added,

creating a background signal in all samples. Promoter methylation of CHFR was detected in samples with 100 to 500 cells per 80 milligram of stool, even up to 72 hr incubation at room temperature.

In conclusion, against a background of human non-tumor DNA, and DNA from other sources like bacteria, tumor DNA can be detected with high sensitivity. Therefore, detection of aberrant methylation in stool holds promise to be implemented in a screening modality.

#### B05

### LARGE GENOMIC DELETIONS OF SMAD4, BMPR1A AND PTEN IN JUVENILE POLYPOSI

Arnout van Hattem<sup>1</sup>, Lodewijk Brosens<sup>1</sup>, Wendy de Leng<sup>1</sup>, Folkert Morsink<sup>1</sup>, Sylvia Lens<sup>2</sup>, Ralph Carvalho<sup>2</sup>, Francis Giardiello<sup>3</sup>, Johan Offerhaus<sup>1</sup>  
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<sup>2</sup>MRC-Holland, Amsterdam, The Netherlands  
<sup>3</sup>The Johns Hopkins Hospital, Baltimore, USA

**Introduction:** Juvenile polyposis syndrome (JPS) is a rare autosomal dominant disorder characterized by multiple gastrointestinal juvenile polyps and an increased risk of colorectal cancer. This syndrome is caused by germline mutation of either SMAD4 or BMPR1A, and possibly ENG. PTEN, originally linked to Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, has also been associated with JPS. By direct sequencing, germline mutations are found in only 30-40% of patients with a JPS phenotype. Therefore, alternative ways of inactivation of the known JPS genes, or additional genes predisposing to JPS may be involved. In this study, a comprehensive genetic analysis of SMAD4, BMPR1A, PTEN and ENG is performed through direct sequencing and multiplex ligation-dependent probe amplification (MLPA) in JPS patients.

**Materials and methods:** Archival material of 29 patients with JPS from 27 families was collected. Direct sequencing and MLPA analysis were performed to search for germline defects in SMAD4, BMPR1A, PTEN and ENG.

**Results:** A germline defect in SMAD4, BMPR1A or PTEN was found in 13 of 27 (48.2%) unrelated JPS patients. Nine mutations (33.3%) were detected by direct sequencing, including six (22.2%) SMAD4 mutations and three (11.1%) BMPR1A mutations. MLPA identified four additional patients (14.8%) with germline hemizygous large genomic deletions, including one deletion of SMAD4, one deletion of exons 10 and 11 of BMPR1A, and two unrelated patients with deletion of

both BMPR1A and PTEN. No ENG gene mutations were found.

**Conclusion:** Large genomic deletions of SMAD4, BMPR1A and PTEN are a common cause of JPS. Using direct sequencing and MLPA, a germline defect was detected in 48.2% of JPS patients. MLPA identified 14.8% (4/27) of these mutations. Since a substantial percentage of JP patients carry a germline deletion and MLPA is a reliable and user friendly technique, we conclude that MLPA is a valuable adjunct in JPS diagnosis.

#### B06

### ROLE OF DNA METHYLATION IN HEPATOCELLULAR CARCINOMA (HCC) CELL LINES IN RESPONSE TO HYPOXIA

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Hypoxia is a common occurrence in many solid tumours, including hepatocellular carcinoma (HCC). It is proposed that tumour cells may undergo genetic or epigenetic changes in response to hypoxia that allow them to progress towards a more aggressive phenotype and an increased resistance to treatment. Under hypoxic conditions, hypoxia-inducible factor-1 alpha (HIF-1a) is the major transcriptional activator that activates target genes by binding to cis-acting hypoxia-responsive-elements (HREs). The conserved HREs contain a CpG dinucleotide that is known to be a potential target of cytosine methylation. It has been reported that aberrant CpG island hypermethylation is an early and frequent event which accumulates during hepatocarcinogenesis. We thus propose that a particular subset of genes with DNA methylation at the HRE site during the development of HCC might abolish the binding of HIF-1a, resulting in an attenuated response to hypoxic stimuli. In this study, Hep3B, HepG2 and PLC/PRF/5 were cultured for three days in the presence or absence of a demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC), and then further cultured for 24 hours under hypoxic or normoxic conditions in freshly replenished media. Affymetrix GeneChip analysis was employed to investigate the changes of global gene expression in triplicate experiments. We identified 91 putative HIF-1a target genes with aberrant DNA methylation which were found to have additive induction in at least two cell lines after treatment with 5-aza-dC followed by hypoxia, as compared to 5-aza-dC treatment alone or hypoxia treatment alone. Furthermore, 32 of the 91 genes include H19 noncoding RNA, CITED2, SERPINE1, IGFBP3,

cyr61 and SLC2A3, were down-regulated in cancerous tissues compared to surrounding non-cancerous liver tissue in 27 HCC patients. Our findings suggest that DNA methylation may contribute to the development of HCC by attenuating the response to hypoxic stimuli, thereby enhancing survival and proliferation of cancer cells or promoting a more aggressive phenotype.

#### B07

### CYP'S GENE POLYMORPHISMS IN BREAST CANCER: INVESTIGATION OF ASSOCIATION WITH CHEMOTHERAPY RESPONSE

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**Background:** Breast cancer is the most abundant women cancer type. The majority of patients with breast cancer receive chemotherapy as a component of multimodality treatment. However, effect of cytotoxic treatment varies considerably in individuals. It is known that many anticancer agents such as Vinca-alkaloids, antracyclines, alkylating agents, taxanes, tamoxifen are substrates for cytochrome P450 superfamily enzymes. Variant alleles coding these enzymes cause individuals to differ considerably in metabolic capacity leading to drug sensitivity alteration of tumor cells. We investigated the association of CYP3A5\*3, CYP3A4\*2, CYP3A4\*1B, CYP2C8\*2, CYP2C8\*3, CYP2C9\*2, CYP2C9\*3, CYP2C19\*2, CYP2B6\*5 polymorphisms with chemotherapy effect in breast cancer patients.

**Methods:** We studied the genotypes of CYP's in blood samples of 300 women from Tomsk Oncology Center. Patients received neoadjuvant therapy (CAF, FAC or CMF protocol) and were divided in two groups according to the response of chemotherapy: effect was estimated as positive in case of complete or partial remission and as negative in case of stabilization or progression disease.

**Results:** We have found the following mutant alleles frequency: CYP3A5\*3 - 0.89; CYP3A4\*2 - absent, CYP3A4\*1B - 0.02, CYP2C8\*2 - 0.01, CYP2C8\*3 - 0.09, CYP2C9\*2 - 0.10, CYP2C9\*3 - 0.10, CYP2C19\*2 - 0.26, CYP2B6\*5 - 0.11. The genotypes in patients were similar to those of healthy European Caucasians described in literature. The odds ratio (OR) was applied to estimate association of CYP's genotypes with chemotherapy resistance risk and was calculated for combined group (mutant homozygous and heterozygous) in comparison with wild genotype group: CYP3A4\*1B (OR=0.56, p=0.69), CYP2C8\*2 (OR=1.22, p=0.70), CYP2C8\*3 (OR=1.56, p=0.65),

CYP2C9\*2 (OR=0.8, p=0.71), CYP2C9\*3 (OR=0.59, p=0.29), CYP2C19\*2 (OR=1.43, p=0.30), CYP2B6\*5 (OR=1.03, p=0.92). The odds ratio for CYP3A5\*3 OR=0.52, p=0.44 was calculated for mutant homozygous group in comparison with heterozygous group because of wild genotype individuals absence.

**Conclusion:** There are no associations of CYP's genetic polymorphisms with neoadjuvant chemotherapy (CAF, FAC or CMF) response in patients with breast cancer.

## B08

### ACGH ANALYSIS OF 30 MALE BREAST CANCERS (MBC)

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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 we analysed a series of 30 MBC by 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. Thirty patients who 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  $\log_2(\text{ratio})$  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 and with Gene Ontology ([www.geneontology.org](http://www.geneontology.org)) to identified specific pathways influenced by alterations. Preliminary results on 30 patients showed the presence of a wide range of chromosome alterations spanning all the genome. The most frequent chromosome bands involved in gains were 2q32.2-q32.3, 3p11.2, 3p14,

3p24.3, 3q24-q26, 4q24-q25, 5p13-p15, 5q22-q23, 6q35.2, 7p14-p15, 9q33.3, 10q11.21, 12q21.3, 13q21-q22, 16p11.2; the most frequent chromosome bands involved in losses were 1p31.1, 1p34, 1p36, 2q24.1, 3p21.31, 3q13-q25, 4p14, 4p15, 4q35.2, 5q11.2-q13, 6p21, 6q23, 7q11, 9q34.11, 16p12-p13, 18q21.1. These regions resulted specific for male breast cancer, as assessed comparing our results with data of the literature. The genes present in that loci are involved in specific pathways, such as regulation of transcription, lipid metabolism and nucleus-cytoplasm transport. Our approach allows to identify somatic genetic changes that are specific MBC. We think it is important to report these regions because our data could show some possible association functions in tumor development.

## B09

### GENETIC ANALYSIS OF SPORADIC AND FAMILIAL HEAD AND NECK PARAGANGLIOMA

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Paragangliomas (PGLs) of the head and neck are hypervascular, mostly benign neoplasms of the autonomic nervous system. Familial PGLs have recently been shown to be associated with germline alterations in succinate dehydrogenase genes (SDHB, SDHC and SDHD), and occasionally with VHL and RET. SDH mutations are also found in 8-25% of sporadic patients, representing in fact occult familial cases. Little is known about the genetic changes involved in the development of PGL. The aim of this study was to compare genome wide DNA copy number changes in tumours of familial and sporadic origin.

Eight familial and 16 sporadic head and neck PGL (carotid body, yugular and vagal) were studied for germline mutations in SDH genes, VHL and RET by direct sequencing and possible exon deletions in SDH genes and VHL by MLPA. Microarray CGH analysis was applied to DNA extracted from formalin-fixed, paraffin embedded tumours.

All 8 familial cases and 7 sporadic cases (i.e. occult familial) carried a mutation in SDHB (n=8) or SDHD (n=7). Mutations and exon deletions in SDHC, VHL or RET were absent. All 5 cases with multiple PGLs had a SDHD mutation. Array CGH detected abnormalities in

10 of 18 cases, and failed in the other six tumor samples. Most frequent were deletions of exactly those chromosomal regions where the genes SDHB, SDHC and SDHD are located: 1p (8 of 10), 1q (6 of 10), and 11q (3 of 10). However, they appeared unrelated to the SDH mutation status. Intriguingly, PGLs without any chromosomal aberrations were mostly sporadic and PGLs with chromosomal aberrations were mostly germline mutation carriers.

In five cases the germline mutation in SDHB or SDHD coincided with a loss of chromosomes 1 or 11, respectively, suggesting that they may be the second 'hit', inactivating succinate dehydrogenase activity in the tumor. On the other hand, eight cases exhibited either a germline mutation or a somatic deletion and five cases showed neither mutation nor deletion. Additional genes and mechanisms may need to be studied to elucidate the molecular pathogenesis of PGL.

#### **B10 CIS-ACTING GENOMIC ELEMENTS OF THE PAS1 LOCUS CONTROL KRAS MUTABILITY IN LUNG TUMORS**

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Inbred mouse models display different susceptibilities to spontaneous or carcinogen-induced lung cancer. Genetic linkage analyses have uncovered a number of Quantitative Trait Loci (QTLs) modulating lung cancer risk and development in mice. In particular, Pas1 (Pulmonary Adenoma Susceptibility 1) locus, located in the distal region of chromosome 6, appears to play a major role in the inherited predisposition to spontaneous and chemically induced lung tumorigenesis in mouse models. Pas1 locus displays two different haplotype: a susceptible one (A/J-like) and a resistant one (C57BL/6J-like). Many reports suggested that Kras, one of the six gene mapping in Pas1 core haplotype, is a good candidate for Pas1 locus function. In order to investigate Kras role in lung tumorigenesis, we propose a new mouse model developed by replacing Kras gene with Hras transcript in a Pas1 susceptible background and crossing these mice with either a resistant (C57BL/6J) and a susceptible (A/J) strain. After lung cancer induction with urethane treatment, we observed that, in both crosses, heterozygous mice carrying the Hras-replacement gene were more prone to develop lung

tumors than wide-type mice, indicating that Hras-replacement gene not only supplies Kras functions but it is also more active. Furthermore, most of the lung tumors carried a Gli61Leu substitution in Hras-replacement gene, whereas no mutations were observed in the endogenous Hras gene. Thus we suggest that Pas1 locus context is able to drive ras genes mutability. Moreover, in tumors obtained from mice carrying Hras-replacement gene, the mutation frequency affecting the wild-type Kras gene was higher when this gene was located in the susceptible (A/J) than in the resistant (C57BL/6J) Pas1 locus context (12% versus 0%, -log P=5.0). These findings indicate that cis-acting elements located in Pas1 locus are the functional components modulating Kras gene mutability and controlling susceptibility to lung tumorigenesis in mouse strains.

#### **B11 IDENTIFICATION AND FUNCTIONAL VALIDATION OF CANDIDATE ONCOGENES IN COLORECTAL CANCER**

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**Introduction:** Colorectal carcinomas are characterized by a specific pattern of chromosomal imbalances. Among others, chromosome 13q is frequently gained. However, the genes representing targets of this recurrent chromosomal aberration still remain unknown.

**Material and Methods:** To detect minimal regions of amplification on chromosome 13q, we performed high resolution microarray CGH of 31 primary colon carcinomas. To identify deregulated genes mapping to these regions, we then profiled a subset of these tumors using whole-genome expression microarrays. Additionally, we re-analyzed gene expression data previously published by our group comparing primary colon cancer and corresponding mucosa samples as well as primary rectal cancers and corresponding mucosa samples. Next, we validated the expression levels of the over-expressed genes in 25 colorectal cancer cell lines using real-time PCR. Subsequently, we conducted an RNAi screen in a colorectal cancer cell line to assess the resulting phenotypic consequences.

**Results:** We detected two minimal regions of recurrent amplification located on chromosome 13q. We then separated 88 genes within these amplicons that were

over-expressed in the tumors compared to normal samples. Using real-time PCR, we identified those genes that were also highly up-regulated in 25 colorectal cancer cell lines. For a subset of these genes, we observed a decreased cellular viability as a consequence of mRNA silencing (ranging from a 20% to a 70% reduction). A secondary screen was performed using two additional siRNAs to confirm these results. The identified genes represent transcription factors, ATP and zinc ion binding proteins, receptor activity proteins, nuclear pore complex proteins, and protein/DNA binding gene products. The function of some of these genes has yet not been discovered.

**Discussion:** Our experimental strategy led to the identification of genes that were amplified and/or over-expressed in primary colorectal cancers. We surmise that some of these genes represent potential oncogenes residing in chromosome 13q.

## B12

### META-CLUSTERING OF GASTRIC, SMALL INTESTINAL AND COLORECTAL ADENOCARCINOMAS BASED ON DNA COPY NUMBER PROFILES

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**Background:** Adenocarcinomas of the stomach, small intestine and colorectum share several clinical and phenotypical characteristics. In addition, the majority of these adenocarcinomas tumors show chromosomal instability, which results in DNA copy number aberrations that can be measured by array comparative genomic hybridization (aCGH). To which extend these three tumor types also share DNA copy number profiles is unknown. Previously, we and others evaluated the chromosomal gains and losses by array CGH of colorectal and gastric tumors. This demonstrated that in colorectal tumors gains of chromosomes 8q, 13q en 20q and losses of 8p are frequent. Among gastric tumors gains of 6p, 8q, 13, 20q gain and losses of 6q and 13 are frequent. Small intestinal cancer is rare in comparison to colorectal and gastric cancer and high resolution aCGH

data not previously reported to our knowledge. Previously, our group succeeded to classify 373 epithelial tumors according to their organ of origin based on their aCGH profiles by hierarchical clustering (Jong et al., *Oncogene* 2007). Within this meta-cluster of primarily epithelial tumors, the gastrointestinal profiles split into a cluster with primarily colon tumors and one cluster with both gastric and colon tumors. No small intestinal tumors were included.

Aim of the present study is to investigate to what extent gastric, small intestinal and colorectal adenocarcinomas tumors share DNA copy number profiles.

**Materials:** aCGH data from forty gastric, forty colorectal and forty small intestinal tumors were selected from different in house datasets, based on array quality and clinical data. From each tissue type thirty tumors were randomly selected as training set and 10 as validation set. Before clustering, smoothed data were called by the calling algorithm CGHcall. Next DNA copy number data were resampled to one hundred data points on equal distance per chromosome. Both called and segmented data were analyzed with hierarchical clustering as well as other methods.

**Results:** First results indicate substantial overlap of DNA copy number profiles between small intestinal and colorectal cancer and less overlap of these two categories with gastric cancer.

## B13

### TGFB1 +29T>C GENETIC POLYMORPHISM IS ASSOCIATED WITH NON-SMALL CELL LUNG CANCER

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**Background:** Non-small cell lung cancer (NSCLC) remains a major health problem worldwide. NSCLC development is a complex and multi-step process, resulting from exposure to environmental and genetic factors. A direct relationship between tobacco smoke and lung cancer has been thorough established, although only a fraction of smokers will develop NSCLC, which supports a role for the individual genetic profile in susceptibility differences. TGF-beta 1 (TGFb1) plays an important role in cell cycle regulation, promoting cellular differentiation and apoptosis in the early phase of cancer development. A functional polymorphism in TGFB1 gene, responsible for a T-to-C substitution at nucleotide 29 has been associated with higher levels of TGFb1 in circulation.

**Aim:** To investigate the influence of TGFBI +29T>C functional polymorphism in NSCLC susceptibility.

**Material and Methods:** DNA was extracted from peripheral blood cells of 696 individuals: 235 patients histopathologically diagnosed with (78.5% smokers, 21.5% non-smokers) and 461 healthy individuals without evidence of neoplastic disease. Genotyping of TGFBI +29T>C polymorphism was performed by Real-Time PCR allelic discrimination method.

**Results:** The frequency of the TT genotype was lower in NSCLC patients than in controls (0.27 and 0.35, respectively). Conversely, we found an overrepresentation of C carriers in NSCLC group in comparison with normal controls (0.73 and 0.65, respectively,  $P=0.035$ ). Carriers of the C allele have an increased risk for NSCLC development (OR=1.45, 95%CI=1.03-2.04). The risk for being diagnosed with NSCLC is increased in women, who are simultaneously carriers of C allele (OR=2.55, 95%CI=1.12-5.78,  $P=0.023$ ), although no similar association was found for men ( $P=0.270$ ). Interestingly, there was a significant risk for developing distant metastasis in homozygous T carriers (OR=1.33, 95%CI=1.02-1.74,  $P=0.017$ ) in NSCLC patients who are active smokers or past heavy-smokers compared to non-smokers.

**Conclusion:** TGFBI +29T>C functional genetic polymorphism might influence NSCLC susceptibility. Gender seems to modulate the association of TGFBI and lung cancer. Smoke-associated metastases risk in TT carriers warrants further research to clarify the interaction between TGFBI and tobacco smoke in metastases development.

#### B14

#### CYP3A4 AND CYP3A5 GENOTYPES: MOLECULAR MARKERS FOR PROSTATE CANCER AGGRESSIVENESS?

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**Background:** Prostate cancer is the second most common malignant disease among men. A number of factors like age, ethnicity, diet and exposure to androgens, have been associated with an increased risk for this disease. It has been suggested that the ethnic differences in incidence and mortality of prostate cancer are related to genetic variation in genes that control the androgen metabolism. Testosterone is an important hormone implicated in prostate growth and differentiation, and genes that control its metabolism, may have a key role in prostate cancer susceptibility.

The enzymes CYP3A4 and CYP3A5 are responsible for the oxidation of testosterone to less biologically active forms of this hormone. Polymorphisms in CYP3A4 and CYP3A5, such as CYP3A4\*1B and CYP3A5\*3 (both A to G transitions), could alter individual susceptibility to prostate cancer. The aim of our study was to evaluate the influence of these polymorphisms in the development of prostate cancer.

**Methods:** DNA samples were extracted from peripheral blood cells of 698 patients with prostate cancer and 401 healthy blood donors. The polymorphisms were analysed through PCR-RFLP. Analysis of data was performed using the computer software SPSS for windows. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measure of the association between CYP3A4 and CYP3A5 and prostate cancer.

**Results:** We found significant statistical differences between the control group and the patients with a Gleason grade equal or superior to 7 (OR=1.61; CI95%: 1.06-2.43;  $P=0.019$ ) and the patients with metastasis (OR=1.80; CI95%: 0.99-3.28;  $P=0.038$ ). Individuals carrying one, two or both less common variants of CYP3A4 and CYP3A5 (G and A respectively) have an increased risk for more aggressive prostate cancer.

**Conclusions:** Our preliminary results suggest that carriers of the less common variants of CYP3A4\*1B and CYP3A5\*3 polymorphisms, which appear to decrease the availability of testosterone, are at higher risk of developing aggressive prostate cancer. Some prostate cancer cells are androgen dependent and some are androgen independent. A lower concentration of testosterone could allow the androgen independent cells to proliferate more than the dependent ones, resulting in the development of a more aggressive prostate cancer.

