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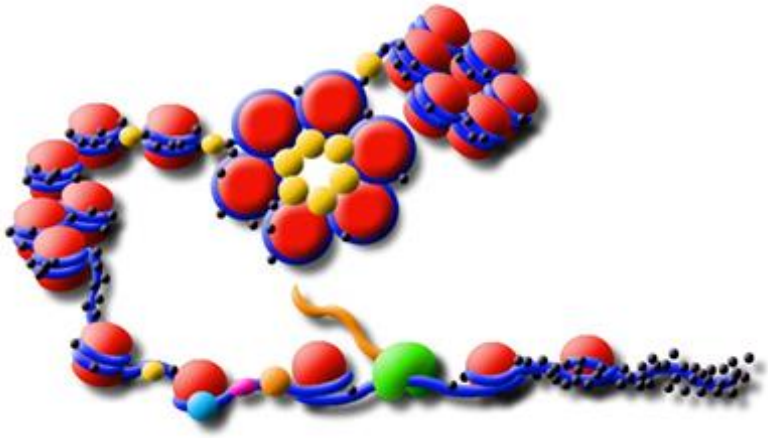

Program
&
Abstract Book

CLEpSo Meeting 5th – 6th/03/2015

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Background and Aim: Natural Killer cells (NKs) are an important line of defense against virally-infected and tumor cells. NKs activation status is determined by the balance between signals from its activating and inhibitory receptors. An imbalance in these signals is often evidenced in many cancers including hepatocellular carcinoma (HCC); enabling tumor cells to evade the NK cell mediated immune responses. MiR-182 was reported to be oncogenic in HCC cell lines. Bioinformatics data showed that it may target both the activating receptor NKG2D and the inhibitory receptor NKG2A. Therefore we aimed for the first time at investigating the role of miR-182 in regulating the expression of NKG2D and NKG2A in HCC. Methods: Peripheral blood mononuclear cells were separated from the peripheral blood samples of 25 HCC patients and 12 healthy controls using the ficoll separation technique. Subsequently, NKs were isolated using magnetic-assisted cell sorting. Isolated NKs were transfected with miR-182 oligonucleotides. Total RNA was extracted and the expression levels of miR-182, NKG2D, NKG2A and Perforin-1 were quantified using qRT-PCR. Results: NKs of HCC patients showed miR-182 overexpression (p=0.0146) compared to healthy controls. NKG2D and NKG2A were found to be upregulated (p=0.0217) and downregulated (p=0.0153) respectively, in the NKs of HCC patients. Significant correlations were found between miR-182 & NKG2D expression levels (r=0.6818, p=0.0104) and between miR-182 & NKG2A expression levels (r=0.7832, p=0.0013). Upon forcing miR-182 expression in the NKs of HCC patients, a significant upregulation of both NKG2D (p=0.0459) and NKG2A (p=0.0230) was observed. Finally, miR-182 was found to induce the NKs cytotoxicity represented in the upregulation of Perforin-1 expression (p=0.0194). Conclusion: Our findings suggest that miR-182 can be used to modulate the expression of two important NK cell receptors to enhance the NK cell activation and cytotoxicity, paving the road towards an effective HCC-immunotherapy.

Epigenetics of Gastrointestinal Malignancies

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Gastrointestinal cancers such as colorectal and pancreas cancers are the leading causes of cancer death. Beyond genetic changes, the contributions of the epigenome are increasingly recognized as cancer drivers in these cancers including changes in DNA methylation and the histone code. Recent findings from TCGA and multiple other studies have identified subsets of gastrointestinal cancers marked by marked increased predilection of widespread methylation termed CpG island methylator phenotype. These findings have implications in understanding the biology of these cancers but also in improving treatment of cancer patients by developing biomarkers for early detection and prognostic and predictive biomarkers. The talk will also discuss data from the first generation of epigenetic therapeutic trials in gastrointestinal cancers and future studies.

