Materials and Methods: To define the role of the KDELR-Golgi-SFK signalling cascade in cell invasion I assessed the ability of A375MM melanoma cells to degrade the extracellular matrix (ECM). Cells were transfected with different constructs to activate/inhibit KDELR and then plated on a fluorescent gelatin matrix in the presence of BB94, a broad spectrum metalloprotease inhibitor. After 16 hours, BB94 was washed out and cells were fixed after 3 hours and processed for immunofluorescence. The degradation areas, visible as dark "holes" in the fluorescent gelatin matrix, were quantified by the LSM510−3.2 software (Zeiss).
Results: The chronic activation of the KDELR by either over-expression of the KDELR itself, which induces its autoactivation, or transfection of artificial secretory proteins that act as KDELR agonists increases ECM degradation. In contrast, the over-expression of the KDELR mutant KDELR-D-193-N leads to a decrease in ECM degradation. The increase in ECM degradation correlates with an increase in SFK activation. Moreover, upon KDELR activation there is an increase in the phosphorylation levels of two SFK substrates, the ARF GTPase activating protein ASAP1 and the focal adhesion kinase FAK. These data indicate that KDELR-Golgi-SFK signalling affects ECM degradation through the involvement of ASAP1 and FAK.
Conclusions: We have investigated whether KDELR–Golgi–SFK signalling could regulate tumour cell invasion. Our data indicate that the chronic activation of this cascade promote ECM degradation. We believe that the Golgi complex has a crucial role in cell invasion, not only by providing hydrolytic enzymes for ECM degradation but also through KDELR-initiated-signalling. Our investigation is important because it highlights a new signalling cascade involved in the regulation of cell invasion thus expanding our knowledge of the metastatisation process and possibly providing new druggable targets to exploit for anti-metastasis therapy.

402 The endogenous EPO/EPOR system contributes to glioma cell proliferation both in vitro and in vivo

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Background: The biology of erythropoietin (EPO) has been recently re-evaluated following the discovery of its receptor (EPOR) on numerous cancer cells [1]. Concerning brain tumours, although the expression of the EPOR has been described on glioma cells [2], data from the literature remain descriptive and controversial [3] and to date no clear demonstration of a potential effect of EPO on controlling tumour growth has been described. Accordingly, the aim of this study was to evaluate and compare both in vitro and in vivo, the effect of two complementary strategies developed to block the biological effect of EPO/EPOR on glioma cell growth.
Material and Methods: Human U87 and U251 glioma cells were genetically modified by RNAi to stably invalidate EPO or EPOR expression. Full-length EPOR (9L-EPORF) or a truncated variant used as a negative dominant for EPOR (9L-EPORT) were stably overexpressed by rat 9L glioma cells. Effects of genetically modified cells were studied both in vitro on cell proliferation by automatic cell counting and in vivo by a longitudinal MRI follow-up of tumour growth and a survival study after intrastriatal implantation of these cells in Fischer rats (for 9L cells) or nude mice (for U87 and U251 cells).
Results: On one hand, the invalidation of either EPO expression (with shRNA EPO) or EPO availability (with soluble EPOR) on distinct human glioma cell lines led to a sustained decrease in cell proliferation from day 1 to day 4 (at day 4, U87-shEPO: 27±5% versus U87 control, $n = 3$, $p < 0.05$). On another hand, the invalidation of EPOR expression or EPOR signaling also induced a reduction of glioma cells number in vitro. Accordingly, in vivo, we observed on day 34 a decrease in tumour volume for the mice bearing U87-shEPOR ($59 \pm 16\ mm^3$), as compared to U87-Control mice ($107 \pm 30\ mm^3$; $n = 4$ for each group, $p = 0.05$). In addition, we also measured by MRI at day 13, a significant reduction of the tumour volume for animals bearing 9L-EPORT cells ($5.4 \pm 1.5\ mm^3$) in comparison to control rats (9L cells: $77.7 \pm 34.8\ mm^3$, $n = 4$ both groups, $p < 0.05$). An increase in the animal survival median was also observed for 9L-EPORT (28 days) in comparison to 9L rats (21 days, $n = 6$ both groups, $p < 0.05$).
Conclusions: Collectively, by the use of two strategies of EPO/EPOR inhibition on glioma cells, our results support that the autocrine EPO/EPOR loop on tumour cells might be a critical mediator of cell proliferation which might influence tumour outcomes of patients with brain tumours.
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403 Mitochondrial superoxide dismutase overexpression changes neuroendocrine, proliferation and apoptosis resistance features in prostate cancer cells

