503 In vivo metastatic profile of breast cancer cell lines expressing hormonal receptors versus triple negative

M. Carvalho[1], A.M. Abrantes[2], M. Laranjo[2], F. Falcão[3], S. Cabrita[4], M.F. Botelho[2], C.F. Oliveira[5], I. Torgal[6]. [1]*Gynaecology Service University Hospitals of Coimbra Biophysics and biomathematics Institute IBILI CIMAGO, Faculty of Medicine of Coimbra University, Coimbra, Portugal*, [2]*Biophysics and biomathematics Institute IBILI CIMAGO, Faculty of Medicine of Coimbra University, Coimbra, Portugal*, [3]*Gynaecology Service University Hospitals of Coimbra, University Hospitals of Coimbra, Coimbra, Portugal*, [4]*Experimental Pathology Institute, Faculty of Medicine of Coimbra University, Coimbra, Portugal*, [5]*CIMAGO, Faculty of Medicine of Coimbra University, Coimbra, Portugal*, [6]*Gynaecology Service University Hospitals of Coimbra, Faculty of Medicine of Coimbra University, Coimbra, Portugal*

Background: Triple negative (TN) tumours are a group of breast cancers with severe clinical behavior. The early metastization is one of the factors that contribute to the aggressiveness. Some clinical studies suggest a differential metastatic spread and a preference for hematogeneous dissemination. Despite these, it is not established a metastatic profile characteristic of each breast cancer type.
The aim of this study is the characterization of metastatic spread *in vivo* of breast cancer cell lines that express hormonal receptors (HR) comparing with TN after injection in the tail vein. The objective is to determine a specific metastization profile, particularly considering the lung and liver.
Material and Methods: It was performed injection in the tail vein of female mice Balb/c nude with 4−6 weeks of age with 1.5×10^6 cells of each breast cancer cell line (MCF7 and HCC1806). Eight weeks after cells injection, the animals were sacrificed and the lungs, liver, kidneys, brain and all sites with suspicious lesion were collected for histological analysis. For morfometric studies were used a histological image analysis focusing on regions of interest (ROI), in order to obtain lesion areas in pixels.
Results: The necropsy revealed a macroscopic pelvic tumour and a bone metastasis in mice injected with MCF7. Considering lung analysis, in all animals injected were found metastatic foci on histological study. On one hand, the number of lung foci was not significantly different considering MCF7 and HCC1806 injection. On the other hand, the mean area of lung metastasis in MCF7 cases were significantly higher than in HCC1806 ($p = 0.023$). The histological study of liver showed 47% of metastasis. The number of liver foci was higher in the group injected with HCC1806 than MCF7, reaching statistical significance ($p = 0.006$). The mean area of liver metastasis was not different in the groups considered (p = n.s.). The logistic regression revealed a potentiating model for liver metastasis with HCC1806 (odds ratio = 16; $p = 0.03$). The number and area of lung metastasis foci were not predictive for liver dissemination.
Conclusion: The mice injected with HR positive breast cancer cells in the tail vein were associated with huge lung metastatic areas. Liver metastization foci were more relevant in TN than HR positive cell lines. TN cells seem to potentiate liver metastasis. The lung metastization does not influence the presence of hepatic metastasis foci after injection in tail vein.

504 WAVE-3 knock-down results in reduced invasion and motility in prostate cancer cells via reduced phosphorylation of paxillin

M. Moazzam[1], L. Yee[2], H. Kynaston[1], W. Jiang[2]. [1]*University Hospital Wales, Urology, Cardiff Wales, United Kingdom*, [2]*University Hospital Wales, Surgery, Cardiff Wales, United Kingdom*