**B15**  
**DIFFERENT PATTERNS OF GENOMIC**  
**INSTABILITY IN GASTRIC CANCERS OF WEST**  
**EUROPEAN AND SOUTH AFRICAN PATIENTS**

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**Background:** Gastric cancer is the second most common cause of cancer death worldwide, but incidence and mortality rates show large variations across different counties. Previous studies have addressed differences in environmental and dietary factors influencing gastric cancer risk, but not much is known about the biological differences in gastric cancers from different geographic locations. Therefore, we aimed to compare genomic differences in gastric cancers obtained from patients from Western Europe and South Africa.

**Material & Methods:** Fifty-eight gastric cancers from 33 Western European and 25 South African patients were included in the study. DNA was isolated and analysed for microsatellite (MIN) and chromosomal (CIN) instability by PCR and microarray comparative genomic hybridisation, respectively.

**Results:** Tumours from South Africans showed significantly more frequent microsatellite instability tumours compared to tumours from West European patients ( $P < 0.05$ ). In addition, microsatellite stable tumours from both groups could be separated by unsupervised hierarchical cluster analysis ( $P = 0.001$ ). Chromosomal regions 1q31, 3p24-26, 7p21 and 21q21 contributed most to this separation.

**Conclusion:** Gastric cancers from South African and European patients showed differences in their genomic instability patterns, indicating possible different biological mechanisms underlying the disease.

**B16**  
**IDENTIFICATION OF TUMOUR SUPPRESSOR**  
**GENES BY NMD INHIBITION IN GASTRIC**  
**CANCER**

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**Background and Aim:** Gastric cancers frequently show chromosomal alterations which can cause activation of oncogenes, and/or inactivation of tumour suppressor genes. In gastric cancer several chromosomal regions have been described to be frequently lost, but in most of these regions, no tumour suppressor genes have been described yet. The present study aimed to identify tumour suppressor genes inactivated by nonsense mutation and deletion in gastric cancer by means of GINI (gene identification by nonsense mediated decay) and whole genome DNA copy number analysis.

**Material and Methods:** Two non-commercial gastric cancer cell lines, GP202 and IPA 220, were transfected with siRNA directed against UPF-1, to specifically inhibit the NMD pathway, and with siRNA directed against non-specific siRNA duplexes (CVII) as a control. Microarray expression experiments were performed in triplicate on 4x44 K agilent arrays by hybridizing UPF-1 transfected cells against non-specific CVII transfected cells. In addition array CGH of the two cell lines were performed on 4x44K agilent arrays to obtain the DNA copy number profiles.

**Results and discussion:** Integration of array CGH and microarray expression data provided multiple candidate genes inactivated by nonsense mutation and deletion. Currently these candidate genes are being sequenced for confirmation of mutations. Subsequently, mRNA expression levels of these candidate genes will be tested in a panel of primary tumours.

### B17 PROMOTER HYPERMETHYLATION OF MAL IN GASTRIC CANCERS

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**Background:** T-lymphocyte maturation associated protein MAL has been described as a tumour suppressor gene with diagnostic value in colorectal and oesophageal cancers. Moreover, previous studies have shown inactivation of this gene by promoter hypermethylation. The aim of the present study was to analyse promoter hypermethylation of MAL in gastric cancers.

**Material and methods:** Bisulphite-treated DNA isolated from formalin-fixed and paraffin embedded tissues of 100 gastric adenocarcinomas and 24 normal gastric mucosa samples was subjected to real-time quantitative methylation specific PCR. Two regions (-680 to -573 bp and -92 to -7 bp before the transcription start site) within the MAL promoter were analysed.

**Results:** Of all gastric adenocarcinomas, 73% showed methylation of both promoter regions and an additional 12% showed methylation of only one of the promoter regions. Methylation was absent in 15% of the tumours. In normal gastric mucosa methylation of a single promoter region was found in 25% of the samples, but only 4% of the normal gastric mucosa tissues were methylated in both promoter regions.

**Conclusion:** Promoter hypermethylation of MAL is frequently observed in gastric cancers but not in normal gastric mucosa. These results indicate that MAL promoter hypermethylation may be a valuable diagnostic marker for gastric adenocarcinoma.

### B18 DNA COPY NUMBER PROFILING OF EARLY ONSET COLORECTAL CANCER

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Several familial colorectal cancer (CRC) syndromes have a known genetic cause, the most common are familial adenomatous polyposis and Lynch syndrome. Early onset CRCs with absence of a known cancer predisposition may harbour specific genomic gains or losses that are indicative of yet undetected susceptibility loci. Mutations in the tumour suppressor gene TP53 are known to be associated with chromosomally unstable (CIN) CRCs. In this study high resolution microarray-based comparative genome hybridization (array-CGH) is applied to obtain a genome-wide DNA copy number profile, with a relative probe spacing of 6 kbp throughout the genome. General DNA copy number profiles at such a high resolution are scarce from CRC. A series of 27 early onset CRCs, not in compliance with the known genetic CRC syndromes, and with average age at onset being 44 years (range 28-53), and a matching group of 20 patients with average age at onset being 78 years (range 69-87) were analysed. The set of late onset CRCs was selected to achieve comparable distributions of tumour stage, localization in the bowel, and gender within the two sample series. Skewed distributions between early and late onset CRCs for losses of genomic regions include 1p36 (33 % vs. 15 %; not significant), 2p11 (30 % vs. 0 %;  $p = 0.014$ ), 4p11 (63 % vs. 20 %;  $p = 0.007$ ), 4q28 (52 % vs. 15 %;  $p = 0.014$ ), and 9q12 (37 % vs. 0 %;  $p = 0.003$ ). The regions from chromosomes 2, 4, and 9 are located in close proximity to centromeres, but have their smallest regions of overlapping deletions measured by oligos targeting outside of the core centromeric regions. The three regions span 0.8 Mbp, 0.6 Mbp, and 1.1 Mbp of non-repetitive DNA covering several transcribed sequences each. Among the commonly gained genomic regions in the early onset CRC group (> 40 %; 8q, 13q, and 20q), none showed statistically significant difference in frequency between the two patient groups. Tumours with mutated TP53, as compared to tumours with wild-type TP53, showed a significantly higher frequency of loss in genomic areas where losses are

known to be associated with the CIN phenotype. In conclusion, we have identified several genomic regions which seem to be preferentially lost in tumours from young at onset patients. This is indicative of tumour suppressor loci and as such promising in the search for additional CRC predisposition genes.

#### B19

#### INTEGRATION OF GENE DOSAGE AND GENE EXPRESSION IN NON SMALL CELL LUNG CANCER; IDENTIFICATION OF HSP90 AS POTENTIAL TARGET

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Lung cancer causes approximately 1.2 million deaths per year worldwide, and non-small cell lung cancer (NSCLC) represents 85% of all lung cancers. Understanding the molecular events in non-small cell lung cancer (NSCLC) is essential to improve early diagnosis and treatment for this disease.

In an attempt to identify novel NSCLC related genes, we performed a genome-wide screening of chromosomal copy number changes affecting gene expression using microarray based comparative genomic hybridization and gene expression arrays on 32 radically resected tumor samples from stage I and II NSCLC patients. An integrative analysis tool was applied to determine whether chromosomal copy number affects gene expression. We identified a deletion on 14q32.2-33 as a common alteration in NSCLC (44%), which significantly influenced gene expression for HSP90, residing on 14q32. This deletion was correlated with better overall survival ( $P=0.008$ ), survival was also longer in patients whose tumors had low expression levels of HSP90. We extended the analysis to three independent validation sets of NSCLC patients, and confirmed low HSP90 expression to be related with longer overall survival ( $P=0.003$ ,  $P=0.07$  and  $P=0.04$ ).

Furthermore, in vitro treatment with an HSP90 inhibitor had potent antiproliferative activity in NSCLC cell lines. We suggest that targeting HSP90 will have clinical impact for NSCLC patients.

## Poster session C: Cancer prevention and prognosis research

#### C01

#### QUANTITATIVE FLUORESCENCE DETERMINATION OF LONG FRAGMENT DNA IN STOOL AS A MARKER FOR THE EARLY DETECTION OF COLORECTAL CANCER

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Screening for colorectal cancer (CRC) has great potential to reduce morbidity and mortality from the disease and new tests based on molecular alterations have been evaluated as an alternative to current diagnostic examinations for the development of a non-invasive diagnostic approach. The results from these studies have shown that multiple mutation analyses could improve test sensitivity, albeit with a corresponding increase in costs and test execution time that could hamper the use of some of these methods in standard clinical practice. DNA integrity analysis of genomic DNA extracted from stool, using a fluorescence long DNA (FL-DNA) method developed in our laboratory, represents a moderately inexpensive and relatively rapid test that showed interesting results in a previous pilot case-control study. Our aim was to validate the diagnostic accuracy of FL-DNA analysis for non-invasive CRC detection. This confirmatory case-control study was conducted on 100 healthy subjects and 100 patients at first diagnosis of colorectal cancer,

recruited from 2005 to 2006. Human long fragment DNA in stool was quantified by fluorescence primers and a standard curve and expressed in DNA nanograms. Sensitivity was only slightly lower, reaching 84% at the lowest cut-off and maintaining a good level (65 %) at the higher cut-off values. Moreover, we validated the 25-ng value, which emerged as the best cut-off in the pilot study, obtaining 79% (95% CI, 71-87%) sensitivity and 89% (95% CI, 83-95%) specificity. Specificity was very high for all the cut-off values from 15-40 ng, ranging from 78% to 96%. Diagnostic potential was independent of gender, age and tumor site, and no statistical differences were observed for tumor stage. Fecal DNA analysis is a non-invasive and relatively simple test that shows high diagnostic potential. These characteristics, together with the small amount of stool required, make it a potential alternative to current non-invasive screening approaches.

**C02**  
**NATURAL FLUORESCENCE SPECTROSCOPY OF HUMAN BLOOD PLASMA FOR CANCER DETECTION: FEASIBILITY STUDY AND PRELIMINARY RESULTS**

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**Background:** Fluorescence spectroscopy of biomolecules is considered a promising method to in vivo discriminate normal tissue from malignant tissue in various sites, including breast, cervix, lung, and colon. However, only few studies have been reported on the feasibility to exploit fluorescence spectroscopy of biofluids to characterize pathological changes usable in diagnostic oncology. In this study, the value of natural fluorescence of human blood plasma in discriminating patients with colorectal cancer from subjects of a control population has been investigated.

**Methods:** The study involved 480 subjects, including 240 blood donors, with no evidence of pathological disease, 196 patients bearing colorectal adenocarcinoma, 15 patients with local relapse, 18 patients with FAP and 11 with single adenoma. Blood samples were collected from all the subjects. Serum CEA level was determined using commercial kits while plasma fluorescence spectrum was analyzed using a conventional spectrofluorimeter.

**Results:** The intensity of the fluorescence emission peak around 615-635 nm, reasonably ascribed to endogenous porphyrins, was significantly different between patients

bearing colorectal cancer and blood donors, with the minimum p level at 623nm ( $p < 0.0001$ ). Interestingly, fluorescence emissions of plasma of patients with FAP were characterized by having the greatest mean intensity. No correlation was found between intensity of fluorescence at 623nm and age ( $r=0.030$ ); on the contrary the intensity of fluorescence resulted strongly correlated to the disease dimension ( $r=0.778$ ). The diagnostic capability of the fluorescence intensity at 623nm as a parameter to discriminate blood donors from patients bearing colorectal diseases, was tested by ROC analysis, which resulted in an AUC of 0.72 significant greater than that obtained with CEA (0.66).

**Conclusion:** According to our results, a possible application of the fluorescence measurements of blood plasma in patients at high risk would seem justified. Work is in progress to assess the true clinical value of the test on a larger series, also considering the renormalization of the intensity of fluorescence at 623nm after successful therapeutic treatment as well as the native fluorescence spectral features of blood plasma when different diseases are concerned.

**C03**  
**EVALUATION OF ROLE OF DIFFERENT HELICOBACTER PYLORI STRAINS (WILD TYPE, CAGA OR VACA NEGATIVE) ON APOPTOSIS INDUCTION IN AGS GASTRIC ADENOCARCINOMA AND HEF FIBROBLAST CELL LINES**

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*Helicobacter pylori* is a gram negative bacterium that infects human stomach. It is one of the most common pathogens affecting humans and is the major environmental factor in the development of gastric cancer. It is reported that the risk of carcinogenesis increases 4 to 6 folds due to this infection. Variations in cancer risk among *H. pylori* infected individuals may correlate to difference in *H. pylori* strains, variable host characteristics and specific interactions between host and microbial determinants.

To determine the effect of different strains of *H. pylori* on cellular apoptosis we designed an in vitro model using AGS and HEF cell lines.

After specified time intervals total cell proteins was extracted and subjected to SDS-PAGE and immunoblotting using anti poly ADP - ribose polymerase (PARP) antibody. Decrease in densitometric value of PARP was indicative of higher level of apoptosis.

The ability of apoptosis induction in AGS and HEF cell lines by wild type, *cagA* negative and *vacA* negative strains were significantly different. The assessed apoptosis in AGS cell line co-cultured with wild type strain was  $88\% \pm 27.9$  in 24 hours,  $91.6\% \pm 19.61$  in 48 hours, and  $92.4\% \pm 19.6$  in 72 hours of incubation time. Similar assessment with *cagA* negative strains in AGS cells was  $26.65\% \pm 9.7$  in 24 h.,  $38.5\% \pm 11.32$  in 48 h., and  $48.39\% \pm 8$  in 72 h. incubation. A variation in apoptotic potential between the *H. pylori* strains on two cells (AGS and HEF) was observed.

Based on our results, it is concluded that *H. pylori* strains as well as target cell types are important in pathogenesis and induction of apoptosis during a specified time interval.

#### **C04 SUCCESSFUL OXYTOCIN SUPPORTED HARVESTING OF NIPPLE FLUID**

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**Background:** A large need remains for new non-invasive breast cancer screening modalities. Nipple fluid, that contains breast epithelial cells, is produced in small amounts in the breast ducts of non-lactating women and can be collected by non-invasive vacuum-aspiration. Previous studies failed to obtain nipple fluid in a considerable proportion of women.

**Methods:** We assessed the feasibility of performing oxytocin supported nipple aspiration on 67 healthy female volunteers. Nipple fluid was aspirated through use of a vacuum system after administration of oxytocin nasal spray.

**Results:** Participants were 18 to 60 years old (mean 29 years). 12% of the women were postmenopausal. Nipple aspiration was successful in 63 out of 67 women (94%). For 13 women (19%), fluid was only obtained unilaterally, 50 women (75%) yielded bilaterally. The only predictor for fluid yielding during aspiration was a history of spontaneous nipple discharge ( $p < 0.005$  and  $p = 0.05$  for right and left breast, respectively). Previous breastfeeding, age, family history, history of breast lumps or surgery, hormonal and reproductive factors showed no predictive value for either the ability to yield fluid or the volume of nipple fluid that was produced. Volumes ranged from 5 to  $>100 \mu\text{l}$ , containing 50-200 ng/ $\mu\text{l}$  DNA, which showed to be largely enough for performing a quantitative methylation specific PCR for multiple genes. The procedure was very well endured. Mean discomfort-rating during different stages of the

procedure was 1.3 (on a 0-10 scale), compared to 1.9 for breastfeeding and 4.3 for mammography.

**Conclusion:** Oxytocin supported nipple aspiration provides a valuable tool for accessing mammary epithelium, providing sufficient DNA for a broad spectrum of analysis in the large majority of women.

*This work was funded by the Dutch Cancer Society, the "Integraal Kankercentrum Midden-The Netherlands (IKMN)", the "Visser Bearda Stichting" and the American Women's Club of The Hague.*

#### **C05 IDENTIFICATION OF MOUSE COLON TUMOR SECRETOME AS PUTATIVE BIOMARKERS FOR BLOOD-BASED SCREENING OF COLORECTAL CANCER**

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Colorectal cancer (CRC) is the second leading cause of cancer death in the Western world. Detection of CRC at an early stage of disease is associated with a much better prognosis for the patient, and is a realistic approach for reducing the high number of CRC deaths. The aim of this study is to identify biomarkers that can be used to develop a blood-based test for early diagnosis of CRC. The tumor secretome comprises proteins that are either secreted, shed by membrane vesicles (exosomes), or externalized due to cell death, and provides a new avenue for discovery of blood-based biomarkers. Using a mouse model for sporadic CRC (conditional truncated *Apc*), colon tumors and healthy control tissues were excised, washed briefly, and incubated in a small volume of PBS at 37°C for 1 hour. Tissues, cells, debris, and insoluble proteins were removed by centrifugation steps. The remaining soluble fraction, the "secretome", was analyzed by in-depth (GeLC-MS/MS) proteomics. Mouse colon tumor secretomes (n=3) were compared to secretomes of healthy colon tissues (n=3) from age- and gender-matched mice. This procedure revealed 2174 different proteins, 146 of which exhibited more spectral counts in each tumor sample than in any control sample. Of these, 80 proteins exhibited an average of 4-fold more spectral counts in tumor samples than in controls (semi-quantitative measurement), including haptoglobin

which was previously reported as a plasma marker for mouse APCMin tumors, thereby confirming the validity of our approach. We are currently examining by Western blotting which of these putative secretome biomarkers can be found at increased levels in blood of tumor-bearing mice, and may serve as promising candidate biomarkers for further validation in human blood from CRC patients and control subjects.

*This research was supported by the 1st AEGON International Scholarship in Oncology, and by the Cancer Center Amsterdam.*

#### C06

### **IL-1 -31T>C PROMOTER POLYMORPHISM IS ASSOCIATED WITH GASTRIC STUMP CANCER BUT NOT WITH EARLY-ONSET AND CONVENTIONAL GASTRIC CANCERS**

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**Background:** It has been reported that IL-1 genes play a crucial role in the genetic predisposition to gastric cancer although there is no information about their role in different sub-types of gastric cancer. Our study investigates the role of the IL-1 -31T>C promoter polymorphism as a risk factor for the development of gastric cancer.

**Material and Methods:** We performed the single nucleotide polymorphism (SNP) analysis using real-time PCR and sequence analysis of 241 gastric cancers including early-onset gastric cancers (EOGC), conventional gastric cancers and gastric stump cancers (GSC) as well as 100 control patients, using real-time PCR and sequence analysis.

**Results:** We found that the C allele was present in 60% of EOGCs, 59% of conventional gastric cancers and

90% of GSCs, in comparison to 62% in the control group. Interestingly, there was no difference in the distribution of the IL-1 -31T>C polymorphism in early-onset and conventional gastric cancer. A statistically significant difference in the presence of the C allele was found in patients with gastric stump cancer (p=0.008) compared to the control group. The T allele is therefore associated with protection against gastric stump cancer. There was no correlation between the presence of the C allele, histology and location of tumor in the gastric cancer sub-types.

**Conclusions:** In summary, we have showed that the IL-1 -31C allele promoter polymorphism is significantly associated with gastric stump cancer when compared to the control group. Although several molecular differences have been identified between conventional gastric cancer and early-onset gastric cancer, the IL-1 -31 allele distribution is similar between these two groups.

#### C07

### **PREDICTIVE ROLE MOLECULAR SUBTYPES OF CYCLOOXYGENASE-2 IN BASAL-LIKE BREAST CANCER PATIENTS TREATED WITH ADJUVANT ANTHRACYCLINE-BASED CHEMOTHERAPY**

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High levels of cyclooxygenase-2 (COX-2), an inducible enzyme involved in prostaglandin biosynthesis, have been shown to correlate with a worse prognosis in patients with several types of tumors including breast cancer (BC). Moreover, epidemiological, experimental and clinical studies have collectively identified COX-2 as a relevant target for the prevention and treatment of a variety of epithelial malignancies overexpressing this enzyme. Purpose of our study is to evaluate the prognostic value of COX-2 in the context of chemoresponsiveness in BC patients submitted to anthracycline-based adjuvant therapy. We further analyzed the potential differences in treatment effect due to COX-2 expression within the BC molecular subtypes, namely luminal A, basal-like, HER2, normal-like, as defined by immunohistochemistry. To this end, 186 stage I-IIA-B BC patients, enrolled in a prospective clinical trial using epirubicin plus cyclophosphamide in the adjuvant setting, were investigated using immunohistochemical methods. COX-2 overexpression was significantly associated to nodal status (p=0.01) and proliferation index (Ki67) (p=0.009). We evidenced a lower percentage of COX-2 positive tumors in the basal-

like subtype than in the other three groups. This difference was statistically significant ( $p=0.022$ ). Multivariate analysis (COX model) indicated that COX-2 was an independent prognostic variable influencing disease free survival (DFS) (HR=3.82, CI=1.54-9.50,  $p=0.004$ ), but not overall survival (OS). We also estimated the DFS and OS within the four BC subtypes according to COX-2 overexpression demonstrating that a significant higher probability of relapsing, but not of dying was observed in COX-2 positive luminal A ( $p=0.03$ ) and basal-like ( $p=0.05$ ) BC. In contrast, the outcomes of HER2 ( $p=0.11$ ) and normal-like ( $p=0.66$ ) subtypes were not affected by COX-2 overexpression. The current data suggest that, in our series, COX-2 up-regulation may be strongly informative in predicting response to adjuvant anthracycline therapy mainly in two subtypes, luminal A and basal-like, usually characterized by significant differences in prognosis and response to therapy. In this context, treatment with selective inhibitors of COX-2 may be an additional therapeutic option.