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Prostate cancer (PC) is a tumour greatly associated with aging and oxidative stress one of the most accepted theories to explain aging. Therefore antioxidant enzymes such as MnSOD/SOD2 have been studied in relation to androgen-independent transition and resistance to radio/chemotherapy treatments. The main role of MnSOD is superoxide radical scavenging and thus this mitochondrial enzyme is induced, among other stimuli, as a radiation-adapted signal. Neuroendocrine (NE) cells are postmitotic cells and the less frequent cell type in the prostate gland that show androgen independence and resistance to treatment. Interestingly, NE differentiation is a common process along with PCa progression.
In order to study the role of MnSOD in NE cells, we stably overexpresed androgen dependent LNCaP. In these clones we first confirmed the presence of NE markers and apoptotic-related proteins by western blotting. Then we tested (1) proliferation rate; (2) the ability of cells to grow in androgen deprived medium both by cell counting; (3) apoptosis resistance to chemotherapeutic treatment by MTT viability assay; and (4) the potential capacity of MnSOD-expressing cells-conditioned media of inducing cell growth in androgen independent PC-3 cells by DNA staining with Hoechst.
MnSOD overexpressing LNCaP clones, namely MnSOD-S4 and S12, show typical NE morphology when compared to mock clones (pcDNA-P2), which was also confirmed by levels of Synaptophisin, a widely used NE marker. Surprisingly, a higher proliferation rate was observed in MnSOD-S4 and S12 clones, indicating that MnSOD overexpression keeps proliferation capacity. In androgen-stripped media, MnSOD-S12 clone displays a significantly higher proliferation rate than pcDNA-P2 while protein levels of androgen receptor are decreased in both, MnSOD-S4 and S12 clones. Regarding apoptosis resistance, MnSOD-S12 clone showed higher resistance etoposide-induced cell death Finally, PC-3 cells maintained with conditioned medium obtained from MnSOD-S4 showed higher levels of proliferation compared with pcDNA-P2.
The increase of MnSOD expression is enough to induce most of the reported morphological and biochemical characteristics of androgen-independent NE-like cells in PC. This would indicate that redox balance mediated by MnSOD could be a key step in androgen dependent-independent transition in PC.

404 A novel role for JAM-A as a crucial regulator of breast cancer cell motility through downstream effects on Rap1GTPase and Beta1-integrin

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Introduction: The cell-cell adhesion protein junction adhesion molecule-A (JAM-A) influences epithelial cell morphology and migration. We have previously demonstrated that high JAM-A levels in breast cancer tumour cells are significantly associated with poor prognosis in breast cancer patients (McSherry, 2009). In addition, we have recently shown that functional inhibition or knockdown of JAM-A decreases MCF-7 breast cancer cell migration, likely due to reduced levels of β1-integrin. The aim of our ongoing studies is to define the pro-migratory signalling cascades at play in breast cancer cells from JAM-A (at the cell-cell interface) to β1-integrin (at the cell-matrix interface).
Materials and Methods: MCF7 cells following siRNA-mediated JAM-A gene knockdown (JAM-A KD cells) were investigated via western blot and immunoprecipitation analysis to determine protein expression of putative signalling pathway proteins (downstream of JAM-A). In addition, JAM-A KD cells were assayed for alterations in cancer cell migration and cell adhesion. As Rap1 is a known activator of integrins, cell migration of MCF7 cells was determined following treatment with either a Rap1GTPase pharmacological inhibitor or a b1-integrin inhibitory antibody. Analysis of active Rap1 was performed in MCF7 cells following either JAM-A gene knockdown or JAM-A protein antagonism. Affects of JAM-A antagonism on PDZGEF2 (a crucial Rap1 activator) was assessed by immunofluorescence and western blot analysis.
Results: We have demonstrated that following JAM-A gene knockdown the protein expression of Rap1 and the Rap1 regulator AF-6 is decreased, and direct association of JAM-A and AF-6 is altered. In addition, the activity of Rap1 is reduced upon either JAM-A knockdown or inhibition. Furthermore, we demonstrated that breast cancer cell migration is decreased upon inhibition of both Rap1GTPase and b1-integrin. Finally, we demonstrate that PDZGEF2 localisation is altered following JAM-A inhibition.
Conclusion: We suggest that JAM-A over-expression can initiate a signalling cascade involving AF-6, PDZGEF2, Rap1GTPase and b1-integrin, to promote

breast cancer cell migration. Furthermore, we have evidence that an inhibitory antibody to JAM-A reduces breast cancer cell migration *in vitro*. Therefore, it is tempting to speculate that, akin to Herceptin targeting the HER2 receptor, targeting JAM-A may represent a new therapeutic modality for future breast cancer treatment.