Novel DNA methylation biomarkers in cholangiocarcinoma and their clinical potential

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Cholangiocarcinoma is a rare cancer of the bile ducts with a high mortality due to late clinical presentation. Diagnosing cholangiocarcinoma is challenging, particularly among patients affected by primary sclerosing cholangitis (PSC). Here we present the identification and evaluation of novel epigenetic biomarkers for cholangiocarcinoma. From epigenome-wide analyses, we have identified 13 genes frequently methylated in cholangiocarcinomas compared to controls. These markers were further analyzed in ERCP derived biliary brush samples using qMSP, and their performance were compared to those of brush cytology. A four-gene panel consisting of *CDO1*, *CNRIP1*, *SEPT9*, and *VIM* outperformed conventional cytology, with a combined sensitivity of 85% and an area under the ROC curve of 0.944. A combination of the biomarker panel and conventional cytology identified 94% of the cancer patients with 96% specificity. The high performance of this biomarker panel suggests presents potential for the suitability of monitoring PSC patients for development of cholangiocarcinoma.

Inhibition of class I HDACs promotes de-differentiation of pancreatic acinar cells.

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Background: Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer mortality, with a 5 year survival rate of 4% and resistance to conventional chemotherapy. PDAC cells show aberrant up-regulation of different isoforms of histone deacetylases (HDACs). As HDACs regulate proliferation, differentiation and apoptosis, targeting these molecules with selective inhibitors (HDACi) constitutes a promising anti-neoplastic strategy. However, clinical trials showed minimal beneficial effects of HDACi treatment of PDAC patients. Here we investigated whether the limited efficacy of HDACi in PDAC could be linked to undesired pro-cancer effects following HDAC inhibition. Methods: Regulation of acetylation levels and HDAC expression were analyzed *in vivo*, using transgenic mice harboring the activating KrasG12D mutation that leads to spontaneous formation of pancreatic pre-malignant lesions, and *in vitro*, using 3D cultures of explanted acinar cells and pan-

creatic cancer cells. The effects of selective inhibitors of class I HDAC were evaluated using biochemical, qRT-PCR and imaging techniques. Results: Class I HDACs were selectively up-regulated in both KrasG12D mice and explanted acinar cells in a time dependent manner, correlating with acinar de-differentiation and development of pre-malignant lesions. Treatment with HDACi reduced the proliferation of acinar cells *in vivo* and *in vitro* and down-regulated the expression of late cyclins. The cytostatic effect of HDACi treatment was associated with induction of the cell cycle inhibitor p21WAF1/Cip1, while expression of other cell cycle inhibitors of the Ink4 and Cip/Kip families was not affected. Unexpectedly, HDACi treatment of pancreatic cancer cells decreased their replication rate but up-regulated the expression of de-differentiation markers. Conclusion: Our *in vivo* and *in vitro* results indicate that pharmacological inhibition of class I HDACs effectively reduces cell replication, but it is associated with increased de-differentiation of pancreatic cancer cells. Further investigations will elucidate whether the de-differentiation phenotype is associated with EMT transition and increased risk of metastasis.

Dysregulation of KDM4A-D Lysine Demethylases Promotes Chromosomal Instability

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Lysine demethylases (KDM), a new and exciting front of biological research, are implicated in multiple cellular processes including transcription, DNA damage response, cell-cycle regulation, cellular differentiation, senescence and carcinogenesis. KDM4A-D members demethylate H3K9 and H3K36 methylation marks. Recent studies show that various types of human cancers exhibit amplification or deletion of KDM4A-D members and thus implicating their activity in promoting chromosomal instability and carcinogenesis. *How misregulation of KDM4 members promotes chromosomal instability remains largely unknown*. In this meeting, I will present three novel functions of KDM4 family that provide molecular insights into the mechanisms by which KDM4 misregulation leads to chromosomal instability. The 1st is related to a new role of KDM4 protein in regulating the fidelity of mitotic chromosomes segregation. The 2nd highlights a novel role of KDM4D in the repair of double-strand breaks. The 3rd shows that KDM4 overexpression triggers H3K36me3 demethylation and disrupts the integrity of DNA mismatch repair. Our findings advance the link between cancer-relevant networks of epigenetic regulation and genome stability

Lung Cancer Epigenetics: Lessons from the mouse embryo to develop novel approaches for early diagnosis and therapy of lung cancer.

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Several genes that are relevant during embryonic lung development share a similar gene structure with two distinct promoters driving the expression of two different transcripts. Expression of both transcripts from the same gene is complementary and is differentially regulated during embryonic development, with one transcript been expressed during early stages of lung development (embryonic isoform) and the other transcript been expressed at later stages and in adult lung (adult isoform). Interestingly, the embryonic isoforms are enriched in lung tumors of mice as well as of human, making them good candidates for lung cancer diagnosis. In addition, forced expression of the embryonic isoforms in murine adult lung is sufficient to induce hyperplasia with characteristics of lung adenocarcinoma supporting their oncogenic potential. Moreover, we found that the complementary expression of both isoforms from the same gene is mediated by differential promoter usage that is regulated by mechanisms that involve changes in the chromatin structure as specific histone modifications and dynamic DNA methylation. In conclusion, we suggest that an embryonic specific phenotype is acquired during lung cancer progression and this can be used for early diagnosis of lung tumors. In addition, approaches to suppress embryonic specific isoforms can be exploited to develop therapeutic strategies against lung cancer.