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**C08**  
**PERINECROTIC HIF-1 EXPRESSION AND NECROSIS PREDICT PROGNOSIS IN PATIENTS WITH ENDOMETRIOID ENDOMETRIAL CARCINOMA**

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**Background:** Hypoxia-inducible factor 1 (HIF-1) plays an essential role in the adaptive response of cells to hypoxia, triggering biologic events associated with aggressive tumour behaviour. Hypoxia and its key regulator HIF-1 play an important role in endometrial carcinogenesis, but contradictory results have been published as to the prognostic value of HIF-1 expression in endometrial carcinoma, probably due to varying methodology and patient groups. The aim of this study was therefore to re-evaluate the prognostic value of HIF-1 expression in a large representative group patients with endometrioid endometrial cancer using well established methodology.

**Methods:** In 98 patients with endometrioid endometrial cancer, expression levels of HIF-1 and p27 were analyzed by immunohistochemistry. Presence of necrosis, and type of HIF-1 expression (perinecrotic, diffuse, or mixed) were noted. Univariate and

multivariate recurrence free survival analysis was performed by Kaplan Meijer/log rank test and Cox regression, respectively.

**Results:** Stage, grade and depth of invasion showed prognostic value as expected, underlining the representativeness of the patient group. Indicators of poor prognosis were presence of necrosis ( $p=0.005$ ) and perinecrotic type of HIF-1 expression ( $p=0.03$ ). In patients with perinecrotic type of HIF-1 expression, high p27 expression was an additional prognostic factor. In Cox regression, HIF-1 was an additional prognostic factor to stage.

**Conclusion:** In patients with endometrioid endometrial cancer, necrosis and necrosis related expression of HIF-1 are important prognostic factors. In view of the proposed role of hypoxia and HIF-1 in endometrial cancer, HIF-1 is thereby an attractive therapeutic target.

**C09**  
**IDENTIFICATION OF GENES THAT MARK ACTIVITY LEVELS OF METABOLIC PATHWAYS IN COLORECTAL ADENOMA-TO-CARCINOMA PROGRESSION**

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Tumor development is a step-wise process in which accumulation of multiple genetic alterations affects normal regulation of biological processes, e.g. as described by Hanahan and Weinberg (2000). As a consequence, changes in metabolic processes are required to provide sufficient 'growth and fuel signals' to support these physiological alterations. Colorectal carcinomas (CRCs) develop from normal colon epithelium through a non-malignant adenoma stage. Importantly, only 5% of adenomas are estimated to progress to malignancy, indicating that adenoma-to-carcinoma progression requires yet additional changes that differ significantly from early alterations in adenoma development. In this study, we aimed to identify what metabolic pathways are affected during adenoma-to-carcinoma progression, and what genes within these pathways mark their activity levels. Genome-wide mRNA expression profiles from 37 colon adenomas and 31 colon carcinomas were compared to each other for 191 predefined KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways - predominantly metabolism and signaling pathways -

using the pathway analysis program Pathway Level Analysis of Gene Expression (PLAGE). This analysis identified 24 metabolic KEGG pathways with significantly different activity levels in adenomas and CRCs. These metabolic pathways could be subdivided in carbohydrate metabolism (6 pathways), glycan biosynthesis and metabolism (5 pathways), lipid metabolism (4 pathways) and amino acid metabolism (4 pathways). PLAGE analysis also revealed what genes within these pathways exhibited most effect on the pathway activity, indicating that mRNA expression levels of AOC2, ADH1C

### C10 PERFORMANCE CHARACTERISTICS OF FAECAL OCCULT BLOOD TESTS: WHICH TEST TO USE FOR COLORECTAL CANCER SCREENING?

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**Introduction:** Guaiac-based faecal occult blood tests (FOBT's) in a colorectal cancer screening setting are commonly hampered by a poor specificity and positive predictive value, resulting in many (futile) follow-up colonoscopies. Hence, immunological FOBT's with apparently better clinical performance, absence of dietary restrictions and only one faecal sample required, have been proposed as a more efficient screening tool.

**Aim:** To compare an immunology-based (OC sensor®, Eiken chemical Co, Japan) and a guaiac-based (hemocult®, Beckman Coulter, Inc. USA) FOBT in consecutive patients undergoing colonoscopy in terms of clinical yield of colorectal cancer and advanced adenomas.

**Methods:** All patients aged  $\times$  18 years and scheduled for a colonoscopy in participating hospitals (N=5) were asked to perform both FOBT's in the week prior to colonoscopy. Exclusion criteria were: a history of IBD, failure to complete both tests and an incomplete colonoscopy. A haemoglobin concentration of  $\times$  100ng/ml in the test sample was considered a positive

result. McNemar's test was used for the comparison of correlated proportions.  $P < 0,05$  was considered statistically significant.

**Results:** After appliance of exclusion criteria, 1331 eligible patients were included. Colorectal carcinoma and advanced adenomas (i.e.  $\times$  1 cm in diameter and/or villous architecture and/or high-grade dysplasia) were found in 3,4 % and 8,6 % of the patients, respectively. Small adenomas, colitis and other lesions were identified in 55,2 % of the patients. No lesions were found in 32,8 % of the patients. The hemocult® test and OC sensor® test showed positivity rates of 5,9 % and 11,9 %, respectively. Test characteristics for both FOBT's are shown in Table 1.

	hemocult® CRC	OC sensor® CRC	hemocult® Advanced adenomas	OC sensor® Advanced adenomas	Hemocult® Neoplasia <sup>a</sup>	OC sensor® Neoplasia <sup>a</sup>
Sens	64.4%	93.3%	13.9%	26.4%	28.1%	52.5%
Spec	96.1%	90.9%	94.8%	93.8%	97.1%	93.6%
PPV	36.7%	26.4%	20.3%	36.5%	37.8%	52.8%
NPV	91.7%	99.7%	92.1%	99.4%	90.1%	96.5%

<sup>a</sup>advanced neoplasia consists of advanced adenomas and cancer

Table 1. Test characteristics

**Conclusions:** Although the sensitivity and specificity of the OC-sensor test in detecting colorectal cancer was high in this patient group, the sensitivity to detect high-risk, pre-cancerous lesions was disappointing. The sensitivity of the Hemocult test is poor for both CRC and advanced adenomas. Moreover, the low positive predictive value of both tests in pre-cancerous patients might hamper the introduction of either one of these tests in a screening setting. A larger cohort is currently being investigated.

**C11  
SCREENING THE AVERAGE POPULATION  
FOR CRC: NUMBER OF COLONOSCOPIES CAN  
BE TUNED TO CAPACITY BY ADJUSTING  
CUT-OFF VALUES OF IMMUNOLOGICAL  
FOBT**

Frank Oort<sup>1</sup>, Jochim Terhaarsive Droste<sup>1</sup>, Mike Craanen<sup>1</sup>, René van der Hulst<sup>2</sup>, Henk van Heukelem<sup>3</sup>, Ruud Loffeld<sup>4</sup>, Eric Wesdorp<sup>5</sup>, Gerrit Meijer<sup>1</sup>, Chris Mulder<sup>1</sup>

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**Introduction:** Immunological faecal occult blood tests (i-FOBT's) have been proposed for population based colorectal cancer screening. i-FOBT has advantages over guaiac-based FOBT's: only one faecal sample is needed and no dietary restrictions exist because of specificity for haemoglobin of human origin. i-FOBT gives a quantitative result which enables adjusting the threshold for calling a test positive and referring a screenee for colonoscopy. This can be relevant in a phase of gradually implementing a CRC screening program because of limited colonoscopy capacity. Different cut-off values may also influence cost-effectiveness. The optimal cut-off value of i-FOBT has not been clarified yet, but cut-off values of 50-100 ng/ml are currently used.

**Aim:** To assess to what extent test characteristics of i-FOBT (OC sensor®, Eiken chemical Co, Japan) are influenced by adjusting the cut-off value in terms of clinical yield of colorectal cancer and advanced adenomas.

**Methods:** All patients aged  $\times 18$  years scheduled for colonoscopy in one of five participating hospitals, were asked to perform an i-FOBT the day prior to colonoscopy. I-FOBT was analyzed with the "OC-SENSOR  $\mu$ " desktop analyser. Cut-off values of  $\times 50$ ,  $\times 100$ ,  $\times 150$  and  $\times 200$  ng haemoglobin per ml, respectively, were used and outcomes compared to colonoscopy. All cases of CRC and advanced adenomas (i.e.  $\times 1$  cm in diameter and/or villous architecture and/or high-grade dysplasia) were scored. Patients with known IBD, patients in whom the caecum was not visualized and/or bowel cleansing was insufficient, were excluded.

**Results:** 1424 patients were eligible and included in this interim analysis. On colonoscopy, advanced neoplasia was found in 11.7% of the patients (CRC in 3.3% and advanced adenomas in 8.4%). The OC sensor® was positive in 8.8%, 9.9%, 11.7% and 13.6% at cut offs of 200, 150, 100, and 50 ng/ml, respectively (Table 1), resulting in 48,000 less colonoscopies between highest and lowest cut offs on a screening population of 1,000,000 while NPV for cancer remains largely stable.

CRC	50ng/ml	100ng/ml	150ng/ml	200ng/ml
Sens	93.6%	93.6%	87.2%	85.1%
Spec	89.1%	91.1%	92.7%	93.8%
PPV	22.7%	26.5%	29.1%	32.0%
NPV	99.8%	99.8%	99.5%	99.5%

Table 1.

**Conclusions:** A higher cut off for i-FOBT can reduce strain on colonoscopy capacity in the start up phase of CRC screening program, while NPV for cancer remains largely stable.

**C12  
QUANTITATIVE MEASUREMENT OF THE  
DISTRIBUTION OF THE PROLIFERATING  
CALLS IN PRE-NEOPLASTIC LUNG LESIONS**

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Lung cancers are believed to arise after a series of progressive pathological changes (known as preneoplastic or precursor lesions) in the respiratory mucosa. An in depth understanding of all the molecular changes involved in the pre-neoplastic development is key to understanding and altering the neoplastic process for therapeutic purposes.

Among the few reports that have examined the use of biomarkers to determine the biological behaviour of dysplastic lesions, many have depended only on qualitative or semi-quantitative assessment of expression of biomarkers on a global basis and ignored potential sources of information in the distribution pattern of these biomarkers within the tissue structure.

Uncontrolled proliferation is a hallmark of cancer and a biologically plausible risk biomarker for preneoplastic epithelium. In this report, we focus on the objective and quantitative analysis of the distribution of proliferating

cells marked by Ki-67 immunohistochemistry in preneoplastic lung lesions.

Our dataset consists of 744 lung bronchial biopsies from a large lung cancer chemoprevention study. Sections adjacent to the H&E section used for pathological interpretation were labeled with Ki-67. The field of view containing the most positive cells in each immunostained section was imaged. For each section, the basement membrane, the epithelial surface, and centers of all cell nuclei were marked and positive cells were labeled by the user.

In this work we implement methods that using the above information, the spatial arrangement of the cells, and positions of Ki-67 positive nuclei within the arrangement automatically extract a number of quantitative features per image such as: the distance of the nuclei to the basement membrane and to the epithelial surface both in terms of the number of cell layers and image coordinates, the percentage of Ki-67 positive nuclei per epithelial cell layer, etc. These features mathematically quantify the pattern of the proliferating cells within the different pathology grades of preneoplasia. To demonstrate that this pattern is biologically controlled and non random, we performed randomization tests on these features on a case by case basis, by permuting the labels of the cells a number of times and extracting the features each time.

### **C13 IDENTIFICATION OF CELL SURFACE PROTEINS AS BIOMARKERS FOR MOLECULAR IMAGING OF COLORECTAL CANCER**

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Colorectal cancer (CRC) is the second leading cause of cancer death in the Western world. Detection of CRC at an early stage of disease (high-risk adenomas) is associated with a much better prognosis for the patient, and is a realistic approach for reducing the high number of CRC deaths. The aim of this study is to identify biomarkers, that discriminate low-risk colon adenomas from high-risk adenomas and adenocarcinomas and that can be applied for molecular imaging. Based on microarray expression analysis, we previously obtained a list of genes of which mRNA expression is significantly increased in adenocarcinomas compared to

low-risk adenomas, further referred to as candidate biomarkers. From this list, genes encoding proteins with extracellular domains would have most potential to be imaggable biomarkers. Therefore we set out to identify cell surface proteins of colon cancer cell lines and correlate them with the candidate biomarkers. Colo205 cells were cultured until 70-80% confluency and incubated with sulfo-NHS-SS-Biotin to biotinylate cell surface proteins, which were isolated from the whole cell lysate. Western blotting confirmed that samples were enriched for extracellular proteins. Protein mixtures were fractionated using 1D SDS-PAGE and further processed for in-depth proteomics analysis by liquid-chromatography followed by tandem mass spectrometry (LC-MS/MS). A total of 1046 proteins were identified upon analysis of three biological replicates of colo 205 cells, 563 of which were reproducibly detected in all three samples. The biotinylated protein fractions were clearly enriched in membrane proteins when compared to the remaining non-biotinylated cell fractions. Integration of the cell-surface proteomics data with the microarray expression data showed that 98 of the 1046 proteins matched with genes that showed mRNA overexpression in adenocarcinomas compared to low-risk adenomas. These preliminary data indicate that this strategy resulted in the successful identification of cell surface proteins that may serve as imaggable biomarkers for high-risk adenomas and CRCs. Currently we are extending our experiments to several other CRC cell lines, including RKO, Caco2, HCT116 and HT29.

### **C14 THE SENSITIVITY, SPECIFICITY AND EFFICIENCY OF MEAN NUCLEAR AREA (MNA) MEASUREMENT IN FNAB SAMPLES OF THE BREAST**

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We studied 39 FNAB samples fixed in 50% ethanol with nuclear area measurement of the most prominent cells among cell groups and in the free cell category. Samples were collected at the African Oncology Institute in Sabratha. The nature of the lesions was verified by histopathology after surgical removal of the lesion. There were 22 malignant cases, and 17 benign cases (fibroadenomas, fibrocystic disease). In morphometric evaluation apocrine metaplastic nuclei were not measured to avoid overlap with malignant

cases. On average 60 nuclei were measured from cell groups, and from the free cell category. The computerized interactive measurement was based on the Prodit morphometry program, and the measurements were calibrated with a micrometer scale in two perpendicular directions.

The table below demonstrates the power of the method. MNA was the most distinctive morphometric feature of all features available. The size variables were significantly different between malignant and benign groups, but shape features did not help in distinction of the groups.

	Cutpoints (micromete r <sup>2</sup> )	Sensitivi ty (%)	Specifici ty (%)	Efficien cy (%)
<b>Free cells</b>				
MNA >=	63	90.9	100.0	94.9
MNA >=	58	95.5	94.1	94.9
MNA <=	54	100.0	88.2	94.9
<b>Cell grou ps</b>				
MNA >=	62	90.9	100.0	94.9
MNA >=	60	95.5	94.1	94.9
MNA <=	58	100.0	94.1	97.4

On the basis of the results in this material the potentially risk-free guidelines are: 1) Samples with MNA>= 63 (free cells) or MNA>=62 (cell groups) are always malignant, and samples with MNA<=54 (free cells) and MNA<=58 (cell groups) are benign. Values falling between 55-62 (free cells) or 59-61 (cell groups) should be considered uncertain and subject to additional studies, such as repeat biopsies, DNA cytometric analysis, core biopsy, cDNA array analysis of gene expression, or - depending on circumstances - removal of the whole lesion followed by frozen section diagnosis. Further studies from different laboratories will be necessary for producing universally more applicable guidelines.

### C15 CELLULAR SOCIOLOGY IS PREDICTIVE FOR THE RECURRENCE OF BASEL CELL CARCINOMAS

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**Introduction:** Basal cell carcinomas (BBCs) usually follow an indolent clinical course. A part of them, however, recurs after treatment causing local tissue destruction. Therefore, it is important to study additional morphological variables predictive for the outcome of these tumors. The aim of our study was to investigate whether an analysis of the cellular sociology of routinely stained HE histologic sections of the resection specimen would be able to identify patients with a high risk for BCC recurrence.

**Materials and Methods:** Our study was based on patients with surgically excised BBCs with a follow-up of at least 60 months. In 24 patients the tumor had recurred. In all cases (n = 56) the primary tumor was excised without compromised resection margins. Specimen and tumor diameters, as well as the minimal distance between tumor and the resection margins were measured. In every case a minimum of 10 randomly selected digitalized images containing at least 150 tumor cells were captured near the tumor invasion front. The nuclei were marked semi -interactively. Based on Delaunay triangulations, the minimal spanning tree and the mean distances to the first 20 nearest neighbors were determined. In order to get a predictive model for tumor recurrence, we calculated univariate and multivariate Cox-regressions regarding event-free survival.

**Results:** In univariate analyses the mean length of the vertices of the minimal spanning tree as well as the neighborhood distances of the first until the 20th order were of prognostic significance, with shorter distances indicating a higher risk of tumor recurrence. In the multivariate analysis, when testing together with the variables "minimal distance to the resection margin" and "histological tumor type", the final model contained only the variables "distance to first neighbor" and the "minimal distance to the resection margin", i.e. BCCs with more closely packed neoplastic cells or a smaller distance to the resection margin were at higher risk for recurrence. A prognostic index separating high-, intermediate- and low-risk BCCs, was created.

**Conclusion:** Cellular sociology of routinely stained HE sections is a simple and rapid method with predictive value for BCC recurrence.

### C16 USING GENE EXPRESSION PROFILING TO DIFFERENTIATE BREAST AND COLON TUMORS

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Classification of human cancers into distinct molecular groups based on their gene expression and alternative splicing profiles rather than their histological appearance may prove to be highly relevant to specific cancer diagnoses and cancer treatments. The overall goal of our studies was to identify and analyze patterns of mRNA splice variants as cancer specific or cancer-enriched biomarkers that can be used to improve diagnosis, prognosis, and prediction of breast and colon cancers. First, using in silico analysis we identified a vast number of potentially cancer-specific or cancer-enriched alternatively spliced mRNA transcripts. Subsequent qPCR analysis performed in a set of breast and colon cancer clinical samples confirmed that many alternatively spliced transcripts can be used as diagnostic or prognostic markers of breast and colon cancer. Based on these findings we developed a qPCR based multianalyte test to diagnose breast and colon cancer using 15 alternatively spliced mRNAs in biopsy samples. Our results show that using expression of alternatively spliced mRNAs as biomarkers we were able to develop a molecular detection system for breast and colon that could be adopted for clinical use for managing patients with breast and colon cancer.

### C17 APPROACH TO EARLY DIAGNOSIS OF LUNG CANCER IN RURAL ACHAIA IN GREECE

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**Aim:** The early diagnosis of lung cancer remains a difficult challenge. The majority of lung cancers diagnosed today are not curable at the time of diagnosis. Studies have shown that subjects who have smoked 30 or more pack-years, and who have any degree of airflow obstruction, results in four to six times more lung cancer

than if airflow is normal, with all other risk factors being equal. Our aim was to find if the frontline practitioner can help with the diagnosis of early lung cancer with a very easy approach.

**Methods:** The files of 303 Health Services adult were examined, 232 males and 71 females. Patients with symptoms of cough, dyspnea and wheeze, strong smoking history, documented airflow obstruction (COPD) and an abnormal spirometry were recorded as High risk - Lung Cancer patients (HR-LC). Patients with similar symptoms and history and a normal spirometry were recorded as Medium risk - Lung Cancer patients (MR-LC). All the patients made chest x-ray.

**Results:** HR-LC: 167 patients (55,11%), 137 male and 9 female. MR-LC: 136 patients (44,89%), 95 male and 62 female. All HR-LC patients were send for (CT) which 2,39% (4 patients) were found to have carcinoma in situ or invasive carcinoma; another 25,15% (42 patients) had moderate dysplasia which yielded additional carcinomas over the subsequent follow-up period.

**Conclusion:** Simple, handheld, accurate office spirometers should be used by all primary care physicians. Spirometry should have all smokers over 45 years old, patients with a family history of lung cancer or COPD, and any patient with cough, inappropriate dyspnea, wheeze or excess mucus production.