405 p38a is required for cancer-specific metabolism and survival

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Background: Increased glycolysis, even in the presence of high oxygen tension, is a common feature of tumour cells, a process known as 'Warburg effect'. A key mechanism sustaining aerobic glycolysis is the stabilization and activation of the transcription factor HIF1α. The p38 pathway is often activated by stress-associated stimuli and cytokines leading to diverse and sometimes opposite cell type-specific responses, such as cell survival and apoptosis. Earlier reports indicate that p38α is responsible for the stabilization of HIF1α in hypoxic MEFs and in a pancreatic cancer cell line. Our previous studies indicate that p38α inhibition induces cell cycle arrest, autophagy and cell death in cancer cells, suggesting that this kinase might be involved in the regulation of cancer-specific energy balance.
Material and Methods: Colorectal, ovarian and prostate cancer cells were treated with p38α inhibitors and characterized by cellular and molecular approaches to evaluate survival, death, autophagy, and protein and gene expression.
Results: Colorectal cancer cells treated with p38α inhibitors showed reduced levels of ATP, together with reduced glucose uptake and lactate extrusion. These results correlated well with the reduction of HIF1α protein levels and the down regulation of a specific subset of HIF1α target genes, which encode for glycolytic rate-limiting enzymes, thus affecting the most important steps of this metabolic pathway. The use of inhibitors of prolyl hydroxylases (i.e. DFO), whose enzymatic activity triggers HIF1α proteasomal degradation, led to the recovery of HIF1α protein levels and activation of its transcriptional program, indicating that p38α is required for HIF1α protein stability. These results were reproduced in both ovarian and prostate cancer cells. The p38α-dependent energetic imbalance triggered the activation of FoxO3A transcriptional program, which promotes energy retrieval for survival; however, prolonged activation of p38α led to autophagic cell death.
Conclusions: We showed that p38α is required for HIF1α stability, probably through a mechanism involving prolyl hydroxylases. Thus, p38α blockade inhibits cancer-specific aerobic glycolysis by switching off the expression of glycolytic rate limiting enzymes. The energetic balance is first maintained by the activation of energy producing pathways; however, prolonged inhibition of p38α eventually leads to autophagic cell death.
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406 The BH4 domain is required for proangiogenic function of bcl-2 protein

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Background: We previously demonstrated that the antiapoptotic protein bcl-2, in addition to act as an antiapoptotic protein, cooperates with hypoxia to promote Vascular Endothelial Growth Factor (VEGF)-mediated tumour angiogenesis, through a mechanism involving the transcription factor Hypoxia Inducible Factor 1 (HIF-1). In the present work we analyzed in which domain(s) map the proangiogenic function of bcl-2.
Materials and Methods: Human melanoma cell line M14 was used for stable and transient trasfections of expression vectors encoding *wild type* or mutated forms bcl-2 protein. Cells expressing different forms of bcl-2 was characterized for secreted VEGF protein level (*ELISA assay*), HIF-1 protein expression (*Western blot*) and transcriptional activity (*Reporter assay*). Conditioned media from cells expressing *type* or mutated forms of bcl-2 were tested for their angiogenic activity using *in vivo* (*Matrigel plugs assay*) and *in vitro* (*endothelial cells proliferation and morphogenesis*) assays.
Results: We showed that removal of or mutations at BH4 domain abrogate bcl-2 ability to induce VEGF secretion, HIF-1α protein expression and HIF-1 trascriptional activity under hypoxic conditions. Conditioned medium from cells expressing bcl-2 deleted of the BH4 domain under hypoxia markedly reduced *in vitro* angiogenesis-related endothelial cell functions and *in vivo* neovascularization when compared to the effect induced by conditioned medium from cells overexpressing *wild type* bcl-2. By contrast BH1 and BH2 domains are not required for the activation of proangiogenic signaling by bcl-2. We also found that the exposure to a cell-permeable form of BH4 domain of bcl-2 is sufficient to induce HIF-1/VEGF protein expression in melanoma cells under hypoxia. Finally, transient overexpression of wild type or deleted forms of bcl-2 extends this observation to other melanoma cell lines and tumour cell lines with different origin.
Conclusions: These results lead to elucidation of the importance of HIF-1 in bcl-2-mediated angiogenic response under hypoxia and show a regulation of angiogenesis by bcl-2 through a mechanism that requires its BH4 domain.