Eradication of asbestos tumors in vivo with histone deacetylase inhibitors-polymer conjugate nanoparticles for acid-responsive drug delivery

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The study reports the synthesis of acid–responsive polymeric nanoparticles (NPs) consisting of polymer-histone deacetylase inhibitors conjugate. An innovative aspect lies in the NP conjugation mode of histone deacetylase (HDAC) inhibitors introduced with a clickable acid-responsive prodrug during monomer synthesis, prior to polymerization. The other novelty is due to the selected norbornene (NB)-polyethylene oxide (PEO) macromonomer allowing standardization of the polymerization process by Ring-Opening Metathesis Polymerization (ROMP) and functionalization through azide-alkyne click chemistry. It has been demonstrated that the synthesized polymer gave 300 nm core-shell spherical nanoparticles with low dispersity, high water dispersability thanks to the PEO shell and well controlled HDAC inhibitor prodrugs loading. Bioluminescence Resonance Energy Transfer (BRET) assay in living cells and viability experiments demonstrated efficient cellular internalization without additional chemistry, drug release inside cells with restoration of the HDAC inhibition and induction of apoptosis. Using combination of decitabine and our HDAC inhibitors functional NPs we were able to eradicate mesothelioma cancer cells in vivo.

Anti-tumor effect of new benzofuranone HDACi on a murine model of mesothelioma.

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Since several years, epigenetic drugs such as Histone deacetylase inhibitors (HDACi) appear as promising agents to treat cancers as shown on a large number of malignant cells. However, HDACi are poorly specific, display important toxicities many

have very low half-lives in the plasma. Moreover, clinical trials showed a poor efficiency on solid tumors. To increase the therapeutic potential of HDACi, the development of new compounds is needed. We previously described a new HDACi, named NODH, with improved pharmacological and anti-tumor properties in vitro. In this work, we characterized the antitumor properties of this HDACi, compared to the FDA approved HDACi SAHA, in a murine model of mesothelioma. Results obtained demonstrated a strong antitumor activity of NODH compared to SAHA while a lower dose of NODH was used. No toxicity was observed and according to immunochemistry, the NODH anti-tumor property is correlated to a decrease of Ki67 and VEGF staining. This anti-tumor effect was also associated with an increase of histone H3 acetylation and activated caspase-3 staining in tumor tissues. These results suggest that NODH could be a promising candidate for mesothelioma therapy.

Is *MGMT* methylation a good prognostic factor in *IDH* wild-type high grade glioma?

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Recent genome-wide studies have been developed to support histologic classification of gliomas and different molecular abnormalities have been linked to tumor grading and disease progression. We investigated the quantitative *MGMT* promoter methylation, *MGMT* loss of heterozygosity (LOH) and *IDH1*/*IDH2* mutations in tumor specimens from 132 patients with high grade gliomas (HGGs) and in 32 with low grade gliomas (LGGs) to explore: 1) the correlations with grading and among biomarkers, 2) the biomarkers prognostic value. Results:-Pyrosequencing analysis of *MGMT* promoter showed that methylation levels decreased from low to high grade tumors ($p<0.005$). -*IDHs* mutations were associated with LGGs ($P<0.0001$), while *MGMT* LOH was more frequent in HGGs ($p<0.005$). As previously reported, *MGMT* methylation (methylation level ≥9%) correlated with *IDHs* mutation ($P<0.0001$). -*MGMT* LOH significantly correlated with *IDHs* wild type status ($P<0.0001$), but it did not show any correlation with *MGMT* methylation status. -Kaplan-Meier analysis in HGGs confirmed that *MGMT* methylation level ≥9% and *IDH* mutations were associated with a better prognosis. *MGMT* LOH did not affect survival. -Focusing on *IDHs* wild type tumors, no significant survival difference was present between patients with methylated and unmethylated *MGMT* tumors. Conclusions: *MGMT* LOH probably does not mimic the epigenetic silencing of the gene and it is not a prognostic factor. Interestingly, in our cohort *IDHs* status emerged as the primary marker of good prognosis due to longer survival and it is independent from *MGMT* promoter methylation.