## Poster session D: Cancer cytometry / Bioinformatics

### D01 GENE REGULATORY LINK BETWEEN THE FANCONI ANEMIA/BRCA PATHWAY AND E2F TRANSCRIPTION FACTORS

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Fanconi anemia (FA) is an autosomal recessive disorder characterized by congenital abnormalities, bone marrow failure, chromosome fragility, and cancer susceptibility. To date, at least eleven members of the FA gene family have been identified using complementation experiments. Each FA has its own characteristic features, but their functions commonly belong to the same categories, such as DNA damage repair or S phase progression. In the present work, we investigated the common transcriptional regulatory factors that regulate FA genes. For this purpose, we evaluated the possible

link between E2F transcription factors and FA genes by analyzing the FA gene promoters in silico followed by a luciferase-based promoter assay. These analyses, combined with a public microarray data search, allowed us to identify a novel aspect of the FA pathway that is partially regulated in a cell cycle-dependent manner via E2Fs. The discovery that both FA genes and BRCA1 are under the control of E2Fs suggests that the FA/BRCA pathway is an effector of E2F-regulated cell cycle progression and DNA damage repair signaling. In conclusion, we found that the FA/BRCA pathway is regulated by activator E2Fs responsible for the execution of the DNA damage repair pathway. Most importantly, this pathway enables mechanistic links between E2F1 and FA genes, illuminating the molecular basis of DNA damage repair and S phase progression. We propose that the present analysis might be used as a research working model to approach systems biology, in combination with in silico and functional analyses, for a comprehensive characterization of cellular events in any given organism.

## D02

### FLOW CYTOMETRY CHARACTERIZATION OF STEM CELL LINEAGES IN THE LIVER OF PATIENTS WITH HEPATIC TUMORS

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Recent papers documented the identification of a tumorigenic cancer stem cell population bearing the CD133 marker in solid tumors (brain, prostate and colon) and hepatocellular carcinoma (HCC) cell lines.

In this study, we characterized stem/progenitor cell compartments of liver tissue from patients affected by HCC or cholangiocarcinoma (CC). For each patient, we evaluated cell suspensions obtained after enzymatic digestion of both intranodular (IN) and extranodular (EN) hepatic tissue. Since we were investigating very rare events, we used a six-color flow cytometry approach to analyze at least  $1 \times 10^6$  cells. We studied 14 patients with liver tumors (11 HCC; 3 CC) undergoing surgical resection and 9 multiorgan donors. Liver cell suspensions were evaluated for the expression of CD133 and others stemness-related antigens (CD34, c-Kit, Thy-1, ABCG2). Based on previous evidence that

CD133 antigen is also expressed on hematopoietic and endothelial progenitors, lineage commitment was also evaluated by specific markers (CD45 and CD146).

In IN tissue, the percentage of total stem cells (i.e. positive for CD133, CD34, Thy-1, c-Kit or ABCG2), was significantly higher than in EN and in organ donor tissues (mean  $\pm$  SD,  $4.84 \pm 3.88$  vs.  $2.29 \pm 1.64$  vs.  $1.73 \pm 2.06$  respectively;  $p < 0.05$ ). Particularly, the following subpopulations of stem cells were increased in IN vs. EN tissues: CD133+ ( $1.3 \pm 1.8$  vs.  $0.7 \pm 0.9$ ;  $p < 0.049$ ), CD34+ ( $0.6 \pm 0.5$  vs.  $0.3 \pm 0.2$ ;  $p = 0.008$ ), and ABCG2+ ( $0.8 \pm 0.7$  vs.  $0.3 \pm 0.2$ ;  $p = 0.013$ ) (Wilcoxon signed rank test). Most CD133 positive cells coexpressed either CD146 (range: 1-42%) or CD45 (range: 23-90%). The lineage commitment of these cells were confirmed by cell culture assays. In conclusion, liver tumors show an increase of stem cells as compared to EN tissues. However, not all hepatic tumors show the same stem cell profile. Unlike in highly purified cell lines, CD133 alone cannot be used to identify genuine tumor-initiating cells, because of the concomitant presence of progenitors committed to non epithelial lineages. Additional markers are therefore needed to the identification of hepatic cancer stem cells: in this regard, ABCG2+ and CD34+ subpopulations should be object of future studies.

## D03

### ARCHITECTURAL DISORDERS OF NORMAL AND MALIGNANT COLONIC MUCOSA DUE TO MALIGNANT TRANSFORMATION AND PROGRESSION: A MORPHOLOGICAL-BASES APPROACH

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We present a morphological based approach of architectural disorders due to malignant transformation and progression. With this aim in view, samples from 80 sporadic colorectal cancers were compared with non malignant (normal and dysplastic) colonic mucosa samples.

First, owing to histomorphological parameters (significantly increased thickness, low density of normal crypts, an increase of abnormal crypts), normal mucosa adjacent to cancer was distinguished from distant mucosa. Yet proliferating cells marker (Ki-67),

-catenin and Cyclin D1 immunolabelling showed no significant difference. Those data suggest a balancing mechanism to preserve normal crypt cells rather than a genuine proliferative process.

Secondly, immunolabelling with Ki-67, -catenin and Cyclin D1 antigens were done on malignant and non malignant samples, to determine cycling cells. Then, we used Voronoï's diagramm, a tool of Cellular Sociology, to quantify tissular architecture disorders within cycling and non cycling cells. Compared to normal mucosa cycling cells, malignant cycling cells show significant spatial rearrangement disorders, which is not the case of non cycling cells.

Finally this study stresses tumor cells heterogeneity ; tumor cells heterogeneity was analyzed by « virtual spotting », demonstrating markers-dependant labelling heterogeneity. This heterogeneity was quantified through a novel mathematical tool: a spatial heterogeneity index that was previously applied to Breast cancer tissues. This analysis showed that dysplasic mucosa samples and well differentiated tumors demonstrate higher levels of heterogeneity than poor differentiated cancers.

#### D04

##### A REVIEW OF STATISTICAL NUCLEAR TEXTURE ANALYSIS IN CANCER RESEARCH

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Nuclear texture analysis gives information about the spatial arrangement of the pixel gray levels in a digitized microscopic nuclear image, and is a quantitative tool for diagnosis and prognosis of human cancer.

In the present work we have reviewed nuclear texture analysis in cancer research, with emphasis on 1) the statistical texture analysis methods, 2) methods for feature evaluation and feature set selection, classification methods and error estimation and 3) the recent literature in the field, focusing on diagnosis and prognosis related applications.

Features from the gray level cooccurrence matrix and gray level run length matrix are the most commonly used statistical texture features in nuclear image analysis. These features are strongly dependent on parameters like inter-pixel distance and number of quantization levels in the image. They are also influenced by the pre-processing of the images, e.g., histogram-matching. Only a few of the published studies have reported which values of the parameters they have

utilized, and which type of histogram-matching (if any) that is performed.

In order to find nuclear features that discriminate between cases from different diagnostic or prognostic classes, a statistical evaluation of the features needs to be performed. It is common to evaluate a large number of nuclear features on a limited data set, without the proper independence between training and test data. This easily leads to over-optimistic results. We point out that only a few studies have evaluated their results on relatively large and independent data sets.

In nuclear image analysis, there is a real need for common and well defined feature sets and standard methods for statistical evaluation of the classification results. In future studies on nuclear image analysis it should be a requirement that the experimental design is clearly described, that the features are well defined, and that the results are properly evaluated on independent data sets. Only then can the results of different approaches be compared and evaluated. We believe that standardizing the methods of feature extraction and statistical evaluation is the only way to get some of the best of the exciting new methods of nuclear image analysis into applied medicine.

#### D05

##### NUCLEAR SEGMENTATION FOR SILVER STAINED CYTOPATHOLOGICAL SPECIMENS

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**Introduction:** The ribosomal RNA is replicated within the nucleolar organizer regions (NORs) of a cell. Hence, they are of central importance for the protein synthesis of a cell, which in turn reveals the proliferation activity of the cell. Proteins associated with nucleolar organizer regions are stained by silver nitrate. The AgNORs indicate active rDNA and, hence, regulatory processes of the cell growth mechanism. The AgNOR count and size per nucleus has been utilized for cancer diagnostics. To automatically detect AgNORs a robust delineation of the nuclei is required.

**Methods:** We have stained 23 FNAB-smears of the thyroid gland according to Feulgen. With a Leica DMLA microscope (63x objective, oil immersion) we have acquired 8617 nuclei images. These Feulgen stained nuclei have been automatically segmented and finally peer reviewed by a cytopathologist. After destaining and silver staining we have once again

acquired images of these cells, now in silver stain. These images have been segmented by a mean shift segmentation, i.e., by a clustering in a feature space. A nuclear segmentation now is obtained from this over segmentation by grouping segments together to achieve the desired nuclear delineation. To this end, we implemented a model-guided region-grouping, i.e., an ellipse model incorporated into an overall energy description of the segmentation.

**Results:** The algorithm is able to automatically segment nuclei in silver stained specimens. To evaluate the segmentation accuracy we compared the segmentation in the silver stain to the previously acquired segmentation in the Feulgen stain. The segmentation algorithm achieved a mean areal segmentation error of  $dA=12\text{micrometer}^2$ .

**Conclusion:** We have shown that an automated segmentation of the nuclei in silver stained specimens can be achieved by a two-step approach of first calculating an over segmentation followed by a model-guided region-grouping. An ellipse model, incorporated into a global energy term, achieved satisfactory results while still allowing shape deformations. Hence, this algorithm allows a stand-alone automated AgNOR analysis without a prior Feulgen stain. However, so far the algorithm fails to segment touching or overlapping nuclei. The latter case is irrelevant for the diagnosis, while the former is a straightforward extension of the algorithm.

#### D06

##### IMAGE REGISTRATION FOR MULTIMODAL CELL ANALYSIS

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**Background:** Single cytochemical stains often exhibit either high diagnostic sensitivity or specificity but not both. Combination of stains thus improves the diagnostic accuracy. Fusion at a single cell level raises the diagnostic significance further. Yet simultaneous application of stains and their analysis is limited. Multimodal cell analysis grounds on the fact that, provided adequate preparation, specimens can be repeatedly destained and restained. Computer-controlled microscopes principally allow to image corresponding spots on a slide repetitively. However, the removal of the slide for the restaining process and imprecisions of

the stage mechanics induce a position error which must be corrected for. Sub-micron accuracy is required to overlay nuclear segmentations from one stain on another. In a high throughput scenario as for screening purposes, the required image registration technique must be robust and efficient.

**Methods:** An intensity-based, multi-scale framework was used for registration. We deduced the adequacy of mutual information as image similarity criterion from staining characteristics and quantitatively compared it with other criteria. Tests were performed on 100 image triples from 40 specimens of pleural effusions. Each triple consisted of May-Grünwald-Giemsa, Feulgen Pararosaniline, and silver nitrate stained images of identical cells. We measured precision, runtime, and the curve's smoothness. Interpolation artefacts derogate registration performance. From the available strategies for artefact suppression, the computationally negligible prior rotation of images was chosen and evaluated. It was shown that structural information for registration of images of cytologic specimens is mainly contained in the edge regions of cell nuclei. An appropriate restriction on regions of interest was implemented.

**Results:** Mutual information performed best among the tested criteria. Interpolation artefacts could be eliminated, whereby three advantages were achieved: increased precision, smoother curves and thus significantly fewer computations during the optimisation, and the potential to employ simple and fast interpolators like nearest neighbour which produce smooth curves if rotation is applied. Regions of interest can be reduced to below 15% of the image area. Both techniques lead to an average speedup of 85% and a median precision of 0.047 microns.

**Conclusions:** The presented solution enables multimodal cell analysis in a high-throughput context.

#### D07

##### DOUBLE-STAINING OF CYTOLOGICAL SAMPLES WITH QUANTITATIVE FEULGEN-THIONIN AND ANTI-KI-67 IMMUNOCYTOCHEMISTRY AS A METHOD OF DISTINGUISHING CELLS WITH ABNORMAL DNA CONTENT FROM NORMAL CYCLING CELLS

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Abnormal amounts of DNA present in a cell nucleus can be indicative of large-scale mutations. Hence, there has been considerable interest in using DNA ploidy for cancer screening. Feulgen-thionin staining is a method

of quantitatively staining the DNA present in a cell. In our experience, this stain gives highly reproducible results. Staining of the HL-60 acute promyelocytic leukemia cell line gives cleanly resolved diploid and tetraploid histogram peaks, each with coefficients of variation (CV) of around 2%. As a screening marker, ploidy determined by Feulgen-thionin staining has been found to perform at least as well as other existing screening technologies, such as conventional cytology or human papilloma virus testing in cervical cancer. Moreover, Feulgen-thionin staining is quick, can be automated, and is relatively inexpensive.

Thus, Feulgen-thionin staining offers a simple way of detecting cells with abnormal DNA content. Normal proliferating cells, though, can have anywhere from 2N to 4N chromatids. To distinguish between these normal dividing cells and truly abnormal cells, we investigated the use of anti-Ki-67 antibodies in a double staining procedure to distinguish actively dividing cells (Ki-67 positive) from non-dividing cells (Ki-67 negative).

Previous attempts to simultaneously determine DNA content and proliferation status have typically involved immunofluorescence and flow cytometry. The method we present here utilizes absorbance stains, allowing us to perform these analyses without the higher costs and photobleaching associated with detecting fluorescence, while avoiding the artifact detection issues surrounding flow cytometry.

We present here some of our results in double-staining for thionin and Ki-67. We found that the antigen retrieval step in immunocytochemistry could remove any thionin staining that had already been performed or drastically reduce the intensity of thionin staining done afterwards. We hypothesized that this was due to the hydrolysis actions of antigen retrieval. Consequently, we adjusted the hydrolysis time in the thionin staining procedure. We have so far been able to doubly-stain HL-60 slides to yield a quantifiable thionin signal, with a CV of about 8%. We hope to further refine this methodology so that it can become a useful screening tool for detecting cells with abnormal DNA content.

## D08

### VARIATION OF CELL POPULATIONS IN THE BLAST GATE IN MYELODYPLASTIC SYNDROMES

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**Introduction:** Bone marrow (BM) lymphoid precursors (blast subset with a low SSC) are decreased in myelodysplasia (MDS). This has been proposed to be a diagnostic feature for this group of disorders. Moreover, an SSC increase in myeloid precursors has been characterized as an adverse prognostic factor.

**Material and Methods:** Using multiparameter flow cytometry we examined the composition of cells in the blast gate in patients with MDS, diagnosed according to WHO criteria. BM from donors for allogeneic transplantation was used as controls. The blast population was gated in the CD45/SSC plot. Two subsets of cells were separated according to SSC: subset 1 with low and subset 2 with high SSC.

**Results:** We analysed 17 normal BMs and 39 cases of MDS (2 refractory anemias, 25 refractory cytopenias with multilineage dysplasia, and 9 refractory anemias with excess of blasts -RAEB). In controls, 2 subpopulations with a different SSC were found: subset 1 with a mean SSC value of 38 (range 28-49) and subset 2 with 113 (range 80-147). In MDS patients, subset 1 was found only in 24/39 cases with SSC values similar to that of normal BM. Subset 1 was decreased in low-grade MDS, but increased in RAEB: 0.21% (0.05%-0.6%) in normal BM; 0.15% (0%-0.99%) in low grade MDS and 0.69% (0%-1.9%) in RAEB ( $p=0.006$ ). The quotient between subset 2 and 1 increased with a higher IPSS and with lower hemoglobin values. The mean SSC based on all cells in the blast gate was positively correlated with the proportion of CD34-/CD117+ cells.

**Conclusion:** FCM is able to separate myeloblasts from lymphoid precursors by SSC. The proportion of subset 1 was decreased in low grade MDS but increases with disease progression. Therefore we hypothesize that the subset with low SSC may contain, besides the lymphoid precursors, also immature stem cells with the potential to differentiate into several other cell lines, since the increase may be due to stem cells committed to the lymphoid series but also not yet committed stem cells.

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### D09 AUTOMATED DETECTION OF AGNORS BY HIGH DYNAMIC RANGE MICROSCOPY

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**Introduction:** The proliferation activity of a cell or, in turn, of a tumor, is a diagnostically highly relevant feature. To quantify this, the count, size, and distribution of AgNORs in silver stained specimens have been shown to be very sensitive diagnostic parameters of the cellular growth behavior. However, since the silver stain exhibits strong intensity and contrast variations an automated, robust, and reliable detection of AgNORs could not be achieved so far.

**Methods:** Due to these variations in the silver stain, the material covers a large dynamic range. This dynamic range, in general, cannot be acquired with conventional digital cameras in a single image. Therefore, the dynamic range of the camera has to be extended. To this end, we acquire a set of images of the same cell at different exposure settings. After linearization of these images, these are combined into one high dynamic range image, which covers the full dynamic range of the cellular material under investigation. Now, the image of each individual nucleus is adapted such that the image becomes independent of the staining variations. Within the thus obtained normalized nuclei images the detection and quantification of the AgNORs is achieved by mean shift segmentation and knowledge-based classification.

**Results:** We have applied this algorithm to 55 nuclear images of four FNAB-smears from the thyroid gland and two specimens from pleural effusions, which have been manually rated to exhibit worst stain and contrast variations by an experienced cytopathologist. An experienced cytopathologist reviewed the segmentations and the proposed algorithm achieved a satisfactory segmentation and detection of individual AgNORs in 90.6%. Due to failed segmentations only 3.6% of the cells must be discarded as diagnostically unusable.

**Conclusion:** Silver stained specimens show strong intensity and contrast variations even on the same slide and the limited dynamic range of conventional digital cameras is insufficient to acquire the whole dynamic range covered by the biological material. However, high dynamic range microscopy, not only removes the dynamic range limitations, but furthermore the variations from the staining procedure can be

compensated. This allowed a robust automated segmentation of AgNORs in silver stained specimens.

### D10 AUTOMATED DETECTION OF IMMUNOCYTOCHEMICAL MARKER DEMONSTRATIONS FOR CANCER SCREENING

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Germany*

**Introduction:** Cytopathological specimens can be obtained without discomfort for the patient, i.e., without a biopsy and, hence, can be utilized for screening applications. Staining with immunocytochemical markers, e.g., p16ink4a for cervical smears, allows to classify the majority of specimens as healthy, decreasing the amount of more time-consuming and expensive methods. We automatically analyze immunocytochemically stained specimens and investigate the influence of the intensity of a counter stain.

**Methods:** Seven specimens of cervical smears have been stained with p16inka4 and hematoxylin counter stain with differing intensities. One more specimen was stained with p16ink4a only. All images have been acquired with a Leica DMLA microscope (5x objective) and a JAI CV-M90 3CCD camera. To acquire images of the whole slide, we have developed a tracking autofocus which ensures sufficient image quality for all images based on the sum modulus differences (SMD) focus criteria evaluated on the counter stain. Focus tracking is achieved by only one additional image per neighboring slide position.

All thus acquired images are now split into two color components, one for the marker stain and one for the counter stain. This is achieved through a Gaussian-Mixture model of the two color components in the color plane of the Lab-color space. The Gaussian distribution of the marker stain is determined from the exclusively p16ink4a marker stained specimen.

**Results:** The autofocus achieved satisfactory results and acquired images of sufficient sharpness throughout each slide. However, increasing the intensity of the counter stain decreases the detection rate of the immunocytochemical marker stained cells. Marker detection specificity ranged from 72%-95%, and for the sensitivity from 41%-91% for high to low intensities of the counter stain respectively.

**Conclusion:** We have shown that a fully automatic acquisition of images from a cytopathological specimen of a cervical smear can be achieved. However, to distinguish the colors of immunocytochemical marker and counter stain a low contrast counter stain is demanded. Otherwise color mixtures turn out to become indistinguishable black. Consequently, ensuring a low counter stain intensity, automated immunocytochemical marker detection with high sensitivity of >90% and specificity >70% are obtainable.

### **D11 QUANTITATIVE AND MOLECULAR PATHOLOGY AND DIAGNOSTIC OR THERAPY-ASSOCIATED CLINICAL DECISIONMAKING**

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The view of quantitative pathology has been changing recently. It used to be thought that measurements done in clinical, cytological, or histological laboratory, or on microscopic images in general were the only subjects of quantitation. Today's view is different. The most fruitful view, no doubt, is that the term quantitative pathology means any disease-related quantitative analysis including the quantitative analysis relevant in the clinical decisionmaking. So quantitative pathology also includes the application of quantitative methods which describe the power of any method in clinical decisionmaking. Such quantitative analysis is relevant in respect to deciding of the presence/absence of disease (differential diagnosis), to evaluating the expected survival, to evaluating the disease free survival after treatment, and to deciding about the potential responsiveness of the disease to specific therapy (prediction of therapy response).

The methodological implementation should include the quantitative evaluation of the usefulness of any method in situations where decisions are made on the mode of therapy. This is done by defining the relevant method characteristics in decisionmaking. The most illustrative characteristics are sensitivity, specificity, and efficiency of the method used. In comparing methods of various kinds, e.g. molecular methods with DNA cytometric methods, a corresponding approach should be taken. Such an approach will also be able to compare various types of cDNA-arrays, and the ways they are read, with each other in clinical decisionmaking.

### **D12 CHALLENGES, STATE-OF-THE ART AND PERSPECTIVES OF COMPUTER-SUPPORTED CELL ANALYSIS**

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The lecture will present an overview on state-of-the-art and emerging computer applications in cytopathological cancer diagnostics. Three major trends are to be observed that partly unfold synergetic impacts.

**(1) Second-generation cell image analysis:** Improved microscope optics, digital image sensors, and image processing algorithms are leading to novel approaches to the digital analysis of cellular and sub-cellular features. Typically, these are measuring processes that deliver quantitative results in contrast to the qualitative assessment of a visual inspection. Features to be measured are derived from models that are built on cell biological and molecular hypotheses. Examples are DNA image cytometry, which is founded on the chromosomal aberration hypothesis, or the assessment of AgNORs by means of measurements of their size and distribution. A striking new technology, multimodal cell analysis, traces individual cells and merges measuring results of complementary analyses. Their correlation yields a further enhancement of diagnostic relevance.