Introduction: WAVE-3 is a member of Wiskott-Aldrich syndrome proteins family, regulating cellular migration through actin related protein complex. WAVE-3 activity is controlled by Rho GTPases. Paxillin serves as a nexus for the control of the Rho family of GTPases for their essential role in regulation of actin cytoarchitecture and adhesion dynamics. This study investigates the possible role of paxillin in the changes induced by WAVE-3 knock-down in prostate cancer cells.
Methods: Expression of WAVE-3 was knocked down through a transgene consisting of hammerhead ribozyme and antisense specific to WAVE-3, cloned in to a PEF6 expression factor and transfected in to PC-3 cell line through electroporation. After confirming knock-down, *in vitro* assays were used to assess cell growth, adhesion, motility and invasion. Expression and phosphorylation status in different cell lines was assessed by PCR, immunoblotting and immunofluorescence cytology.
Results: Stably transfected $PC3^{WAVE\text{-}3\ KD}$ cells exhibited reduced expression of WAVE-3 at both mRNA and protein levels. PC-3 $^{\Delta WAVE3\ KD}$ cell line showed reduced invasion ($P < 0.01$) and motility ($P < 0.01$). The active phophorylated form of paxillin was significantly reduced ($P < 0.01$) in PC-3 $^{\Delta WAVE3\ KD}$ cells on western blotting as compared to $PC\text{-}3^{WT}$ & $PC\text{-}3^{pEF}$ cells and failed to show any significant increase in phosphorylation following growth factor stimulation with HGF.
Conclusion: Optimal levels of phosphorylated paxillin are reduced following elimination of WAVE-3 in prostate cancer cells and contribute to a reduction in the invasive phenotype of prostate cancer cells.

505 Novel potential markers of tumour-iniziating cells in colon cancer

M.A. Puglisi[1], A. Sgambato[2], M. Barba[1], E. Giorda[3], N. Saulnier[1], A. Boninsegna[2], A.C. Piscaglia[1], R. Carsetti[3], A. Cittadini[2], A. Gasbarrini[1]. [1]*Politechnical University "A. Gemelli", Internal Medicine, Rome, Italy*, [2]*Politechnical University "A. Gemelli", General Pathology, Rome, Italy*, [3]*Bambino Gesù Paediatric Hospital, Cytofluorimetry Laboratory, Rome, Italy*

Background and Aim: "Cancer stem cells" (CSC) represent a minority of cancer cells responsible for tumour initiation, maintenance and spreading. Several in vitro assays have been used to identify CSC, including Hoechst dye efflux properties which define the side population (SP), or by the expression of cell surface markers, such as CD133. However, each of these methods has potential pitfalls that complicate interpretation of the results. Our research group have previously demonstrated the presence of a significant CD133+ cell fraction in human primitive and metastatic colon cancer (CC). In the present study, using the CaCo-2 cell line, we wanted to confirm that CD133 expression is a valid method for isolating CSC in CC and identify new antigens in order to increase the specificity of this marker.
Methods: CD133+ and CD133− cells were isolated from CaCo-2 cell line by FACSorter and the tumour-initiating potential of CD133+ cells was assessed *in vitro*, by soft-agar colony formation assay, and *in vivo*, upon transplantation into nude mice. Furthermore, the gene expression profile of CD133+ versus CD133− CaCo-2 cells was compared by the means of microarray analysis. Then, in the effort to identify a common "tumour stem cell" signature for CC, the most relevant transcripts resulting from gene expression profiling on CD133+ cells was assessed by real-time PCR on SP-fraction isolated from the same cell line.
Results: Using the CaCo-2 cell line, we showed that only CD133+ cells have a tumour-initiating potential in vitro and in vivo. Furthermore, microarray analysis of CD133+ versus CD133− CaCo-2 cells revealed a significant overexpression of various transcripts involved in cell proliferation, invasion and stemness in CD133+ cell fraction. Phenotypic analysis displayed that CD133 expression was higher in the SP fraction than no-SP fraction. Comparison of the transcripts by real-time PCR revealed that the genes of Endothelin-1, Smad-7, S100P and NR4A2 are highly expressed in both CD133+ cells and in SP fraction.
Conclusion: Overall, we showed that only CD133+ cells exert a tumour-initiating potential *in vitro* and *in vivo*, confirming that CD133 is a good marker for colon CSC. Furthermore, microarray analysis revealed a unique molecular profile of the CD133+ cells. In particular, four genes are highly expressed in both CD133 + cells and in SP fraction. These genes are involved in regulating cell proliferation and metastasis, so they may be excellent markers to increase the specificity of CD133 in identifying CSC in CC.