Specific transcripts of *DNMT3A* are important for differentiation of hematopoietic stem and progenitor cells

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DNA-methyltransferase 3A (DNMT3A) is a *de novo* DNA-methyltransferase that is alternatively spliced in a tissue- and disease-specific manner, but little is known about the function of its transcripts. *DNMT3A* is frequently mutated in acute myeloid leukemia (AML) and a recent study from our group indicated this mutation can be mimicked by epigenetic dysregulation within the *DNMT3A* sequence: about 40% of AML samples revealed aberrant hypermethylation at this region and this was associated with shorter overall survival. In analogy to *DNMT3A* mutations, this "epimutation" seems to interfere with normal expression of *DNMT3A* transcripts. In this study we aim to elucidate the functional role of individual *DNMT3A* splice variants in the development of AML. Single *DNMT3A* transcripts were knocked down by lentiviral expression of short-hairpin RNAs in cord blood derived hematopoietic stem and progenitor cells (HSPCs). Knockdown efficiency of individual transcripts was validated by qRT-PCR. Notably, the knockdown of transcript 2 caused an upregulation of transcripts 1+3 and transcript 4. We also examined the potential of colony formation with a CFU-assay. The overall number of colonies formed in the CFU-assay was significantly decreased in HSPCs with downregulated transcripts 1+3 compared to control HSPCs. Downregulation of transcript 4 leads to a bias towards erythroid progenitors. Subsequently, we analyzed the proliferation and immunophenotype of infected HSPCs. Interestingly, HSPCs with downregulation of transcript 2 and 4 tend to proliferate slower whereas HSPCs with downregulation of transcript 2 maintain higher CD34 expression for more cell Divs. Our results indicate that the different *DNMT3A* transcripts, especially transcript 2, have specific regulatory functions in HSPCs that may be relevant for disease progression in AML patients, which carry either a mutation or epimutation in *DNMT3A*.

Curcumin Modulates 5-FU-mediated Regulation of Epithelial-to-Mesenchymal Transition and Cancer Stem Cells in Tumor Microenvironment Co-Cultures

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The tumor microenvironment is essential for up-keeping and promoting tumor cell proliferation, invasion and metastasis, which are all important factors for tumor malignity. In a 3D-co-culture model we investigated the crosstalk between colorectal cancer (CRC) cells with stromal fibroblasts (MRC-5) and the anti-cancer effects of curcumin or/and 5-Fluorouracil (5-FU), especially on epithelial-to-mesenchymal transition and cancer stem cell survival. Methods: High density tumor microenvironment mono-cultures of CRC cells HCT116 or co-cultures of HCT116 and MRC-5 were cultured with/without curcumin or/and 5-FU. Results: In high density tumor microenvironment co-cultures, synergistic cross talk between HCT116 and stromal fibroblasts, markedly increased tumor-promoting factors (NF-kB, MMP-13), TGF-β3, enhanced CSC survival (characterized by up-regulation of CD133, CD44, ALDH1) and up-regulation of EMT-factors (increased vimentin and Slug, decreased E-cadherin)

compared to HCT116 mono-cultures. These synergistic cross talk effects were even more pronounced in the presence of 5-FU, but decreased in the presence of curcumin or anti-TGF-β. Finally, curcumin induced biochemical changes to mesenchymal-epithelial transition (MET), thereby sensitizing HCT116 to 5-FU treatment. Conclusion: Activation of CSCs, EMT and tumor-promoting factors in tumor microenvironment co-cultures was mediated, at least in part through TGF-β. Modulation of this functional cooperation in co-culture by curcumin might be a potential therapy for CRC and suppress metastasis.