**(2) Vertical integration:** A great deal of knowledge has been piled up on the specific disease-related levels such as epidemiology, patient, organ, tissue, cells/nuclei, and, finally, the genome. Level-specific databases, in the past, were mostly isolated, experts talked different lingo, and knowledge was exchanged inside particular communities. Meanwhile, however, it is understood that improved computer models can be developed by the combination of knowledge on complementary levels. Suchlike comprehensive models will lead towards improved prevention plans, diagnostic strategies and treatment protocols. An expanded approach will strengthen the awareness of the relevance of the cellular/sub-cellular level, and thus will boost the role of cytopathological diagnostics.

**(3) The digital pathology:** Digital analysis processes invading the cytological labs, growing throughput of specimen, pressure of time and costs, and the demand for conformity with hospital IT environments are heralding the era of the "fully-digital (cyto)pathology".

In a networked system of computerized workplaces the image material will immediately be digitized by front-end image acquisition stations. New developments of high-precision slide digitizers will partly replace today's microscopes. Digital image archives and work-flow architectures will complement the IT work environment. These trends are enforced by the European Community's ICT-Health research programme with actual foci on improved modelling by the 'virtual physiological human' initiative, and on extended exploitation of existing models by explicitly demanding for 'vertical integration'.

### **D13 IMAGE CYTOMETRY FOR ORAL CANCER SCREENING**

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Oral cancer like many readily accessible epithelial cancers are much more treatable if caught in their early pre-invasive/minimally invasive stages. In British Columbia we have established an Oral Cancer Prevention Program in which we are evaluating and investigating several technologies and their interactions for the screening and follow-up of oral cancer to be implemented into a population based screening program. These sensitive "field of view" image based screening technologies are generally sensitive for the detection of suspect OPLs (oral premalignant lesions), but can highlight areas whose actual characteristics may be masked by inflammation/ulceration and other conditions. As part of a comprehensive management program we present our pilot data on the use of oral cytological samples collected by targeted brushing and analyzed by a fully automated high resolution image cytometry device (cyto-savant).

For this study we collected 193 cytological samples using targeted brushing of select areas in the oral cavity from individuals with squamous cell carcinoma (SCC), carcinoma in situ (CIS), severe dysplasia, no areas of abnormality and subjects with areas of inflammation/infection etc. All of these samples were spun down onto slides and the DNA quantitatively labeled with a modified Feulgen-Thionin stain and the slides automatically scanned by the cyto-savant. For each object (cell/debris) imaged ~120 features were calculated and used by a cell recognition decision tree (originally trained for cervical cell recognition) to differentiate cells from debris. The 108 samples from

known normal areas and 57 samples from (SCC, CIS and severe dysplasia) were used to determine the appropriate thresholds for the frequency of cells displaying characteristics previously known to be associated with cancer detection (in cervix and lung). Using these thresholds the system correctly identified 89% of the abnormal cases and 84% of the normal cases. When tested on the 28 inflammation/infection cases the system correctly identified 81% of the evaluable samples as non OPLs. These pilot results indicate that image cytometry may have a roll in oral cancer screening.

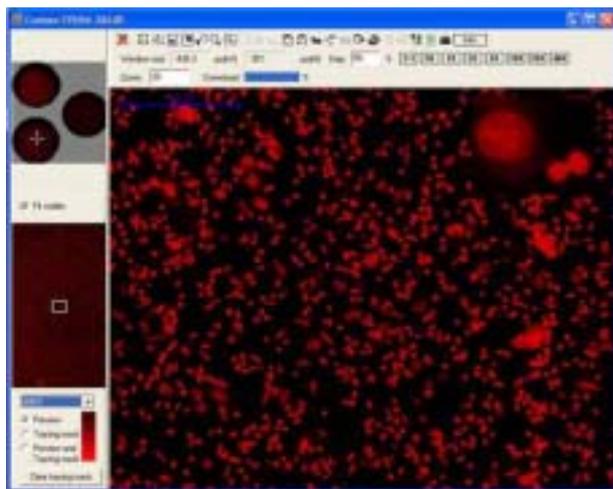
### **D14 HIGH THROUGHPUT SLIDE SCANNER BASED DNA IMAGE CYTOMETRY**

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**Background:** DNA aneuploidy reflects gross genomic changes and can be measured either by DNA flow cytometry (DNA-FCM) or DNA image cytometry (DNA-ICM). With the recent introduction of fully automated slide scanners the advantages of both DNA-FCM and DNA-ICM can be combined. In the present study we investigated whether high throughput DNA-ICM (HT-DNA-ICM) is attainable using a commercially available slide scanner.

**Material and Methods:** Nuclei were isolated from formalin fixed paraffin embedded tissue samples of different cancer types. DNA-FCM was performed with a Partec PAS system and DNA-ICM (Zeiss Axioplan) and HT-DNA-ICM (Zeiss Mirax Scanner) as well as with the traditional PIPE image analysis system. DNA histograms produced by all three methods were analyzed with MultiCycle and results compared.

**Results:** DNA indices of all three methods are comparable. Coefficients of variation of the DNA diploid peaks were lowest for DNA-FCM (average number of nuclei 28200; average CV 3.3%), marginally higher for DNA-ICM (average number of nuclei 612; average CV 4.3%), and in general substantially higher for HT-DNA-ICM (average number of nuclei 52168; average CV 10%). Furthermore, rare-event detection (9C exceeding events) was feasible using HT-DNA-ICM.



Overview of high throughput fluorescence DNA image

**Conclusion:** Advantages of DNA-FCM and DNA-ICM can be combined using HT-DNA-ICM. HT-DNA-ICM is attainable. However, there are obvious differences in certain quantitative parameters between DNA-ICM and HT-DNA-ICM and suitable internal control slides for HT-DNA-ICM to standardize results are necessary. These slides are currently being developed as well as the possibility to use HT-DNA-ICM in fluorescent mode.

#### **D15 GENERATION OF FLOW CYTOMETRY DATA FILES WITH A POTENTIALLY INFINITE NUMBER OF DIMENSIONS**

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Immunophenotypic characterization of B-cell chronic lymphoproliferative disorders (B-CLPD) is associated with the use of increasingly larger panels of multiple combinations of 3 to 6 or more monoclonal antibodies (Mab), which require data analysis to separately performed for each of the different stained sample aliquots. Accordingly, in a sample for which two or more aliquots were separately stained with different combinations of MAb, single cellular events are only associated with part of all information/parameters evaluated; in addition, the correlation between the

patterns of antigen expression observed in one aliquot and those of another staining can not be directly obtained and require of an experienced operator. In some cases, further staining of a new aliquot of the sample with the precise combination of reagents whose evaluation requires a direct correlation in single cells, is needed. This can only be done if the reagents available at that moment in the laboratory are conjugated with different and compatible fluorochromes. In the present paper we describe and validate an automated method for the calculation of flow cytometric data corresponding to single cellular events from several multicolor stainings of the same cell sample, after merging data from different aliquots stained with partially overlapping combinations of Mab reagents (focusing on 1 or more cell populations) into one single data file, as if it concerned a single "super" multi-color staining. Evaluation of the performance of the method described here was performed in a group of 60 B-CLPD studied at diagnosis with 18 different reagents in a panel containing 6 different 4-color stainings, which systematically contained the CD19 reagent for the identification of B-cells. Our results show a high degree of correlation and agreement between originally measured and calculated data about cell surface stainings, providing a basis for the use of this approach for the generation of flow cytometric data-files containing information about a virtually infinite number of stainings for each individual cellular event measured in a sample, despite the use of a limited number of fluorochrome stainings. In summary, we describe and demonstrate the reliability of a new statistical approach that may be used for the automated generation of flow cytometry data-files containing information on single events about a virtually infinite number of parameters. This new strategy opens the door for many applications of flow cytometry for which a largenumber of parameters are needed, particularly for leukaemia immunophenotyping.

**D16**  
**ASSOCIATION BETWEEN THE PROLIFERATIVE RATE OF NEOPLASTIC B-CELLS. THEIR MATURATION STAGE AND UNDERLYING, THEIR MATURATION STAGE AND UNDERLYING CYTOGENETIC ABNORMALITIES IN B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS**

Julia Almeida<sup>1</sup>, Sandra Quijano<sup>1</sup>, Antonio Lopez<sup>1</sup>, Ana Rasillo<sup>1</sup>, Maria Luz Sanchez<sup>1</sup>, Juan Flores<sup>1</sup>, Carlos Fernandez<sup>1</sup>, Jose Maria Sayagues<sup>1</sup>, Pilar Giraldo<sup>2</sup>, Manuel Giralt<sup>2</sup>, Maria Carmen Perez<sup>2</sup>, Jose Manuel Martin-Antoran<sup>3</sup>, Oliver Gutierrez<sup>3</sup>, Luis Perdiguier<sup>4</sup>, Alberto Orfao<sup>1</sup>

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Limited knowledge exists about the impact of specific genetic abnormalities on the proliferation of neoplastic B cells from chronic B-cell lymphoproliferative disorders (B-CLPD). Our aim was to analyse the impact of cytogenetic abnormalities on the proliferation of neoplastic B cells from a series of 432 B-CLPD patients -grouped according to diagnosis and site of sampling, in comparison to their normal counterparts. Proliferation of neoplastic B cells highly varied among the different B-CLPD subtypes, but the greatest numbers of proliferating cells were detected in diffuse large B-cell (DLBCL) and Burkitt's lymphomas (BL). Compared to normal B cells, neoplastic B-CLPD cells showed abnormally increased S+G2M-phase values in mantle cell lymphoma (MCL), B-cell chronic lymphoid leukemia (B-CLL), BL and some DLBCL cases. In contrast, decrease proliferation was observed in follicular lymphoma (FL), lymphoplasmacytic lymphoma (LPL/WM) and some DLBCL patients, while normal levels were found for hairy cell leukemia, splenic marginal zone and MALT-lymphoma patients. Interestingly, in B-CLL and MCL significantly higher percentages of S+G2/M cells were detected in bone marrow (BM) versus peripheral blood (PB) and in lymph node versus BM and PB samples, respectively. In turn, the presence of 14q32.3 gene rearrangements and DNA aneuploidy, but not other cytogenetic changes, were associated with a higher percentage of S+G2/M-phase cells among LPL/WM and B-CLL cases, respectively.

**D17**  
**MINIMAL RESIDUAL DISEASE BY MULTIPARAMETRIC FLOW CYTOMETRY IN B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS- A PROBABILISTIC APPROACH**

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**Introduction:** a new automated strategy for detection of minimal residual disease (MRD) in B-cell chronic lymphoproliferative disorders based on pattern classification tools and the Bayes theorem is described.

**Methodology:** Files containing B-cells gated of five peripheral blood (PB) samples from normal voluntaries were merged in a "normal-B-cells-pool". Each one of the 50 files containing B-cells gated of PB samples from B-cell chronic lymphoproliferative disorders (B-CLPD) were merged with the "normal-B-cells-pool" and projected in two dimensions by using a principal components transformation. In the same way, for each patient "MRD-files" were constructed by merging the "normal-B-cells-pool" with progressively decreasing quantities of neoplastic B-cells events. Afterward, for each case, the nearest normal B-cell subpopulation to the neoplastic B-cell population is identified. The probabilities of each event to belong to the neoplastic B-cell population and to the normal B-cell population were calculated according to the optimal Bayes classification principle.

**Results:** With this approach, we were able to systematically identify MRD by flow cytometry with a minimum sensitivity of 10<sup>-4</sup> (in most of cases - 80% - a sensitivity of 10<sup>-6</sup> was reached). Furthermore, this approach allowed the definition of the sensitivity that will be reached for in each case, a priori, at diagnosis. The specificity was also high; in 86% no events corresponding to normal B-cells were wrongly identified as belonging to the neoplastic B-cell population. In the remaining patients, the maximum of five events (in two cases) were wrongly identified as belonging to the neoplastic B-cell population.

**Discussion:** Our results show that this approach can be applied to virtually all B-CLPD with both a high sensitivity and specificity whenever the search for minimal numbers of neoplastic B-cells similar to those detected at diagnosis.

### D18 REPRODUCIBILITY OF IMAGE ANALYSIS IN VIRTUAL MICROSCOPY

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Virtual microscopy is revolutionising pathology by producing diagnostic quality digital images of the entire slide. This provides enormous opportunities for automated image analysis, particularly for tissue microarray (TMA) immuno-biomarker analysis. However quantitative image analysis of TMA virtual slides must produce consistent intra- and inter-scanner results.

This study aims to assess the reproducibility of morphologic, densitometric and texture data on different slide scanners. A single tissue microarray (TMAs) showing samples of gastric lesions and immunohistochemically stained for Cyclin B1 was scanned 5 times on two different Aperio CS Scanscopes. The same ten tissue cores were selected from each scan and a series of geometric, densitometric and textural measurements made to compare reproducibility on sequential scans on different instruments.

Measurements showed that intra-machine scans produce consistent densitometric results with a coefficient of variation of 0.039 (machine 1) and 0.013 (machine 2) for mean grey level of positively stained tissue over the entire core. A comparison of tissue cores scanned on different instruments shows subtle differences in the visual appearance of the images. This was confirmed in the quantitative evaluation of immunomarker labelling: e.g. a 4% difference was observed between the number of positively stained pixels between machines on a single core and a 32.4% difference in total mean density in positively stained areas.

Consistent results are essential for the quantitative evaluation of TMA immunohistochemistry. This study has shown that sequential scans from a single instrument are reproducible. However, there are obvious differences in the quantitative evaluation of TMAs from different instruments and it may be necessary to develop suitable internal control slides to standardise results.

### D19 MLPA-DAT: DATA ANALYSIS TOOL FOR RELIABLE AUTOMATED NORMALIZATION OF MLPA FRAGMENT DATA

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<sup>3</sup>MRC-Holland, Amsterdam, The Netherlands

**Background:** Multiplex Ligation dependent Probe Amplification (MLPA) is a rapid, simple, reliable and customized method for detection of nucleic acid copy number changes of individual genes at a high resolution and allows for high throughput analysis. This technique is typically applied for studying specific genes in large sample series. The large amount of data, dissimilarities in PCR efficiency among the different probe amplification products, and sample-to-sample variation pose a challenge to data analysis and interpretation. We therefore set out to develop an MLPA data analysis strategy and tool that is simple to use, while still taking into account the above-mentioned sources of variation.

**Materials and Methods:** MLPA-DAT was developed in Visual Basic for Applications, and can accept a large number of file formats directly from capillary sequence systems. Sizes of all MLPA probe signals are determined and filtered, quality control steps are performed, and variation in peak intensity related to size is corrected for. DNA copy number ratios of test samples are computed, displayed in a table view and a set of comprehensive figures is generated. To validate this approach, MLPA reactions were performed using a dedicated MLPA mix on 6 different colorectal cancer cell lines. The generated data were normalized using our program and results were compared to previously performed array-CGH results using both statistical methods and visual examination.

**Results and Discussion:** Visual examination of bar graphs and direct ratios for both techniques showed very similar results, while the average Pearson moment correlation over all MLPA probes was found to be 0.42. Automated MLPA data processing may therefore be of significant use when handling large MLPA data sets, when samples are of different quality, or interpretation of MLPA electropherograms is too complex. It remains however important to recognize that automated MLPA data processing may only be successful when a dedicated experimental setup is also considered.

**Availability:** The algorithm is implemented in VBA and runs in the environment of Microsoft Office 2003. The MLPA-DAT is available at <http://www.mlpa.com/coffalyser>.

*Contact: Coffalyser@mlpa.com & GA.Meijer@vumc.nl*  
*Supplementary information: Supplementary data are available at <http://www.mlpa.com/>.*

## Poster session E: Translational cancer research

### E01 TELEPATHOLOGY IN EMERGING COUNTRIES. PILOT PROJECT BETWEEN ITALY & EGYPT

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Pathological examination includes gross & microscopic examinations at different magnification, although the steps of examination, we obtain a lot of images that can be used for telepathology. Telepathology is the practice of pathology at a distance viewing images on a monitor rather than directly through a light microscope. It can be used for primary diagnosis, second opinion, quality assurance and distance learning. Telepathology is classified into Static, Dynamic, Hybrid and Whole Slide Imaging [WSI].

We have a successful experience in Egypt to apply the static & dynamic techniques of telepathology through a pilot project between the Italian Hospital in Cairo [NPO] and Civico Hospital in Palermo. This project began in 2003 and continued till now. In the next year 2004, Ospedale S. Giovanni e Paolo Hospital in Venice, Charing Cross Hospital in London and University of Pittsburgh Medical Center Health System [UPMC] in USA shared as new participants in our project. Along the past 5 years we consulted a lot of problematic pathological cases with these different specialized pathological centers in Italy, UK & USA. Beside the highly specialized scientific value of consulting the cases and exchanging knowledge, we saved a lot of time & money and offered our patients a better medical service.

Nowadays, we are planning to establish a Digital Telepathology Center [DTC] in the pathology department, Cairo University using the latest technique of telepathology which is Whole Slide Imaging [WSI].

We believe that it will help us a lot to reach the proper diagnosis for our difficult pathological cases and offer our staff a good chance for E-learning throughout the discussion of our cases.

### E02 LIMITED ONGOING LYMPHANGIOGENESIS IN RENAL CELL CARCINOMA BY LYMPHATIC ENDOTHELIAL CELL PROLIFERATION FRACTION, LYMPH VESSEL CHALKLEY COUNT AND MRNA EXPRESSION LEVELS OF LYMPHANGIOGENIC FACTORS

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<sup>1</sup>*University Hospital Maastricht, Maastricht, The Netherlands*

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**Aims:** To assess ongoing lymphangiogenesis in renal cell carcinomas (RCC) by histomorphometry and by quantifying mRNA expression levels of lymphangiogenic factors.

**Methods and Results:** The relative lymph vessel area (LVA) was measured with the Chalkley method following immunostaining of tissue sections with D2-40 (195 RCC and 61 controls). LVA was low but higher in the immediate peritumoural area compared to intratumoural counts (3.7 versus 1.3). In renal tissue at a distance, LVA was the highest (6.8). Lymphatic endothelial cell proliferation fraction (LECPF) was assessed on tissue section double-immunostained for D2-40 and Ki67. In a small fraction of RCC (2.6% and 6.5% (intra- and peritumoural area, respectively)) proliferating endothelial cells were seen. LECPF was small (<1%) without any difference between control, peritumoural and intratumoural tissue. Compared to control renal tissue (n = 9), RCC (25 clear cell RCC) had significantly higher mRNA content of VEGF-A and -C, but lower content of VEGF-D and Prox-1.

**Conclusions:** the data suggest that there is only limited ongoing lymphangiogenesis in RCC with LECPF 10 to 20 times lower than in breast carcinomas. Given the promiscuity of several growth factors, stimulating both angiogenesis and lymphangiogenesis, this observation is worthy of further study.

**E03****MET AMPLIFICATION IN NSCLC: CORRELATION WITH EGFR MUTATIONS AND GENE AMPLIFICATION**

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<sup>3</sup>Department of Medical Oncology, University General Hospital of Heraklion, Heraklion, Greece

**Background:** MET amplification in NSCLC was correlated with acquired resistance to EGFR TKIs, gefitinib and erlotinib. The aim of this study was to investigate whether MET is amplified in NSCLC prior to TKIs therapy and examine the association of MET amplification with EGFR copy number, mutation status and responsiveness to TKIs.

**Methods:** Amplification levels of MET and EGFR genes were determined in FFPE tumor DNA of known EGFR and K-RAS mutation status from 69 patients with advanced NSCLC treated with gefitinib. Quantitative real-time PCR was performed to analyse MET (7q31.2) and EGFR (7p12) gene copy numbers relative to a reference, the TOP3A (17p12-p11.2) gene. Relative copy numbers of the EGFR and MET genes were expressed as the ratio of PCR signals obtained for EGFR or MET and TOP3A. The cutoff for normal copy numbers was established as the 95% confidence upper limit (mean + 1.96 standard deviations) of EGFR/TOP3A and MET/TOP3A determined in DNA of normal matched tissue from 14 patients respectively.

**Results:** Seven (10%) of the 69 primary tumors exhibited increased MET copy number. None of these tumors had an EGFR mutation whereas 3 of them had K-RAS mutation. No significant association was found between MET copy number and TKI-related disease control rate or survival. Among the 7 patients showing MET amplification 2 (28.5%) with EGFR wild type mutation status exhibited progressive disease. EGFR amplification was detected in 5 (7.2%) patients and among them one (20%) showed MET amplification as well. Response rate was not associated with the EGFR amplification. One patient with a Del19 EGFR mutation reached partial response and another one with wild type mutation status achieved stable disease. The patient showing EGFR and MET amplification had not EGFR and K-RAS mutations and exhibited progressive disease.

**Conclusions:** Met amplification is not a common event in NSCLC prior to EGFR TKIs therapy. However the predictive value of chromosome 7 polysomy should be considered with caution.