506 Influence of wild-type MLL on glucocorticoid sensitivity and response to DNA-damage in paediatric acute lymphoblastic leukaemia

A.H. Beesley[1], J.L. Rampellini[1], M.L. Palmer[1], J.Y.S. Lee[1], M.J. Firth[2], U.R. Kees[1]. [1]*Telethon Institute for Child Health Research and Centre for Child Health Research University of Western Australia, Leukaemia and Cancer Research, Perth, Australia*, [2]*Telethon Institute for Child Health Research and Centre for Child Health Research University of Western Australia, Biostatistics and Genetic Epidemiology, Perth, Australia*

Background: Rearrangement of the mixed-lineage leukemia gene (*MLL*) is found in 80% of infant acute lymphoblastic leukemia (ALL) and is associated with poor prognosis and resistance to glucocorticoids (GCs). We have recently observed that GC resistance in T-ALL cell lines is associated with a proliferative metabolism and reduced expression of *MLL*. In this study we have further explored the relationship between *MLL* status and GC sensitivity.
Material and Methods: Studies were performed using a cell line panel comprising nine T-ALL lines derived in our own laboratory from paediatric ALL bone marrow specimens, plus six additional T-ALL cell lines obtained from external sources. Sensitivity of T-ALL cell lines to methylprednisolone (MPRED) and dexamethasone (DEX) was measured using the MTT assay with drugs incubated over four days. For gene expression profiling RNA was extracted from cell lines in exponential growth phase and hybridized to Affymetrix HG-U133A microarrays. Published microarray data used for *in silico* analysis was downloaded from publicly available depositories or authors' websites.
Results: Negative correlation of *MLL* expression with GC resistance in 15 T-ALL cell lines was confirmed by quantitative RT-PCR. The absence of *MLL*-rearrangements in the panel of T-ALL cell lines suggested that this relationship represented expression of wild-type *MLL*. Analysis of *MLL* expression patterns revealed a negative relationship with cellular metabolism, proliferation and anti-apoptotic transcriptional networks. *In silico* analysis of published data demonstrated that reduced levels of *MLL* mRNA are associated with relapse and GC resistance in T-ALL patients and adverse clinical outcome in children with *MLL*-rearranged ALL. RNAi knockdown of *MLL* expression in T-ALL cell lines significantly increased resistance to dexamethasone and gamma

irradiation indicating an important role for wild-type *MLL* in the control of cellular apoptosis.
Conclusions: The data suggests that reduced expression of wild-type *MLL* can contribute to GC resistance in ALL patients both with and without *MLL*-translocations.

507 In vitro analysis of population specific BRCA1 splicing variants

J. Sevcik[1], M. Falk[2], P. Kleiblova[1], F. Lhota[1], P. Pohlreich[1], Z. Kleibl[1]. [1]*Charles University in Prague First Faculty of Medicine, Institute of Biochemistry and Experimental Oncology, Prague, Czech Republic,* [2]*ASCR in Brno, Institute of Biophysics in Brno, Brno, Czech Republic*

Background: The BRCA1 is multifunctional tumour suppressor, important for DNA repair. Mutations in this gene are responsible for the majority of hereditary breast and ovarian cancer cases in the Czech Republic. Beside genetic alterations numerous splicing variants of unknown clinical significance have been detected. In order to test their biological importance, we have developed new, cell line based, *in vitro* system.
Methods: Stable clones of breast adenocarcinoma cell lines expressing population-specific *BRCA1* splicing variants, with/without co-expression of shRNAs that target the wtBRCA1 were prepared. Functionality of used expression systems was confirmed both at the mRNA and protein level by qPCR and Western blotting respectively. Proliferation of cells following g-irradiation (GI) was determined by real time cell analyzer. The kinetic of DNA double-strand break (DSB) repair was quantified by counting γH2AX foci colocalizing with 53BP1 protein (IRIF, Ionizing Radiation Induced Foci) during the post-irradiation time.
Results: We have established stable clones (1) expressing *BRCA1* splicing variants (14+15 del; 17−19 del), (2) down regulating *BRCA1* to <10% relative to control cells, and (3) stably expressing BRCA1 splicing variants 14+15 del and 17−19 del alongside to down-regulated wtBRCA1.
The proliferation of cells examined in relation to GI-induced DNA damage showed that clones up-regulating variant 17−19 del exert increased radio-resistance, contrary to the clones with up-regulated variant 14+15 del that were significantly more radio-sensitive. In accordance, formation and persistence of GI-induced IRIFs was markedly prolonged in clones with shRNA-mediated down-regulation of wtBRCA1 expression as well as in clones with up-regulated expression of BRCA1 17−19 del variant.
Conclusions: Our current *in vitro* results indicate that studied splicing variants of *BRCA*, with affected phosphorylation or BRCT domains, differentially influence growth properties of cells in relation to GI-induced DSB damage in the established model system and may alter DSB repair capacity on the level of IRIF dynamics.
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508 Alternative splicing of Lysyl Oxidase-Like 4 in ovarian carcinoma