A novel quantitative plasma DNA biomarker panel for early diagnosis and prognosis of cervical cancer

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Cell-free plasma DNA have been proposed as a potential invasive diagnosis marker for various human diseases. However, little is known concerning the relationship between plasma DNA levels and grade/stage of cervical lesions. In this study, we investigated the levels of total plasma DNA and two methylated tumor suppressor genes levels in 118 women with cervical CIN1/2/3 or cancerous disease by using duplex real-time PCR with internal standards and quantitative methylation-specific PCR (qMSP). Results: Total plasma DNA levels were significantly increased in CIN2/3 patients (median, 24.7ng/ml) and cervical cancer patients (median, 58.1 ng/ml) compared with the control (median, 18.5 ng/ml) and CIN1 cases (median, 19.3 ng/ml) (P<0.01). Methylated *APC* and *RASSF1A* was detected in none of controls and in only two CIN1 cases. The frequency of plasma *APC*/*RASSF1A* methylation in stage II–IV cancers was 85.7%, significantly higher than in stage I cancer patients 44.4% and CIN2/3 patients 37.2% (P<0.05). Further ROC analysis demonstrated that a high total plasma DNA or methylated *APC*/*RASSF1A* level predicted an elevated risk of cervical cancer with an area under the ROC curve (AUC) value of 0.8606. The overall sensitivity and specificity for the combined use of total plasma DNA and *APC/RASSF1A* methylation markers in detecting cervical precancerous and cancerous disease were 79% and 80%, respectively. The *RASSF1A* methylation frequency was found to be higher in patients with lymph node metastasis (66.7%) than those without metastasis (27.6%). Conclusions: Quantitative detection of total plasma DNA and *APC*/*RASSF1A* methylation may be useful as a noninvasive biomarker panel for the early diagnosis and prognosis of cervical precancerous and cancerous disease.

Methylation of tumor suppressive microRNA in multiple myeloma

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Multiple myeloma (MM) is an incurable blood cancer arising from neoplastic proliferation of plasma cells. Clinically, MM may be preceded by asymptomatic monoclonal gammopathy of undetermined significance (MGUS), which progresses into symptomatic MM at a rate of 1% per year. Symptomatic MM is characterized by the presence of end-organ damages including hypercalcemia, Renal failure, Anemia, and osteolytic Bone lesions (CRAB), and hence a miserable disease. MicroRNA, of 19-25 nucleotides in length, is a form of small non-coding RNA, which leads to repression of its target protein-coding genes by sequence-specific binding to their 3'UTR. Based on the genes they repress, microRNA can be oncogenic or tumor suppressive. DNA methylation refers to the addition of a CH3 group to the 5th carbon position of a cytosine ring, resulting in formation of methylcytosine. Aberrant promoter DNA methylation results in a compact chromatin configuration and hence gene silencing, is an alternative mode of gene inactivation. Therefore, tumor suppressive microRNA may be inactivated by DNA methylation in addition to gene mutation/deletion, fulfilling the Knudson's hypothesis. Herein, we describe the role of aberrant DNA methylation if tumor suppressive microRNA in the pathogenesis or progression of MM.

Correlation between gene-specific methylation in blood DNA and risk factors for late-onset Alzheimer's disease

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A growing body of evidence suggests that changes in DNA methylation levels are likely to be involved in the pathogenesis of Alzheimer's disease (AD). Therefore, there is increasing interest in searching for peripheral epigenetic biomarkers of the disease that could help to clarify the contribution of physiological, genetic, nutritional and environmental factors to the observed changes in DNA methylation levels. In this regard, we collected blood DNA samples from 120 late-onset AD patients and 115 healthy matched controls and analysed the methylation levels of CpG islands in the promoter/5'-untraslated region (UTR) of genes involved in amyloid-beta peptide production (*PSEN1* and *BACE1*), in DNA methylation reactions (*DNMT1*, *DNMT3A* and *DNMT3B*), and in one-carbon metabolism (*MTHFR*), searching for correlation with increasing age, homocysteine levels, B-group vitamins availability, and the apolipoprotein E (*APOE*) ε4 allele, all known or suspected risk factors for late-onset AD. Increasing age correlated with *BACE1* methylation levels, and was inversely correlated with both *DNMT1* and *MTHFR* methylation levels. Inverse correlation between plasma homocysteine and both *MTHFR* and *PSEN1* methylation levels was observed. Positive correlation was observed between serum folate levels and *MTHFR* methylation, and between serum vitamin B12 levels and both *BACE1* and *DNMT1* methylation. *DNMT1* methylation levels were significantly lower in *APOE* ε4 carriers than in *APOE* ε4 non-carriers. The study revealed that age, biomarkers of one-carbon metabolism, and the *APOE* ε4 allele, correlate with gene-specific methylation levels in blood DNA of AD and healthy matched individuals.