**E04****LUNG CANCER DSA: A PLATFORM FOR DISCOVERY OF BIOMARKERS IN LUNG CANCER**

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 Almac Diagnostics, Craigavon, UK

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality worldwide with poor differential diagnosis of the disease and with low response rates to standard chemotherapy treatment. It is therefore a subject of extensive research focused on identification of reliable genomics biomarkers to aid in accurate classification of the disease, predicting its progression and patients' response to both available therapies and those in development. Powerful genomics tools used in this research are however lacking disease focus and thus are likely to miss potentially vital information contained in patients' tissue samples.

Through a combination of large-scale in-house sequencing, gene expression profiling and public sequence and gene expression data mining we have characterised the transcriptome of NSC lung cancer and used this information to create a unique disease focused microarray - Lung Cancer DSA research tool. Built on the Affymetrix GeneChip platform the tool allows for interrogation of ~60,000 transcripts relevant to Lung Cancer, tens of thousands of which are unavailable on leading commercial microarrays. Presented here are the array design process and the results of experiments carried out to demonstrate the array's utility for use in biomarker discovery projects with using NSCLC and normal samples.

**E05**  
**THE TWIST1 ONCOGENE IS A DIRECT TARGET OF HYPOXIA-INDUCIBLE FACTOR-2ALPHA**

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Hypoxia-inducible factors (HIFs) are highly conserved transcription factors that play a crucial role in oxygen homeostasis. Intratumoral hypoxia and genetic alterations lead to HIF activity, which is a hallmark of solid cancer and is associated with poor clinical outcome. HIF activity is regulated by an evolutionary conserved mechanism involving oxygen-dependent HIFalpha protein degradation. To identify novel components of the HIF pathway, we performed a genome-wide RNA interference screen in *Caenorhabditis elegans*, to suppress HIF-dependent phenotypes, like egg-laying defects and hypoxia survival. In addition to hif-1 (HIFalpha) and aha-1 (HIFbeta), we identified hih-8, gsk-3 and spe-8. The hih-8 gene is homologous to the human oncogene TWIST1. We show that TWIST1 expression in human cancer cells is enhanced by hypoxia in a HIF-2alpha-dependent manner. Furthermore, intronic hypoxia response elements of TWIST1 are regulated by HIF-2alpha, but not HIF-1alpha. These results identify TWIST1 as a direct target gene of HIF-2alpha, which may provide insight into the acquired metastatic capacity of hypoxic tumors.

**E06**  
**IN-TRANSIT LYMPH NODE METASTASES IN BREAST CANCER: A POSSIBLE SOURCE OF LOCAL RECURRENCE AFTER SENTINEL NODE PROCEDURE**

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**Background:** In-transit lymph node metastases are a common phenomenon in patients with melanoma, raising more attention since the introduction of the Sentinel Node (SN) procedure. To which extent this also occurs in breast cancer patients has not been studied yet.

**Aim:** To explore the occurrence of in-transit lymph nodes metastases in breast cancer.

**Methods:** Between 1998 and 2001, afferent lymph vessels to the SN identified by blue dye were removed from 18 breast cancer patients during regular SN procedure.

**Results:** Three out of 18 patients showed a lymph node associated with the afferent lymph vessels. One of these lymph nodes (6%) showed a breast cancer metastasis, to be regarded as an in-transit metastasis. This metastasis would normally have been left in situ and could thereby have been a source of local recurrence.

**Conclusion:** In-transit lymph nodes associated with the afferent SN lymph vessels seem to occur in a significant proportion in breast cancer patients, and may contain metastases. As these are a potential source of local recurrence when left in situ, there may be an indication to remove the SN afferent lymph vessels during the SN procedure.

**E07**  
**THE MICROANATOMIC LOCATION OF METASTATIC BREAST CANCER IN SENTINEL LYMPH NODES PREDICTS NON-SENTINEL LYMPH NODE INVOLVEMENT**

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**Background:** The majority of sentinel node (SN) positive breast cancer patients do not have additional non-SN involvement and may not benefit from axillary lymph node dissection (ALND). Previous studies in melanoma have suggested that microanatomic localization of SN metastases may predict non-SN involvement. The present study was designed to assess whether these criteria might also be used to be more restrictive in selecting breast cancer patients who would benefit from an ALND.

**Methods:** A consecutive series of 357 patients with invasive breast cancer and a tumor positive axillary SN, followed by an ALND, was reviewed. Microanatomic SN tumor features (subcapsular, combined subcapsular and parenchymal, parenchymal or extensive localization, and the penetrative depth from the SN capsule) were evaluated for their predictive value for non-SN involvement.

**Results:** Non-SN metastases were found in 136/357 cases (38%). A subcapsular localisation of tumor deposits and limited penetrative depth were associated with a low frequency of non-SN involvement (10%).

**Conclusions:** Microanatomic location and penetrative depth of breast cancer SN metastases predict non-SN involvement. However, based on these features no subgroup of patients could be selected with less than 10% non-SN involvement.

### E08

#### LARGE SCALE GENOMIC INSTABILITY AS AN ADDITIVE PROGNOSTIC MARKER IN EARLY PROSTATE CANCER

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Prostate cancer is the most frequent type of cancer among Norwegian men. The clinical outcome for an individual cancer patient is often difficult to predict, due to lack of reliable independent prognostic biomarkers. We tested DNA ploidy as a prognostic factor for clinical outcome in 186 patients treated with radical prostatectomy. DNA ploidy was measured using an automatic image cytometry system. The results were correlated with preoperative PSA, age at operation, Mostofi grade and Gleason score. The mean follow up time after operation was 73.3 months (range 2 to 176 months). Of the 186 microdissected tumour foci taken from the prostatectomies, 96 were identified as diploid, 61 as tetraploid and 29 as aneuploid. Twenty-three per cent, 36% and 62% of the diploid, tetraploid and aneuploid cases respectively, suffered from relapse during the observation time. DNA ploidy, Gleason score, Mostofi grading and preoperative PSA were all significant predictors of relapse in a univariate analysis. On multivariate analysis, only Gleason score and DNA ploidy proved to be independently predictors of disease recurrence.

Furthermore, among the 68 cases identified with Gleason score 7, DNA ploidy was the only significant predictor of disease recurrence, compared to preoperative PSA, age at operation, Mostofi grade and Gleason 3+4 versus 4+3. Our data strongly suggest that DNA ploidy should be included as an important additive prognostic factor for prostate cancer, especially for patients identified with Gleason score 7 tumours.

### E09

#### CYTOGENETIC ANALYSIS OF CIRCULATING TUMOR CELLS OF METASTATIC PROSTATE CANCER

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Tumor cells in blood of patients with metastatic carcinomas have been associated with poor survival prospects. In this study we evaluate the presence of cytogenetic abnormalities in Circulating Tumor Cells (CTC) detected in patients with metastatic prostate cancer. CTCs were enumerated in 7.5 mL of blood with the CellSearch system<sup>TM</sup>. After enumeration of the Cytokeratin+, CD45-, nucleated CTC, the fluid in the analysis cartridge was removed and the cells were fixed while maintaining their original position. Fluorescence in situ hybridization (FISH) is applied to the fixed cells labeling the centromeric regions of chromosome 1, 7, 8 and 17. After FISH the cartridges were placed on a CellTracks Analyzer<sup>®</sup> equipped with a 40X objective (NA0.60). The previously identified CTCs are revisited and fluorescent images of each of the four chromosomes labels were acquired. Next, the copy number of chromosomes 1, 7, 8 and 17 were determined for each CTC. Leukocytes surrounding the CTC were used as internal controls. 61% of the blood samples taken after initiation of therapy contained  $\times 1$  CTC and FISH was applied to 84 of the CTC containing blood samples from 42 metastatic prostate cancer patients. The 84 samples contained a total of 2932 CTC (mean=5, average= 33.7, SD=76.0). Of the 2932 CTC, 225 (8%) were lost during the fixation and FISH. Of the remaining 2707 CTC no FISH signals were detected in 69% of the CTC. The lack of FISH signals in these CTC is ascribed to apoptosis as the surrounding leukocytes showed good quality FISH signals. The 829 (31%) evaluable CTC contained on average 2.8 copies of chromosome 1, 3.0 copies of chromosome 7, 3.3 copies of chromosome 8 and 2.3 copies of chromosome 17. 664/829 (80%) of the CTC were aneuploid and a higher amplification of the chromosomes 1, 7, 8 and 17 was observed with increasing CTC number. In only 1 patient no aneuploid cells were detected and this patient only had 1 CTC. Heterogeneity in the chromosomal abnormalities was observed between CTC of different patients as well as among CTC of the same patient. In the same samples 885 leukocytes were evaluated as controls and showed an average of 1.9, 2.0, 1.9 and 1.8 copies of chromosome 1, 7, 8 and 17 respectively. Cytogenetic

composition of CTC can be assessed after they have been identified by the CellSearch system. The relation between the presence of aneusomy, the extent of amplification and outcome can now be investigated.

#### E10

##### **BIOBANKING OF FRESH FROZEN TISSUE: EFFECT OF TISSUE WARM ISCHEMIA TIME ON RNA DEGRADATION**

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A great need exists for well preserved and annotated specimens from normal and neoplastic human tissues that can be used for molecular analyses to investigate pathogenetic mechanisms, to classify tumors and to assess disease prognosis and treatment efficacy. However, as retrospective studies use tissue collected over time, it is important to be aware of possible biases of results in studies with a) frozen and b) fixed paraffin-embedded tissues. Measurements of gene expression is based on the assumption that RNA samples closely represent the amounts of transcripts *in vivo*. Partial degradation during cell lysis, warm ischemia time before freezing, artefacts from tissue processing or fragmentation induced by intense cross-linking during fixation may alter the relative abundance of transcripts and artificially modify gene expression profiles. The activity of our Frozen Tissue Bank, initiated in 2002 and currently collecting and storing primary and metastatic lesions with the corresponding normal tissues from different tumour types (about 1700 new cases yearly, which contributed specimens for more than 30 specific research projects), also includes an investigation on quality control procedures for tissue processing and storing. In order to understand the tissue handling bias on gene expression results, at first we focused on timing of tissue procurement, and consequently freezing, that, in our experience is within 60 minutes for the majority of the cases. We analyzed RNA integrity and gene expression in breast cancer specimens that were serially harvested from tumour lumps and snap-frozen at various time point (immediately and 2, 6 and 24 hours) after surgical resection. Overall, RNA does not appear to be rapidly degraded, regardless of time of tissue procurement, even when kept at room temperature up to 24 hours. Conversely, significant changes in gene expression occurred starting from 2 hours following surgical removal in samples maintained at room temperature compared to those snap-frozen in liquid

nitrogen immediately or at least within 2 hours of surgery. Specifically, this tissue processing time effect accounted also for changes in some of the genes included in the breast cancer prognostic signatures recently published .

#### E11

##### **NK CELL EXPANSION IS DEPENDENT ON GENOTYPE**

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Natural Killer (NK) cells are effector cells in the innate immune system. The peripheral blood mononuclear cells (PBMCs) of eighteen healthy individuals were cultured to expand NK cell numbers. The killer cell immunoglobulin-like receptor (KIR) and HLA repertoire were determined to analyse the influence of receptor-ligand interaction. The percentage of NK cell expansion from the total PBMC fraction varied between 5.4% and 71.6%. A significantly better NK cell expansion was observed for individuals homozygous for HLA-C epitope group 2 ( $p=0.05$ ). For evaluation of cytolytic competence of the cultured NK cells, specific killing of an HLA class I expression deficient LCL 721.221 cell line and three 721.221 cell lines transfected with different HLA-C alleles was determined. A significantly better NK cell-induced specific cytotoxicity was observed of the untransfected 721.221 cells compared to the HLA-C transfected 721.221 cells ( $p<0.01$ ). No differences were observed between killing of the three HLA-C transfected 721.221 cell lines as was explained by the KIR repertoire. We have shown that *in vitro* expansion of NK cells is dependant on the expression of HLA-C epitopes. Cytolytic capacities of the cultured NK cells are maintained which indicate that NK cell numbers can be expanded for the application of NK cell-based immunotherapy.

**E12**  
**IMMUNOTHERAPY FOR METASTATIC MELANOMA PATIENTS USING IN VIVO SALMONELLA TYPHIMURIUM VACCINATION AND TREATMENT**

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We report a conceptually new approach in order to develop an effective immunotherapy preventing the ability of tumours to escape the immune system. It is based on in vivo infection of tumoural cells using Salmonella Typhimurium and killing of infected tumour cells by a specific immune response. Two clinical steps were planned: oral vaccination with Ty21a and intratumoral treatment with Ty21a, in order to stimulate an autologous immune response. Since May 2006, 8 patients affected by not operable stage III or IV M1a metastatic melanoma were enrolled in the trial. Two metastases were treated and 1 observed in order to evaluate the indirect effect of vaccination. Among 8 enrolled patients, 1 could not be vaccinated due to the evidence of hepatic metastasis during staging, 1 had a rapid worsening of the performance status during vaccination while 2 didn't develop adequate anti-ST vaccination titre. Of the remaining 4 patients, 1 completed the first cycle, while the other 3 interrupted the treatment due to progressive disease, important hypersensitivity reaction and a worsening of the hepatic function indexes. At injection, all the patients developed a variable degree of lumbar pain, nausea/vomiting, shivering, fever, hypotension easily controlled by treatment with antihistaminics, antipyretics, antiemetics without the use of cortisones. Treated metastases showed all the signs of local inflammation and objective dimensional stabilization or reduction, but systemic disease seem not to be affected by treatment.

**E13**  
**EPIGENETIC MULTIDRUG APPROACH TO MODULATE THE IMMUNO-PHENOTYPE OF MALIGNANT NEUROBLASTOMA**

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Neuroblastoma (NB) is among the most common solid tumors in childhood. Despite the most recent advances

in combined surgical and radio-therapeutic approaches, the overall survival of NB patients has not significantly improved. Thus, new and more effective strategies are urgently needed for this deadly disease.

Both genetic and epigenetic alterations contribute to NB development and progression: in particular, epigenetic modifications (DNA methylation and histone modifications) have been shown to play a major role in the down-regulated expression of several immune molecules in NB cells (i.e., HLA class II, CD40, Cancer Testis Antigens) possibly favouring the immuno-escape of neoplastic cells.

On this background, we evaluated the immunomodulating effects of different schedules of the epigenetics drugs 5-Aza-2'-Deoxycytidine (5-AZA-CdR), a DNA methyltransferase inhibitor, and Trichostatin A (TSA), a histone deacetylase inhibitor, alone or combined, on a panel of 8 NB cell lines. The expression of selected immune molecules (i.e., HLA class I and II, b2-microglobulin and the costimulatory molecules ICAM-1, LFA-3 and CD40) was investigated by FACS analysis. Treatment of NB cells with 1μM 5-AZA-CdR alone, allowed to define two NB cellular subsets with different behaviours: in 5 out of 8 analysed cell lines, 5-AZA-CdR up-regulated the expression of investigated antigens, while in the remaining 3 cell lines investigated it did not. To evaluate whether combining 5-AZA-CdR and TSA was able to break this constitutive "immuno-resistance" to 5-AZA-CdR, the effects of simultaneous treatment with 1μM 5-AZA-CdR and 50nM TSA were investigated on the unresponsive SHSY5Y NB cell line. Our results show that, compared with untreated cells, the exposure to 5-AZA-CdR and TSA significantly ( $P < 0,05$ ) up-regulated the expression of HLA class I, b2-microglobulin, ICAM-1, LFA-3 and CD40 on SHSY5Y NB cells.

These data suggest that combined epigenetic approaches might represent new tools to potentiate the immunogenicity of NB cells and to improve the effects of immunotherapeutic approaches in NB patients.

#### E14 DNA COPY NUMBER BASED PREDICTORS OF RESPONSE TO CHEMOTHERAPY IN PRIMARY COLORECTAL CANCERS

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Beatriz Carvalho<sup>1</sup>, Tineke Buffart<sup>1</sup>, Paul Eijk<sup>1</sup>, Frits  
Peters<sup>1</sup>, Bauke Ylstra<sup>1</sup>, Han van Krieken<sup>2</sup>, Kees Punt<sup>2</sup>

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**Background:** Colorectal cancer is biologically a heterogeneous disease, which gives rise to differences in clinical behavior, including risk of metastasis and response to drug therapy. Obviously, the success of both classical drug therapies as well as novel targeted therapies can be improved by matching the right combination of drugs with different biological classes of CRC.

Aim of the present study was to correlate genome wide DNA copy number status in advanced colorectal cancer with response to systemic chemotherapy.

**Material:** Thirty-two patients with advanced colorectal cancer were selected from the patient series of the CAIRO study of the Dutch Colorectal Cancer Group (DCCG), based on either a good response (n=16) or a poor response (n=16) to first-line combined irinotecan and capecitabine therapy. For all cases, DNA was isolated from formaldehyde-fixed paraffin embedded tissue samples of the primary tumors.

**Methods:** High resolution DNA copy number profiles were determined by means of 30k oligonucleotide-based array comparative genomic hybridization (array CGH).

**Results:** The group of the non-responders had fewer aberrations ( $P<0.2$ ) than the responders, especially for the losses ( $P<0.03$ ). The median number of chromosomal alterations per carcinoma in the group of the 16 responders was 7.0 (range 1-19), with a median number of 3.0 gains (range 1-8) and 3.5 losses (range 0-12). For the group of the 16 non-responders the median chromosomal aberrations was 4.0 (range 0-14), with a median number of 2.5 gains (range 0-9) and 0 losses (range 0-12). The striking difference in aberrations between the two groups were losses 18p ( $P<0.02$ ) and 18q ( $P<0.03$ ), that were more frequent in the CRC of patients which had a good response to chemotherapy.

Hierarchical cluster analysis of the array-CGH data revealed two clusters with cluster 1 containing nineteen tumors and cluster 2 thirteen tumors. Thirteen out of nineteen tumors of cluster 1 consisted of non-responders, while in cluster 2 eleven out of thirteen

tumors were responders. Cluster membership showed a significant correlation with response status ( $P<0.03$ ).

**Conclusion:** Primary tumors of patients with advanced colorectal cancer with a good or poor response to systemic chemotherapy show different DNA copy number profiles. Tumors of patients with a good response to combined irinotecan and capecitabine treatment had overall more chromosomal aberrations, especially losses of 18p and 18q.

#### E15 IMMUNOPHENOTYPE OF DUCTAL CARCINOMA IN SITU IN BRCA GERMLINE MUTATION CARRIERS

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Wall

UMC Utrecht, Utrecht, The Netherlands

**Background:** Invasive cancers in germline BRCA1 and BRCA2 mutation carriers have a distinct morphological and immunophenotype, characterized by high expression of proteins associated with the basal epithelial phenotype (cytokeratins 5/6 and 14), and low expression of the estrogen (ER), progesterone (PR) and HER-2/neu receptors. Further, we have previously described that high expression of the epidermal growth factor receptor (EGFR) is part of this BRCA1/2 germline mutation related cancer profile. Little is known about the immunophenotype of precursor lesions in BRCA1/2 germline mutation carriers. The aim of this study was to examine the immunophenotype of precursor lesions like ductal carcinoma in situ (DCIS) in BRCA1/2 mutation carriers to elucidate whether the characteristic phenotype is already present before the stage of invasion.

**Material and Methods:** DCIS of 6 proven BRCA1 and 4 BRCA2 germline mutation carriers were stained by immunohistochemistry for ER, PR, HER-2/neu, Ck5/6, Ck14, EGFR and Ki67. Only clear membrane staining for EGFR was considered as overexpression. HER2 was scored according to the DAKO system.

**Results:** 4/11 cases (36%) were ER positive, 0/7 (0%) were PR positive, 0/10 (0%) were HER2 positive, 5/10 (50%) were CK5/6 positive, 1/9 (11%) were CK14 positive, and 6/10 (60%) were EGFR positive. Mean percentage Ki67 nuclear staining was 30% (range 0-100). These percentages are similar to those that have been reported for invasive cancers in BRCA1/2 mutation carriers, except for ER that is generally even lower in BRCA1/2 related cancers.

**Discussion:** DCIS in BRCA1/2 germline mutation carriers shows a so called basal immunophenotype with

high proliferation and EGFR positivity similar to that of invasive cancers in such patients. This may be useful to identify “BRCA-ness” in cases of DCIS in diagnostic pathology, and opens up new ways for targeted therapy against EGFR to prevent development of invasive cancer in case of a germline mutation.

*Supported in part by the unrestricted Aegon International Scholarship in Oncology.*

## E16

### **N-MYC DOWNSTREAM REGULATED GENE 4 (NDRG4) PROMOTER METHYLATION IS A SENSITIVE AND SPECIFIC BIOMARKER FOR COLORECTAL CANCER**

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**Background and Aims:** N-Myc downstream regulated gene 4 (*NDRG4*), a gene involved in cellular differentiation and neurite formation, is a member of the *NDRG* family. Here we address the role of *NDRG4* promoter methylation in colorectal cancer (CRC).