R. Reich[1], S. Sebban[1], R. Golan-Gerstl[2], R. Karni[2], B. Davidson[3]. [1]*Hebrew University Medical School, Institute for Drug Research, Jerusalem, Israel,* [2]*Hebrew University Medical School, Biochemistry and Molecular Biology, Jerusalem, Israel,* [3]*National Hospital – Norwegian Radium Hospital, Pathology, Oslo, Norway*

Introduction: Lysyl oxidase (LOX) is an amine oxidase that is usually synthesized and secreted by fibrogenic cells. Four LOX-like (LOXL) genes have been identified so far in mammalian genomes, encoding four different LOX-like proteins: LOXL1, LOXL2, LOXL3 and LOXL4. All members of the LOX family show a highly conserved C-terminus region that contains the catalytic domain. The N-terminus of the LOX isoforms is less conserved among the different members and is thought to determine the individual role and tissue distribution of each isoenzyme. LOXL4, the least studied member of the LOX-like family enzymes, undergoes a process of alternative splicing in cancer, in a site- and stage-specific manner that we have previously shown. The purpose of the current study was to uncover the splicing mechanism that is responsible for this process.
Experimental procedures: I. ShRNAs for four splicing factors: SF2/ASF, SRp55, hnRNP-A1 and hnRNP-A2, were transfected in two cell lines: U-87 MG cell line (human glioblastoma) and NCI-H460 (human large-cell lung carcinoma). II. Over-expression of SF2 was performed in MST0-211H cell line (human malignant mesothelioma), HeLa cell line (human epithelial cervical cancer) and MCF10A cell line (human mammary epithelial line). III. Western blotting for SF2/ASF and tubulin. IV. RT-PCR for LOXL4 full length, splice-variant1 (splv1) and splice-variant2 (splv2) mRNA expression.
Results: We examined LOXL4 expression in U-87 MG cells. When untreated, these cells express the full length and splv2, almost equally. The silencing of two factors, SF2/ASF and hnRNP-A1, resulted in a dramatic changes in the expression pattern of LOXL4. For both silenced factors, LOXL4 full-length mRNA expression was much stronger, while the shortest variant, splv2, completely vanished. The silencing of hnRNP-A2 led to a smaller decrease in splv2, while SRp55 silencing did not seem to change LOXL4 splicing. In NCI-H460 cells, which normally express small amounts of all variants, no significant changes were found following silencing. In an attempt to further establish the splicing factor responsible for LOXL4 splicing, we over-expressed SF2/ASF in MST0-211H cells, which normally express only the full length LOXL4. Expression of SF2/ASF resulted in the appearance of splv2, while dramatically reducing the expression of the full length. Similar results were seen in HeLa cells. Over-expression of SF2/ASF in MCF10A cells, which untreated, have the unique quality of expressing splv2 alone, caused only a slight increase in the expression of splv2.
Conclusions: These results demonstrate for the first time, that LOXL4 is a direct target of the splicing factor SF2 SF2/ASF. Furthermore, in concordance with our previous in-vivo findings, it can be concluded that LOXL4 splicing occurs similarly in other epithelial cancer types, such as breast cancer and mesothelioma.