407 Direct protein and peptide imaging in breast tumour by mass spectrometry

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Background: Matrix-assisted laser desorption/ionization (MALDI)-imaging mass spectrometry (IMS) has been used for detection and verification of peptides or other polymers of biological interest. Direct application of MALDI-time of flight (TOF)-MS on tissue sections makes it possible to obtain specific information on local molecular composition, relative abundance, and spatial distribution for image profiling. The identification directly from tissue sections is important for the diagnosis of tumours, and is the main aim for developing the MALDI-IMS technology.
Material and Methods: MALDI measurements and image analyses were performed using a linear Autoflex instrument equipped with a Smartbeam laser and FlexImaging 2.1 and ClinProTools 2.1 software packages (Bruker Daltonics).
Results: For successful tissue MALDI-IMS results, preparations of the tissue samples are crucial. On testing the condition of tissue preparation embedded in optical cutting temperature polymer (OCT), which are detected in the *m*/*z* 921.7–2,022.4 range, OCT polymer signals suppress the peptide signals and interrupt peptide imaging because of contaminant noisy peaks. With Tissue MALDI-IMS, we obtained protein peaks ranged in 3−17 KDa from sinapinic acid matrix, peptide peaks ranged in 0.8−2.9 KDa from DHB matrix. Peptide images enable to be better as detected more MS peaks than protein peaks. 25 peptide images were obtained in OCT removed tissue and 18 peptide images in OCT embedded tissue, whereas only 10 protein images were produced in the same sample. Therefore, embedding of tissue should be avoided contamination with OCT by using the blade of the cryostat to remove OCT, and embedding in only one side bottom of sample. Peptide imaging is preferable in effectiveness and more information than protein imaging. On PCA analysis to evaluate the quality of tissue spatial features based on MALDI-IMS data, signals with three different regions were extracted and peptide-specific ions was identified. Ions at 2,032 *m*/*z* were most abundant in interface zone (IZ), whereas those at 911.8 *m*/*z* were most abundant in normal zone, and those at 1,542 *m*/*z* were found specifically in tumour burden. We will perform peptide sequencing directly from the tissue section using MS/MS and identify a significant increase peptide of 2,302 *m*/*z* peak in IZ.
Conclusions: We compared peptide with protein imaging patterns by using MALDI-IMS technology in crude samples of fresh-frozen tissue samples based on tumour, normal and IZ. The best imaging results were generated with peptide imaging in OCT removed. Given update of current theoretical concept, molecular margin could be practically developed.

408 The metabolic microenvironment finely regulates invadopodia ECM proteolysis through an ezrin-PKA-RhoA-NHE1 signaling axis

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Background: Degradation of the extracellular matrix (ECM) is one of the critical steps of tumour cell invasion and invadopodia are thought to mediate invasion through focal proteolysis of the ECM. Invasion of the surrounding tissue needs a continuous communication between the tumour cells and the surrounding ECM and, in this context, the tumour microenvironment assumes a fundamental importance since alterations in the ECM, in cytokines and in growth factors can influence cellular behavior. Invadopodia form as a consequence of the tumour cell interaction with the ECM via activation of ECM receptors. However, tumour associated stromal components find themselves in a markedly different environment when in a tumour. In particular the tumour environment is hypoxic and has a low extracellular pH/nutrients. Indeed, it is increasingly clearer that the selective events underlying metastatic progression often involve interactions with elements of both the tumour-specific stromal and metabolic microenvironments. Therefore, a fundamental question is how the invasive mechanism(s) are, in turn, regulated by the other components of the tumour metabolic microenvironment such as low serum and/or hypoxia.
Materials and Methods: Experiments were conducted in the metastatic breast cancer cell line, MDA-MB-231, seeded onto Matrigel containing the quenched fluorescent substrate, DQ-Green-BSA, such that proteolysis produces fluorescence in a dark background. Proteolysis was evaluated microscopically in 3D co-localization analysis with cortactin and/or actin to localize invadopodia.
Results: Here, we observe significant differences in invadopodial-driven focal ECM digestion and cell shape/size when cells on Matrigel are subjected