**Methods:** *NDRG4* promoter methylation was analyzed in CRC cell lines, normal colon mucosa, colorectal adenomas, carcinomas and other neoplasias using methylation specific PCR (MSP) and bisulfite sequencing. *NDRG4* promoter methylation was analyzed in fecal DNA of CRC patients and controls using quantitative MSP. Loss of heterozygosity (LOH) mapping the *NDRG4* locus and mutation analysis using direct sequencing of *NDRG4* were performed. *NDRG4* mRNA and protein expression was studied using RT-PCR and immunohistochemistry.

**Results:** *NDRG4* promoter methylation was observed in 7/8 CRC cell lines. The prevalence of *NDRG4* promoter methylation in CRC tissue was 86% (71/83) compared to 4% (2/48) in normal colon mucosa ( $p < 0.001$ ). An independent series of CRCs confirmed the high prevalence (69%, 127/183) of *NDRG4* methylation. *NDRG4* methylation was also observed in 81% (13/16)

of oesophageal adenocarcinomas and 77% (17/22) of gastric cancers. *NDRG4* promoter methylation analysis in fecal DNA yielded a sensitivity of 76% (16/21) (95% CI, 58%-94%) and a specificity of 97% (65/67) (95% CI 93%-100%). No mutations were found while 30,5% of CRCs showed LOH on the *NDRG4* locus. Expression of *NDRG4* is decreased at the RNA and protein level in CRC when compared to normal tissue.

**Conclusions:** *NDRG4* is frequently methylated in colorectal adenomas, carcinomas and other adenocarcinomas of the gastrointestinal tract. *NDRG4* promoter methylation in fecal DNA can be used as a sensitive and specific biomarker for detection of CRC.

## **Poster session F: Experimental therapeutics and pharmacogenomics**

### F01

#### **CAMPATH-1H: A POTENTIAL THERAPY FOR SKELETAL TUMORS?**

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**Aims:** Bone tumors are rare neoplasms of the skeletal system. Earlier investigations suggested that CD52, a GPI-anchored protein expressed on leucocytes and cells of the male genital tract, is overexpressed in mesenchymal neoplasms of the bone. While the physiological role of CD52 is unclear, an antibody directed to CD52 (CAMPATH-1H) is capable of complement activation and antibody-mediated cellular cytotoxicity leading to lymphocyte depletion. The current study aimed to further analyze the expression of CD52 on several skeletal tumors and to evaluate the potential for treatment of CD52 expressing skeletal tumors with CAMPATH-1H.

**Methods:** RT-PCR, immunohistochemical staining and flow cytometry were used to analyze the expression of CD52. MNNG/HOS osteosarcoma cells were treated

with CAMPATH-1H and proliferation of the cells was measured using MTT-assays.

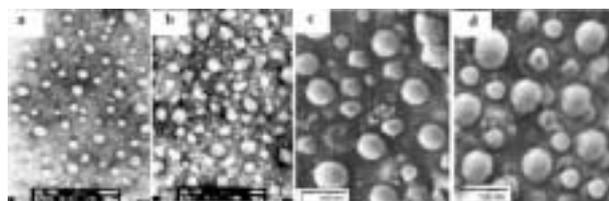
**Results:** We confirmed the expression of CD52 mRNA and protein both in vivo and in vitro in benign and malignant skeletal tumors and their non-neoplastic counterparts. In general, the malignant tumors showed a higher CD52-expression compared to benign entities. Treatment of MNNG/HOS cells with CAMPATH-1H led to a complement- and antibody-dependent reduction of viable osteosarcoma cells. **Conclusion:** In this study we describe for the first time the extra- and intracellular expression of CD52 in mesenchymal tumors. Higher expression of CD52 in malignant tumors suggests a functional involvement of CD52 in tumor progression. Our results obtained in osteosarcoma MNNG/HOS cells showed that CAMPATH-1H, a CD52 antibody currently used in the treatment of chronic lymphatic leukaemia, led to a complement- and antibody-dependent reduction of viable osteosarcoma cells. Thus, CAMPATH-1H-induced reduction of tumor cell growth might indicate a potential use of this antibody in the treatment of skeletal tumors.

**F02  
TARGETED AND INTRACELLULAR DELIVERY  
OF PODOPHYLLOTOXIN USING FOLATE  
ATTACHED POLYMERIC MICELLES**

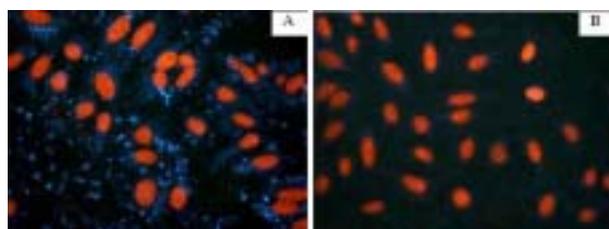
Li Fan, Hong Wu, Hui Zhang, Fei Li  
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Natural Podophyllotoxin (POD) is an effective anti-cancer drug, although a critical disadvantage is its non-targeting nature. To address this issue, chitosan-grafted poly N-isopropylacrylamide was synthesized with different starting monomer ratios via a free radical copolymerization route. Folate was subsequently attached to the hydrophilic segment of the polymer in order to target folate receptors-overexpressing cancer cells. The success of synthesis was confirmed with FTIR and <sup>1</sup>H-NMR measurements. Using a free radical copolymerization method, the polymer was then self-assembled into micelles whose hydrophobic cores could be utilized to encapsulate POD, an extremely hydrophobic compound. The polymer had a low CMC of 20 mg/L in water. Dynamic light scattering further showed that the sizes of blank micelles formed from the polymer were below 100 nm at neutral solution tested and ~150nm upon drug incorporation. More importantly, it was demonstrated that the micelles exhibited a useful pH-sensitivity, such that drug was released more rapidly at pH 5.0 (acidic

endosomal/lysosomal environment) than at pH 7.4 (normal extracellular pH). MTT assay and fluorescence microscopic study certificated one step further that micelles promoted drug release drastically in tumor surroundings while exerted no effect on drug release in physiological condition. In vitro cytotoxicity assays performed against lung cancer A549 cells then provided concluding evidences that the cellular uptake of micelles surface-functionalised with folate was indeed enhanced due to a receptor-assisted endocytosis process. For mice treated with POD-loaded nanoparticles, the decrease in body weight was limited, and tumor regression was significantly observed with complete tumor regression for over 50% of mice. In addition, the life span of tumor-bearing mice was significantly increased when they were treated with POD-loaded nanoparticles. This novel polymeric design thus has the potential to be a useful POD vehicle for the treatment of folate-receptor positive cancers.



*TEM and SEM micrographs of blank and drug-loaded*



*Luminescence of A549 cells with nanoparticles*

**F03  
GENISTEIN PROTECTS P53 WILD TYPE CELLS  
FROM TAXOL**

Sergey Tokalov, Michael Gerlach, Andrij Abramyuk,  
Gunter Wolf, Nasreddin Abolmaali  
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Lung cancer (LC) is the leading cause of death among human malignances and p53 alterations in LC is shown to be associated with resistance to radio-chemotherapy and poorer survival prognosis. At the same time lack of wt p53 gene in cancer cells could be exploited for therapeutic advantage by using a sequence of two antagonistic drugs.

A possibility to selectively kill p53-deficient cells (H1299, FaDu) by taxol and to protect p53 wild type cells (A549) by the prior administration of structurally related flavonoids (apigenin, genistein and quercetin) were investigated in vitro by flow cytometry in comparison to known anticancer drugs (5-fluorouracil, camptothecin, cisplatin, doxorubicin, etoposide) administered in a wide range of doses.

It was found that taxol caused dose dependent mitotic arrest, accompanied by a large fraction of apoptotic cells independent of their p53 status. Pretreatment of A549 cells with doxorubicin or genistein for 24 hours followed by incubation with taxol for additional 24 hours did not increase significantly their mitotic index and protected the cells from the cytotoxicity of taxol. H1299 and FaDu cells responded to the same treatment with mitotic arrest and massive apoptosis. The other compounds revealed less selectivity (5-fluorouracil, apigenin, camptothecin, cisplatin) or demonstrated significant toxicity (camptothecin, quercetin).

It was confirmed, that doxorubicin could induce growth arrest and protect normal p53 wild type cells from the taxol, which simultaneously killed or blocked p53-deficient cancer cells in the radiosensitive mitosis. However, it should be used at toxic doses to protect normal cells and arrested some of FaDu and H1299 cells in G2 phase by p53 independent mechanisms. At the same time doxorubicin might be successfully substituted in this way by less toxic genistein, which could more selectively arrest A549 cell cycle progression either at G1/S or at G2/M boundaries protecting them from the taxol and did not protect p53-deficient FaDu and H1299 cells.

We propose a therapeutic strategy to protect normal cells from taxol while increasing apoptosis selectively in mitotic p53-deficient cancer cells using naturally occurring compound genistein, which may be used without significant toxicity in rather high concentration as present in common diets up to 1 g per day.

#### **F04 PHARMACOGENOMIC PROFILING OF THE PI3K/PTEN PATHWAY IN SPORADIC BREAST CANCER**

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Pharmacogenomics is the study of genetic variations between individuals to predict the probability that a patient will respond to single or multidrug chemotherapy. Breast cancer is one of the most common

cancers among women worldwide. Treatment of breast cancer by the application of biological rationales, give us the ability to match the correct pharmacology to individual tumour genetic profiles.

The ideal of mechanistic precision is beginning to play out in the setting of PI3 kinase inhibitors since breast cancers exhibit multiple anomalies in this pathway such as PTEN loss that can now be put in context of therapy with rapamycin analogues, direct inhibitors of the PI3 kinase catalytic subunit and mTOR. Patients with PTEN-deficient breast cancers also had significantly poorer responses to trastuzumab based therapy than those with normal PTEN. Thus, PTEN deficiency is a powerful predictor for efficacy of rapamycin analogues and trastuzumab resistance.

In this study, PTEN was evaluated by means of polymerase chain reaction, single-strand conformation polymorphism, heteroduplex analysis and sequencing in 72 breast cancer tumors in patients of Isfahan for detection and characterization of mutations.

According to the results of this research, nucleotide substitutions were found in 6/72 (8%) of samples. The sporadic breast cancer patient was found to be heterozygote for the p.D92N, p.C105W, p.D107N, p.W111G, p.A121P and p.R130Q mutations. Two mutations p.D107N and W111G were found for the first time in this study.

The interplay between pharmacological targeting and the breast cancer genome will be most likely addressed in the neoadjuvant setting. Considering the Methylation of the PTEN promoter region, which is associated with reduced PTEN expression, occurs in almost half of sporadic breast tumors; this rate of mutations is an important consideration for novel therapeutic in breast cancer in which biologic efficacy is influenced by the activity level of PTEN.

#### **F05 INFLAMMATORY MEDIATORS-INDUCED LIVER METASTASIS DEVELOPMENT AFTER SURGERY IS PREVENTED BY BLOCKING INTEGRIN 2**

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Currently, surgery is the only curative option for colorectal cancer patients. Unfortunately, many patients will develop liver metastases after curative resection of the primary tumour. Evidence is accumulating, which

suggests that the surgery-associated inflammatory reaction stimulates tumour growth. In this study we investigated the involvement of both adhesion molecules and inflammatory mediators in surgery-induced liver metastases outgrowth.

To investigate liver metastases development we established a novel model, in which the portal vein of rats was catheterized and tumour cells were injected directly in the portal system.

Surgical trauma dramatically enhanced liver metastases development, which was due to increased adhesion of tumour cells. We found that tight junctions between the endothelial cells were disrupted. This could result in exposure of extracellular matrix (ECM) to which tumour cells preferably bind. Blocking of integrin  $\alpha 2$  resulted in decreased CC531s adhesion. Importantly, blocking integrin  $\alpha 2$  prevented surgery-induced liver metastases outgrowth. Inflammatory mediators such as IL-6 were elevated after surgery, which were presumably responsible for the observed alterations in endothelial cells.

Our data indicates that surgery creates permissive circumstances for liver metastasis outgrowth. It may well be that release of inflammatory mediators after surgery disrupts sinusoid integrity and results in enhanced ECM exposure. This may facilitate tumour cell adhesion, which could be inhibited by blocking integrin  $\alpha 2$ . We are currently investigating the exact mechanisms of surgery induced liver metastases outgrowth, which may ultimately lead to development of peri-operative adjuvant therapies.

**F06**  
**NANDIASPONGIOLIDE, A NOVEL MARINE**  
**NATURAL PRODUCT. INDUCES APOTOSIS IN**  
**HUMAN CANCER CELL BYA PKR/eIF2**  
**/CASPASE-12-DEPENDENT PATHWAY**

Daniela Trisciuglio<sup>1</sup>, Badarch Uranchimeg<sup>2</sup>, Jonh II Cardellina<sup>2</sup>, Donatella Del Bufalo<sup>1</sup>, Robert Shoemaker<sup>2</sup>, Giovanni Melillo<sup>2</sup>

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Candidaspongiolide, a novel marine natural product isolated from a sponge of Genus *candidaspongia*, is a potent cytotoxic agent with differential activity toward melanoma and central nervous system cell lines of the National Cancer Institute 60 cell-line screen. However, the underlying mechanism of the cytotoxic activity is poorly understood. We demonstrated that Candidaspongiolide potently induced apoptotic cell death in cancer cell lines, at least in part by a caspase 12-

dependent pathway. In addition, Candidaspongiolide induced rapid and sustained phosphorylation of eIF2 $\alpha$ , which was required for the induction of apoptosis, but was only partially involved in the inhibition of protein synthesis. Indeed, stable expression of a dominant-negative eIF2 allele prevented eIF2 phosphorylation and induction of apoptosis, but not inhibition of protein synthesis by Candidaspongiolide. Notably, Candidaspongiolide inhibited protein synthesis in both cancer cells and normal human fibroblasts, yet only induced eIF2 phosphorylation and apoptosis in cancer cells. Finally, inhibition of PKR, by siRNA or 2-AP, completely blocked Candidaspongiolide-dependent eIF2 phosphorylation and induction of apoptosis. These results demonstrate that Candidaspongiolide triggers two distinct cellular responses in cancer cells, i.e., PKR/eIF2 /caspase-12-dependent apoptosis and inhibition of protein synthesis, which account for a novel and potent mechanism of cytotoxicity, and they may form the basis for future preclinical studies to validate Candidaspongiolide potential therapeutic applications.

**F07**  
**CHANGES IN AMPLIFICATION AND**  
**EXPRESSION OF THE MYCN GENE IN DRUG-**  
**RESISTANT NEUROBLASTOMA CELL LINES**

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MYCN amplification is a powerful predictor of poor prognosis in neuroblastoma. High risk neuroblastoma (HR NBL) is one of the most poorly curable childhood tumours. One of the main causes of failure of chemotherapy is the rise of drug-resistance. We studied HR NBL cell lines resistant to commonly used drugs: doxorubicine, vincristin, cisplatin and substances that demonstrate anticancer effect: BS-RNase and ellipticine. To start, we examined the cell lines using comparative genomic hybridisation (CGH). We showed changes that correspond to HR NBL including MYCN amplification at the sensitive NBL cell lines UKF-NB-4 (S-type) and IMR-32 (N-type). In all drug-resistant cell lines we found the loss of 2p24 that corresponds to the decrease of number of amplified MYCN gene copies. This decrease in number of MYCN copies was verified using FISH. The average number of copies in UKF-NB-4 and IMR-32 was 62 and 54 respectively. In drug-resistant lines, the average number was decreased to 43 copies (t-test, significance at  $p < 0,001$ ) (Fig. 1).

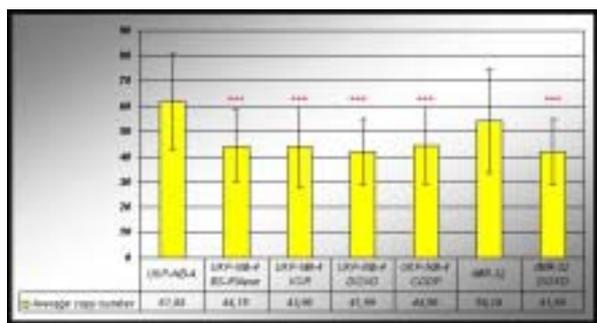


Fig. 1. Average number of the MYCN gene copies

This decrease seems to be related to the sensitivity of the used drug. MYCN amplification is formed in the early stage of tumour development and there aren't any reported changes in amplification in the further development of the disease. We used real-time RT-PCR for determination of the relative expression of mRNA MYCN. In parental cell lines, there is more than 2 logs higher expression than in healthy volunteer leukocytes. In all drug-resistant cell lines, the expression is approximately more than one log higher in comparison to the parental cell lines (Fig. 2).

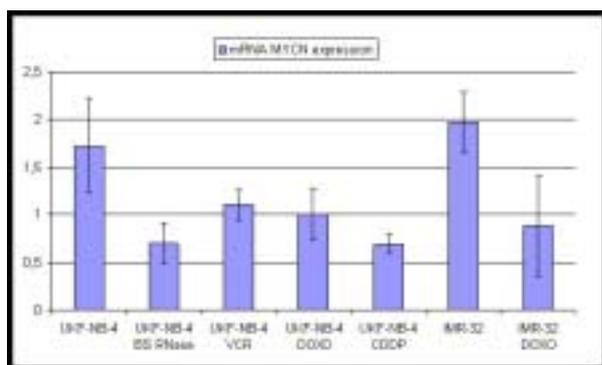


Fig. 2. Relative expression of the mRNA MYCN

We cultivated UKF-NB-4 CDDP for five passages without CDDP and then performed real-time RT-PCR. Already in the first passage without CDDP, the expression of MYCN gene declines to that of UKF-NB-4. Difference between expression of UKF-NB-4 and expression of UKF-NB-4 CDDP in the third passage without CDDP is insignificant. Our results indicate that expression of the MYCN gene in UKF-NB-4 CDDP is dependent on the presence, or absence of CDDP. We assume, that similar changes may occur in patients with HR NBL treated by cytostatics.

## F08 TOPOTECAN INHIBITS ACTIVATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION AND ANGIOGENIC ACTIVITY BY HYPOXIA-INDUCIBLE FACTOR (HIF)-1A AND HIF-2A IN HUMAN NEUROBLASTOMA

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**Background:** Neuroblastoma, the most common pediatric extracranial solid tumor, elaborate angiogenic peptides, and the extent of angiogenesis correlates with tumor progression and poor clinical outcome. Hence, inhibition of angiogenic factor production represents an important modality of therapeutic intervention. One of the major drives to tumor angiogenesis an a challenge of therapy is hypoxia, a decrease in oxygen tension that characterizes the tumor microenvironment.

**Methods:** We investigated the effects of the topoisomerase-1 inhibitor, topotecan, on hypoxia-induced production of the proangiogenic mediator, VEGF, in advanced-stage MYCN-amplified human neuroblastoma cell lines.

**Results:** We demonstrate that pharmacological concentrations of topotecan counteracted hypoxic induction of VEGF. This effect was paralleled by decreased angiogenic activity of conditioned medium from hypoxic NB cells in vivo in the chorioallantoic membrane (CAM) assay. Functional studies of the VEGF promoter utilizing luciferase reporter constructs demonstrated the requirement for both hypoxia-inducible factor (HIF)-1a and -2a in the activation of VEGF gene transcription by hypoxia, because: (i) targeted knockdown of either HIF-1a or HIF-2a accumulation in response to hypoxia by RNA interference counteracted hypoxia ability to activate VEGF transcription and resulted in a parallel inhibition of VEGF mRNA and protein induction; (ii) overexpression of either protein under normoxia by transfection with expression vectors resulted in VEGF promoter transactivation, which was abrogated by mutation in the HIF-1-binding site.

**Conclusion:** Topotecan treatment significantly decreased VEGF promoter transactivation by hypoxia by targeting the expression of both subunits. A similar pattern of

results was obtained in cells treated with the hypoxia-mimetic agent, desferrioxamine. These findings have important implications for neuroblastoma treatment.

**F09**  
**PHARMACOGENOMICS STUDY ON THE**  
**ANTITUMORAL N6-**  
**ISOPENTENYLADENOSINE AND**  
**DEVELOPMENT OF ITS ANALOGUES**

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N6-isopentenyladenosine (i6A) is a member of the family of plant hormones, called cytokinins that regulate plant cells growth and differentiation. This cytokinin is also present in mammalian cells in tRNA-bound or free form. We have previously observed that i6A has a potent in vitro anti-tumor activity toward different types of human epithelial cancer cell lines. Nevertheless, it has been shown that this molecule has only a weak effect in vivo. Several hypotheses on the mechanisms of action of i6A have been made but it still remains to be clarified. Here, we reported a gene expression profile analysis of i6A treated cells, aiming to identify the target genes of this potential anticancer therapeutic agent. We found that i6A treatment induced genes as DDIT3, PPP1R15A, HBPI, SESN2, DNAJB9 and DNAJC3 involved in the negative regulation of progression through cell cycle and reported to be up-regulated during cell cycle arrest in stress conditions, such as endoplasmic reticulum (ER) stress, thus suggesting that i6A may inhibit cell proliferation by inducing ER stress. In addition, here we showed also an attempt to identify biologically active (in vitro as well as in vivo) i6A analogues. Among the six i6A derivatives investigated, only the one in which the double bond of the isopentenyl side chain was saturated has an in vivo anti-tumor effect, weaker than that of i6A. Moreover, we did not find any modulation of the genes up-regulated by i6A after cell treatment with the effective analog, suggesting that i6A biological activity should be strongly linked to its structure. We also carried out an in vivo study on the active analogue compared to i6A, but neither i6A nor its analogue were able to significantly inhibit in vivo cancer cell growth. In conclusion, further studies are needed to clarify the role of i6A in ER stress and, of course, to identify active analogues potentially useful in therapeutic strategies.