509 Evaluation of Human Epididymis Protein 4 in endometrial cancer patients

A. Ravaggi[1], E. Bignotti[1], E. Bandiera[1], L. Zanotti[1], R.A. Tassi[1], C. Romani[1], S. Bergamelli[1], M. Ragnoli[1], F.E. Odicino[1], S. Pecorelli[1]. [1]*Brescia University, Materno Infantile e Tecn. Biom., Brescia, Italy*

Background: Endometrial cancer (EC) is the most common gynaecologic malignancy in Western world. To date, no good marker for screening or disease monitoring for this cancer is available. The aim of this study was to investigate HE4 gene and protein expression in a panel of ECs and normal endometria (NE) and to test the potential utility of HE4 as serum marker for early EC detection. Moreover we correlated HE4 serum levels with clinicopathologic characteristics of the patients.
Material and Methods: Using quantitative real-time PCR we tested a total of 46 ECs and 20 NEs for HE4 gene expression. Protein expression was analyzed by immunohistochemical staining (IHC) in tissue sections from 155 EC patients (40 well-differentiated (G1), 60 moderately-differentiated (G2), 55 poorly-differentiated (G3)) and 33 NE controls. Finally, pre-operative serum samples from 141 EC patients (25 G1, 60 G2, 56 G3) were analysed for HE4 (HE4 EIA-assay, Fujirebio-Diagnostics) and CA125 (Architect-CA125II assay, Abbott-Diagnostics) levels. Serum control samples were obtained from 76 NE patients.
Results: EC patients showed a significant HE4 gene overexpression compared with controls (t-test, $p < 0.0001$), as measured by qRT-PCR. Using IHC, HE4 protein expression was found higher in ECs compared to NEs, with a significant difference between the 2 groups (Mann-Whitney, $p = 0.028$); HE4 immunoreactivity was stronger in G1 ECs and decreased with higher grade (Anova test, G3 vs G1, $p < 0.0001$; G3 vs G2, $p = 0.0062$). Finally, HE4 serum levels (sHE4) were significantly higher in EC patients compared with controls (t-test, $p < 0.0001$), as measured by ELISA. Setting the specificity at 95%, the sensitivities in detecting EC were 67% for HE4, 30% for CA125 and 68% for the combination of both markers. HE4 serum levels significantly increased with higher stage (<IIB vs ⩾IIB, $p < 0.001$), higher grade (G1 vs G2/G3, $p < 0.0001$) and deeper myometrial invasion (M0/M1 vs M2, $p < 0.001$). No significant difference in sHE4 levels was found among different tumour histotypes.
Conclusions: This study highlights that HE4 is overexpressed both at mRNA and protein level in EC and that it is secreted at higher levels in serum of EC patients compared with NE controls. HE4 serum levels are more sensitive and specific compared to serum CA125 levels in distinguishing healthy subjects from malignant disease, regardless of tumour stage and grade. sHE4 levels are positively correlated with high stage and grade, deeper myometrial invasion, lymph node positivity, therefore they could be associated with a more aggressive tumour phenotype.

510 Effects of anti-VEGFR and anti-EGFR agents in glioblastoma

K. Dimitropoulos[1], E. Giannopoulou[1], A. Argyriou[1], I. Kritikou[1], H.P. Kalofonos[1]. [1]*University of Patras, Department of Medicine, Patras – Rio, Greece*

Background: Malignant gliomas are the most common and aggressive primary brain tumours. Sunitinib is an oral, multi-targeting receptor tyrosine kinase inhibitor (TKI), including platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR). Lapatinib is an ATP-competitive dual TKI for epidermal growth factor receptor (EGFR) and HER2/neu (ErbB-2). The aim of the current *in vitro* study was to assess the effect of sunitinib and lapatinib applied either alone or in combination on proliferation, apoptosis, invasion and release of MMPs into the culture medium of U87 and M059K human glioblastoma cell lines. Other parameters analyzed were the effect of lapatinib on the formation of EGFR-integrin b1 complex, as well as the effect of sunitinib on the VEGFR-integrin b3 and PDGFR-integrinb3 complexes formation on U87 cells.
Material and Methods: U87 and M059K cells were treated with Sunitinib and Lapatinib at several concentrations. The proliferation of cells was determined by MTT assay. Apoptosis was evaluated with Annexin binding assay. Migration assays were performed in 24-well microchemotaxis chambers. The release of MMPs into the culture medium of cells was measured by zymography.