MicroRNAs as predictive markers for chemoresistance in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) remains a highly chemoresistant tumor entity for which no reliable predictors of susceptibility to adjuvant first-line chemotherapy with gemcitabine exist. Recently, we identified a panel of microRNAs associated with induced gemcitabine chemoresistance in human PDAC cell lines. In this clinical study we evaluated whether expression of these microRNAs and other molecular markers can predict outcome in resected PDAC patients treated with adjuvant gemcitabine chemotherapy. Methods: Specimens from 98 PDAC patients UICC Stage II with curative pancreatic resection between 2004 and 2012 were investigated using 13 benign, non-inflammatory pancreatic specimens as control. A panel of 10 significantly dysregulated microRNAs in chemoresistant PDAC cell lines was investigated by qRT-PCR. Phosphatase-and-tensin-homolog (PTEN), deoxycytidine kinase (DCK), Hu-antigen-R (HuR), multidrug resistance protein (MRP-1), multidrug resistance P-glycoprotein (MDR-1), breast cancer resistance protein (BCRP), and vascular epithelial growth factor (VEGF-1) expression was analyzed by tissue microarray. Expression data were correlated with clinicopathologic and survival data by uni- und multivariate analyses. Values for $p<0.05$ were considered to be statistically significant. Results: PDAC patients UICC Stage II with high expression of microRNA-21 and three further microRNAs had a significantly shorter overall survival and recurrence-free survival in the adjuvant setting ($p<0.05$). Low expression of PTEN correlated significantly with worse overall survival ($p=0.04$) and recurrence-free survival ($p=0.02$). Tumor stage, size, grading, and adjuvant gemcitabine treatment were correlated with clinical outcome in PDAC patients ($p<0.05$). Conclusion: 4 microRNAs associated with reduced chemotherapy response in PDAC patients were identified, 3 of which are new players in this setting. Further prospective multicenter studies are required to evaluate whether these microRNAs are suitable predictive markers or therapeutic targets in PDAC patients scheduled for adjuvant gemcitabine treatment.

First outcomes of the European Network of Imprinting Disorders – EUCID.net: Common nomenclatures and first steps towards harmonized diagnostic procedures

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Imprinting disorders (IDs) are a group of rare congenital diseases affecting growth, development and metabolism with a life-long impact on patients´ quality of life. Efforts to elucidate the aetiology of IDs have been fragmented across Europe and standardisation of diagnostic and clinical management was lacking. In 2013, our Action, supported by the European COST programme (BM1208), has drawn together researchers, clinicians, SMEs and patients organisations of the known human IDs in an interdisciplinary pan-European network for Human Congenital IDs (EUCID.net), working to advance understanding of the pathophysiology with the major aim of translating this knowledge to improvement of diagnostic and clinical management for the benefit of the patients and their families. The ID network currently consists of 47 groups from 22 countries. After 1.5 years, we are able to report on the first outcomes of our cooperation: We suggest a common nomenclature of IDs (e.g. Temple syndrome for the formerly upd(14)mat syndrome) and overlapping molecular findings (e.g. multilocus imprinting disturbances). A standardised nomenclature for imprinted loci, CpGs and aberrant methylation patterns is currently in progress in cooperation with HGV. For a harmonized patient recording, a common HPO based questionnaire has been developed. Our Action initiated a EMQN quality assessment scheme for Silver-Russell and Beckwith-Wiedemann syndrome, leading to the creation of standard operation procedures. The first consensus on diagnosis and therapy of Silver-Russell syndromes is expected for October 2015, consensus papers for Beckwith-Wiedemann syndrome and pseudohypoparathyroidism Ib will follow in 2016 and 2017. The network decided to use LOVD as a common database to make the data compatible and useful as a springboard for collective research initiatives. After more than a year, the active networking between the groups is documented by a growing number of joint publications, a mutual staff exchange by short term scientific missions, and stimulating training schools. All relevant outcomes are available on the website of EUCID.net (www.imprinting-disorders.eu), and we encourage all colleagues working on imprinting disorders to contact us and to contribute their knowledge and experiences to our Action.

nter-locus as well as intra-locus heterogeneity in LINE-1 promoter methylation in common human cancers suggests selective demethylation pressure at specific CpGs