**F10**  
**HDAC INHIBITOR VORINOSTAT INDUCED**  
**SYNERGISTIC ANTITUMOR EFFECT IN**  
**COMBINATION WITH 5-FLUOROURACIL OR**  
**RALTITREXED IN HUMAN CANCER CELLS BY**  
**MODULATING THYMIDYLATE SYNTHASE**  
**AND P53 EXPRESSION**

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Intrinsic or acquired resistance to chemotherapeutics as well as the existence of multiple and compensatory survival and proliferative signals, reflecting the genetic flexibility of cancer cell genome, are the cause of the limited activity of anti-cancer strategies. We have recently shown preclinical synergistic interaction, as well as feasibility and activity in clinical study, of the combination of raltitrexed (RTX) and 5-fluorouracil (5FU), two anticancer agents targeting, with different mechanisms, thymidylate synthase (TS). TS is an essential enzyme for the de novo synthesis of thymidylate and subsequently DNA synthesis, whose increased expression has been associated with poorer overall survival and the development of both 5FU and RTX resistance. Histone deacetylase (HDAC) inhibitors represent a new class of anticancer agents that by enhancing histone acetylation modulate the expression of genes regulating cell cycle, survival and differentiation, affecting multiple pathways. In this study we have found that the antiproliferative effect induced by the HDAC inhibitor vorinostat, currently in clinical trial, was paralleled by downregulation of TS protein, in both human colorectal cancer (CRC) and head and neck squamous cancer (HNSCC) derived cells. On the basis of this observation we also showed that vorinostat in combination with 5-FU or with RTX, demonstrated a schedule-dependent synergistic antiproliferative interaction independent to p53 status in CRC and HNSCC cells. Moreover we also provide evidences for the first time indicating that vorinostat can overcome resistance to both 5FU and RTX. Down-modulation of TS protein expression induced by vorinostat within 24 h represent a key factor in enhancing the effect of 5FU and of RTX in both sensitive and resistant tumor cells, however we demonstrated that p53, whose functional wild type expression is critical for drug sensitivity to 5FU and RTX, is up-regulated by vorinostat in wt-p53 cells but downregulated in mut-p53 cells, as single agent or in combination treatment, suggesting an additional mechanism of the antiproliferative synergistic interactions observed. Overall this data add new insights

in the mechanism of vorinostat antitumor effect and suggested that the association of vorinostat plus 5FU and/or RTX should be clinically explored.

#### F11

#### **EFFECT OF XYMEDON ON THE PERIPHERAL NEUROPATHY CAUSED BY CISPLATIN**

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Cytostatics administration causes some unwanted side effects. The lasts can declare them selves as a sensory neuropathy. As we have shown earlier in the model of the posttraumatic regeneration of peripheral nerve, the pyrimidin-derivate, xymedon, has an opposite effect, acts as a neuroprotector, and stimulates regeneration and sprouting of nerve fibers. Here we present the investigation of the effect of xymedon on the peripheral neuropathy caused by cisplatin. The peripheral neuropathy was induced among the 16 animals by using the standard technique of intra-peritoneal introduction of cysplatin («Ebeve», Austria). Simultaneously, from the very beginning, 30 mg/kg of xymedon daily were being injected intra-abdominal into the 8 cysplatin treated rats (second group). The third group was presented by 8 intact animals. After 30 days of the experiment, a part of a left sciatic nerve from the rats of all 3 groups were excised under anesthesia. The total number of myelin fibers on semifine sections was counted. After 30 experiments' days the number of myelin fibers (especially thin myelin fibers) decreases in 2.3 times in the first group while increases in 2.1 times in the second (xymedon treated) one. The results of the second group does not differ from the third intact group.

New evidence of the inhibitory effect of xymedon on the peripheral neuropathy caused by cisplatin is obtained and presented.

#### F12

#### **THE ROLE OF HOMOCYSTEINE IN DRUG SENSITIVITY OF LEUKEMIC CELLS**

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Hyperhomocysteinemia (HHc) are found out frequently at patients with cardiovascular and nervous diseases and cancer patients with hepar, colon, breast tumors and leucosis. The crucial moments of the HHc induced

disorders are homocysteine (Hc) influence on cell proliferation and apoptosis that are important factors of tumor drug sensitivity. Moreover increased Hc level can result in the break of SAH/SAM ratio causing hypomethylation of genes associated with drug resistance and reduction of breast and ovary cancer cells drug sensitivity. Because patients with leucosis also can have HHc and chemotherapy is the main tool of their treatment we studied influence of increased doses of Hc on leucosis cells drug sensitivity. Methods. Study was carried out with 0.015-5 mM DL-Hcy to U937 leucosis cell line. Number and viability of cells was counted with trypane blue dye exclusion method in haemocytometer and with propidium iodide (PI) dye in flow cytometer (FC); apoptosis (Annexin V/PI dyes), cell cycle, Ki67, bcl-2, Pgp, GST, MT expression was studied by FC; cell drug sensitivity was assessed with MTT assay. Additional morphological and electron-microscope investigations of cells was carried out. Results. 72 hour cultivation of U937 cells with 0.015-0.3 mM Hc (doses that was equal to Hc level in HHc patients) resulted in 30% decrease of cell sensitivity to doxorubicine (DOX) ( $p < 0,05$ ) but not to CDDP. These changes was accompanied with few decrease of cell proliferation and Ki67+ cells (from  $65,7 \pm 6,1\%$  to  $52,0 \pm 0,7\%$ ;  $p < 0,05$ ). Hc not induced apoptosis in U937 cells, that can be associated with high bcl-2 expression in these cells. Simultaneous increase of P-gp+ (40%,  $P < 0,02$ ), GST (near 20%,  $p < 0,05$ ) and MT+ (10%) cells was observed. Cultivation of cells with high dose of Hc (5 mM) did not result in decrease of cell sensitivity. At the same time it was observed decrease S-phase with simultaneous increase of GO-1 phase of cell cycle and the most significant decrease of Ki67+ cells (35%,  $p < 0,01$ ) in this group. As well we have not found increase of Pgp+, GST+ cells. Conclusion. Hc can induce decrease of DOX sensitivity of leucosis cells as a result of decrease of cell proliferation that are accompanied by rise of proteins associated with drug resistance. So Hc level can be potential factor determinative of DOX efficacy in patients with leucosis.

## Poster session G: Signalling pathways

### G01

#### MOLECULAR ANALYSES OF P53, CCND1 AND MEN1 EXPRESSION IN MCF7 AND MDA-MB-468 BREAST CANCER CELL LINES TREATED WITH ADRIAMYCIN

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Cyclin D1 and p53 are interacting regulatory genes that are both frequently altered in breast cancer. p53, a well-known tumor suppressor gene, is involved in regulation of apoptosis following DNA damage. Cyclin D1, an important regulator of G1 to S phase transition, is over-expressed in 1/3 of breast carcinomas. Men1 is another tumor suppressor gene that has important role in regulation of the cell cycle and apoptosis. Therefore, in this study p53, CCND1 and Men1 mRNA and protein expression in MCF7 and MDA-MB-468 breast cancer cell lines with different ER status were analyzed following Adriamycin (Adr) treatment using RT-PCR and immunocytochemistry, respectively. Cytotoxicity of Adr on tested cell lines was also determined using MTT assay. Adr showed different dose and time-dependent anti-proliferative effects on these cell lines with more growth inhibitory effect on MDA-MB-468 cells (IC<sub>50</sub>=250nM) than MCF7 (IC<sub>50</sub>=500nM). Interestingly, Men1 mRNA expression showed to be higher in MDA-MB-468 than in MCF7 cells. Unlike Men1, mRNA levels of p53 and CCND1 did not change significantly after Adr exposure. Whereas MDA-MB-468 cells showed only a mild increase in cyclin D1 nuclear expression after Adr exposure, MCF7 cells showed strong cytoplasmic expression in addition to nuclear staining. Higher levels of menin and p53 proteins expression were detected after Adr treatment in both cell lines. In conclusion, these cells showed differential molecular responses to Adriamycin that is important in tumor-targeted chemotherapy. In addition, these data emphasize on the requirement of evaluation of new biomarkers in breast cancer such as Men1 to be used in combination with current markers for prediction of response to chemotherapy.

### G02

#### PROSTANOID RECEPTORS IN COLORECTAL CANCER

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Svante Nordgren<sup>1</sup>, Marianne Andersson<sup>1</sup>, Wenhua  
Wang<sup>1</sup>, Christina Lönnroth<sup>1</sup>, Kent Lundholm<sup>1</sup>  
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Sweden

It is well established that levels of prostaglandins are altered in tumor tissue with elevated concentrations of PGE<sub>2</sub>, PGD<sub>2</sub> and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and reduced levels of PGF<sub>2</sub>alpha and PGI<sub>2</sub>. Prostaglandins mediate effects via G-protein coupled transmembrane receptors specific to each prostanoid, DP1-2 (PGD<sub>2</sub>), EP1-4 (PGE<sub>2</sub>), FP, (PGF<sub>2</sub>alpha), IP (PGI<sub>2</sub>) and TP (TXA<sub>2</sub>), whereas some also act on nuclear receptors such as PPARgamma (PGJ<sub>2</sub>, derivate of PGD<sub>2</sub>). Therefore, the aim of this study was to evaluate changes in prostanoid receptor expression in colon tumor tissue compared to normal colon tissue.

Total RNA from 99 untreated tumors and adjacent normal colon tissue (n = 48) was extracted. Quantification of receptor expression was performed by either realtime-PCR (LightCycler, Roche) or PCR (Eppendorf Mastercycler) and related to GAPDH expression. Non-parametric tests were used for statistical evaluations (Kruskal-Wallis, Mann-Whitney and Spearman).

Overall DP1, EP2, EP4, IP and PPARgamma receptor expressions were significantly reduced in tumor tissue (p<0.0001-0.05). Only the expression of TP receptor was significantly elevated in tumor tissue. Tumor tissue expression of prostanoid receptors was not related to tumor stage or tumor cell differentiation. However, multivariate analyses indicate that increased tumor tissue EP2 predicted poor survival (p<0.05).

Our results indicate altered prostanoid receptor expression in colon tumor tissue with complex relationships to tumor progression and patient survival, where EP2 receptor expression predicted disease specific mortality.

**G03****INHIBITION OF PI3K/AKT SIGNALING BY ALKYL-LYSOPHOSPHOLIPID PERIFOSINE SHOWS ANTIPROLIFERATIVE AND PROAPOPTOTIC EFFECTS IN PROSTATE CANCER CELLS ACTIVATING MULTIPLE SIGNALING PATHWAYS**

Claudio Festuccia, Giovanni Gravina, Paola Muzi, Danilo Millimaggi, Silvia Specca, Vincenza Dolo, Enrico Ricevuto, Corrado Ficorella, Carlo Vicentini, Leda Biordi, Mauro Bologna  
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Despite initial positive response to androgen ablation therapy virtually all patients with prostate cancer (PCa) will relapse through the acquisition of an hormone refractory disease with a selective outgrowth of tumor cells and a multi-drug resistant phenotype. To date, no effective therapeutic treatment allows to abrogate the prostate cancer (PCa) progression to more invasive disease forms.

Antiandrogenic therapy was able to increase Akt activity in vitro and in vivo by mechanisms involving PTEN downmodulation and EGFR/Her2 overexpression/activation. Increased Akt activity was associated to increased resistance to radiotherapy and chemotherapeutic agents because PTEN negative cells are unable to down-regulate PI3K/Akt activity. One of the major targets for the therapy in PCa can be the PI3K. Infact, pharmacological PI3K inhibition or PTEN transfection, are able to reduce cell proliferation and to sensitize tumor cells to pharmacological treatments. One of PI3K inhibitors is the oral active lysopholipid derivative (perifosine). Here we demonstrate that PTEN negative PCa cells (PC3 and LnCaP) were sensitive to the antiproliferative properties of perifosine with an IC50 of 0.8 and 1.2  $\mu$ M, respectively. Cell cycle arrest at the G1-S and G2-M boundaries was observed independent of p53 function. Indeed p53 null PC3 and p53 wt LnCaP cells were induced similarly to trigger an apoptotic process involving p21WAF1 increased expression. PTEN negative DU145 and 22rv1 cells were partially resistant to perifosine treatment. However, EGF, which was an Akt inducer in these cells, sensitized versus perifosine ation and EGF-augmented cells were induced to block their proliferation and to trigger a p21WAF-1 mediated cell death. The sensitivity versus chemotherapeutic such as doxorubicine or paclitaxel and versus anti-receptorial therapeutics such as anti EGFR tyrosine kinase inhibitors, Gefitinib and erlotinib, was restored and perifosine synergized with these drugs. These data, therefore, indicate that

perifosine blocks cell cycle progression of prostate carcinoma cells at G1-S and G2-M by inducing p21WAF1, irrespective of p53 function, and may be exploited clinically because the majority of human malignancies harbor p53 mutations alone or in combination with chemotherapeutics and anti-target drugs.

**G04****ANTI-HORMONE TREATMENTS INCREASE AKT PROTEIN EXPRESSION AND ACTIVATION IN PROSTATE CANCER**

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The precise molecular mechanisms by which prostate cancer cells progress to androgen-insensitive status still remain largely unclear. We analyzed the effects of bicalutamide (BCLT) or surgical castration in AR and PTEN positive PCa cell lines and in LuCaP35 xenografts, respectively. We observed that prolonged perturbation of AR signaling was able to reduce PTEN expression and this seemed to be an important/necessary driving phenomenon for the subsequent resistance to anti-androgens. Both DNMT and HDAC activities were increased after the generation of BCLT resistant (BCLTR) cells and in LuCaP35 xenografts after castration. Therefore we analyzed if these enzymatic activities were responsible for PTEN reduction. We observed that DNMT inhibition by azacytidine as well as HDAC inhibition by valproic acid were able to increase the levels of PTEN both in untreated and BCLT treated cells. However, about 30% and 40% of PTEN reduction can be due to increased DNA methylation and HDAC activity, respectively. Moreover we observed that PTEN protein ubiquitin-dependent degradation was also increased in BCLTR cells since the inhibition of this process by lactacystatin slowed down PTEN reduction after BCLT culture. In addition we observed that hormonal therapies were able also to induce the expression/activation of EGFR and Her-2. Taken together these molecular changes sustain Akt activity and protect prostatic cells to apoptosis. Akt inhibition as well as Akt gene knock down obstructed the insorgence of BCLT resistance and similar effects were observed with the EGFR inhibitor, gefitinib. We observed also that Akt inhibition synergized with gefitinib in BCLT resistant cells suggesting that the increased Akt activity was responsible for gefitinib resistance. Thus our study suggests that PCa cells, after any anti-hormone therapy, undergo a series of coordinated molecular changes that

may contribute to the development of androgen independence through: (i) maintaining cell proliferation; (ii) inhibiting apoptosis; and/or (iii) inducing AR activation in a ligand-independent fashion. Therefore combined use of gefitinib and bicalutamide could have potential effects to increase the androgen dependent phase.

#### G05

### Z-CRYSTALLIN IS A NEW STABILIZING BCL-2 MRNA AU-RICH ELEMENT BINDING PROTEIN OVEREXPRESSED IN THE ACUTE LYMPHOCYTIC LEUKEMIA JURKAT T-CELL LINE

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**Background:** An adenine+uracil-rich element (ARE) in the 3'UTR of bcl-2 mRNA which is involved in post-transcriptional control of bcl-2 gene expression by modulating its mRNA stability upon interaction with peculiar ARE-binding proteins (AUBPs) has been identified in our laboratory. Bcl-2 gene is over-expressed in most cancers without obvious gene rearrangement, suggesting involvement of an impairment of its ARE/AUBP-based post-transcriptional control. Here, using the acute lymphocytic leukaemia Jurkat T-cell line as a leukaemia model, we describe identification of z-crystallin as a new bcl-2 AUBP and demonstrate that bcl-2 over-expression results from z-crystallin over-production.

**Methodology/Principal findings:** We have analyzed the mechanism(s) underlying bcl-2 over-expression in cancer using the acute lymphocytic leukaemia (ALL) Jurkat T-cell line as experimental model and activated or PHA-activated T-lymphocytes as normal counterparts. Initially, using affinity-purified bcl-2 ARE mRNP, bi-dimensional SDS-PAGE and mass-spectrometry analysis, we identified in Jurkat T-cells highly detectable z-crystallin, a new bcl-2 AUBP, barely detectable in inactivated T-lymphocytes. We next observed, using western blot and FACS analyses, that z-crystallin and Bcl-2 production were concomitantly enhanced in PHA-activated T-lymphocytes and malignant T cells in

respect to controls. Increased Bcl-2 protein production in Jurkat T-cells resulted from increased bcl-2 mRNA resulting from enhanced binding of z-crystallin to bcl-2 ARE. The direct implication of z-crystallin in bcl-2 post-transcriptional control was assessed by over-expression and silencing experiments of z-crystallin.

**Conclusion/Significance:** Here we demonstrate that overexpression of bcl-2 gene in the human non-translocated Jurkat T-cell line relies on enhanced binding of the novel bcl-2 AUBP z-crystallin to the ARE of its mRNA compared to healthy T-lymphocytes. This results in higher bcl-2 mRNA half-life in malignant cells, strongly suggesting that z-crystallin expression affects the balance between the previously described bcl-2 AUBPs AUF1 and Tino. Our work opens avenues to exploit z-crystallin as a new molecular target for pharmacological tools targeting bcl-2 over-expression in leukaemias.

#### G06

### EGFR INHIBITION CAUSES CELL CYCLE ARREST IN GLIOBLASTOMA CANCER CELLS

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The absence of an effective therapy for glioblastoma multiforme makes necessary a better understanding of its pathogenic pathways. Since one hallmark of glioblastoma multiforme is deregulation of the epidermal growth factor receptor (EGFR) pathway, we have studied its inhibition using the tyrosine kinase inhibitor AG1478.

Our results on GBM cell lines show that AG1478 inhibits EGFR phosphorylation and reduces cell proliferation, being this effect lower in U87 and LN-229 cells. Cell cycle experiments reveal that this reduction is due to a G1 arrest in LN229 and T98 cells or a G2/M arrest in U87 cells. However, A172 cells do not undergo any cell cycle arrest. AG1478 effects on cell cycle are associated with a decrease in steady-state levels of cyclin D1 protein and an increase in p27 and p21 protein levels in LN-229 cells, where the G1 arrest is more apparent, as it is further demonstrated with a decrease in bromo-deoxyuridine (BrdU) incorporation, which confirms that LN-229 cells treated with AG1478 do not enter S phase. Likewise, there is a decrease in cyclin A protein levels in U87 cells that could be associated with the G2/M arrest observed in these cells upon AG1478 treatment.

In addition, we observe a decrease in phosphorylation of MAPK, Akt and STAT3 levels. The decrease in phosphorylation of AKt is an early event, suggesting that the effects of AG1478 are mediated via the PI3K/Akt pathway.

Our results show that inhibition of the EGFR could be an interesting therapeutic strategy for the treatment of patients suffering from glioblastoma.

#### **G07**

#### **RANBP1 LEVELS MODULATE THE RESPONSE OF CANCER CELLS TO CHEMOTHERAPEUTIC MICROTUBULE-TARGETING DRUGS**

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Microtubule-targeting drugs, such as taxol (paclitaxel), are frequently used in cancer therapy. Cells exposed to taxol become arrested during mitosis, due to hyperstable microtubules, and either remain mitotic arrested, or die through apoptosis. Checkpoint-defective cells instead can escape mitotic onset to form multinucleated cells. Also several genes play a role in the dynamics of microtubules and they could therefore show a functional interaction with anti-microtubule drugs used in chemotherapy, by tendering cells more or less sensitive to these drugs. Understanding which (group of) genes can influence the resistance to microtubule-targeting drugs, may help to devise a specific therapeutic strategy, based on the gene expression profile of the cancer cells.

The GTPase Ran is an important regulator of spindle function. There are several indications that the RanBP1 gene, a regulator of the GTPase Ran, is involved in regulating microtubules dynamics. RanBP1 downregulation results in hyperstable microtubules, which is similar to the effect of taxol and induces apoptosis during early mitotic stages.

In this work we have investigated the influence of RanBP1 gene silencing on the sensitivity of cancer cell lines on exposure to taxol. The results demonstrate an increase in apoptosis when cells interfered for RanBP1 were exposed to taxol compared to cells with normal RanBP1 levels (both in HeLa and U2OS cell lines). This was not seen in the MCF7 cell line that is defective for the apoptotic inductor caspase-3. Thus, silencing the gene for RanBP1 sensitizes cancer cells to taxol. This

response is dependent of the type of cell line and more specifically, on the set of apoptotic regulators that they express. It will be important to clarify the “cross-talk” between this emerging pathway and the spindle checkpoint.