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Hypomethylation of LINE-1 has been observed in tumorigenesis when using degenerate assays, which provide an average across all repeats. However, it is unknown whether individual LINE-1 loci or different CpGs within one specific LINE-1 pro-

moter are equally affected by methylation changes. Conceivably, studying methylation changes at specific LINE-1s may be more informative than global assays for cancer diagnostics. Therefore, with the aim of mapping methylation at individual LINE-1 loci at single-CpG resolution and exploring the diagnostic potential of individual LINE-1 locus methylation, we analyzed methylation at 11 loci by pyrosequencing, next generation bisulfite sequencing as well as global LINE-1 methylation in bladder, colon, pancreas, prostate and stomach cancers compared to paired normal tissues and in blood samples from some of the patients compared to healthy donors. Results: Most (72/80) tumor samples harbored significant methylation changes at at least one locus. Notably, our data revealed not only the expected hypomethylation, but also hypermethylation at some loci. Specific CpGs within the LINE-1 consensus sequence appeared preferentially hypomethylated suggesting that these could act as seeds for hypomethylation. *In silico* analysis revealed that these CpG sites more likely faced the histones in the nucleosome. Multivariate logistic regression analysis did not reveal a significant clinical advantage of locus-specific methylation markers over global methylation markers in distinguishing tumors from normal tissues. Conclusions: Methylation changes at individual LINE-1 loci are heterogeneous, whereas specific CpGs within the consensus sequence appear to be more prone to hypomethylation. With a broader selection of loci, locus-specific LINE-1 methylation could become a tool for tumor detection.

DNA methylation in breast cancer:
impact on classification, progression, gene expression, treatment response and prognosis

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Breast cancer is a heterogeneous disease where different subgroups have different clinical characteristics and outcomes. In the early 2000s, breast cancer was divided in five subtypes based gene expression profiles – Luminal A, Luminal B, Her2 enriched, Basal-like and Normal-like. More recently, genome-wide DNA methylation profiles have been determined for breast cancer tumors, which has allowed for subgrouping also based on DNA methylation. Luminal A tumors, a group of tumors that are not easily further subdivided, but where the patients still have varying clinical outcome, could be subdivided using DNA methylation. This subclassification has potential clinical application. Genome-wide DNA methylation analysis of normal breast tissue, ductal carcinoma *in situ* (DCIS) and invasive carcinoma showed that most aberrations in DNA methylation happened between normal and DCIS, that is, early in breast cancer development. More than 5000 genes were differentially methylated between normal tissue and DCIS. In breast cancer, gene expression of about 3000 genes was associated to DNA methylation level of CpGs in or around the genes, further underscoring the importance of DNA methylation in breast tumors. Our group has developed a DNA methylation-based prognostic signature using CpGs where methylation level was associated to gene expression. The prognostic value of this signature was validated in a large publicly available patient cohort. DNA methylation may also play a role in acquired drug resistance. The cell cycle regulators *CDKN2A*, *CCND2* and *CCNA1* were differentially methylated after treatment with doxorubicin and a combination therapy of 5-fluorouracil and mitomycin C. In addition, methylation level of these genes was differentially methylated depending on response to the treatment.

Measuring methylation in archival Bone Marrow smears: exploring the limits

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Every year, 1% of monoclonal gammopathy of undetermined significance (MGUS) patients will develop the bone marrow cancer Multiple Myeloma (MM). Currently, no reliable biomarkers can predict which MGUS patients will transform to MM and which will not. The past decade highlighted the role of epigenetic processes such as DNA methylation in cancer development. Therefore, we aim to identify a panel of epigenetic biomarkers that can predict MM transformation. We will use a historical collection of serial bone marrow smears and cell pellets from the University Biobank Limburg, which contains samples of MGUS patients that have remained MGUS or transformed to MM over time. As the smear collection covers a longer follow up period, it is preferred for biomarker identification. However, the application of these smears for DNA methylation markers has not yet been investigated. To this end, we extracted DNA from smears and pellets, and evaluated DNA yield and integrity. We also assessed different bisulfite conversion conditions and subsequently performed methylation PCR to determine the best condition for biomarker identification. DNA of fresh smears and pellets was of high yield (in microgram range) and integrity. Although 20-year-old smears gave high DNA yields, the DNA was degraded. Subsequently, we tested different bisulfite conversion protocols using input concentrations of 5 to 100ng non-degraded DNA from smears and pellets. The bisulfite converted DNA could be successfully used for methylation PCR, indicating that there were no inhibitions due to sample type, staining or bisulfite conversion protocols. Additionally, no difference between smears and pellets was detected. However, 5ng of DNA proved too little to obtain a reproducibly reliable result. Currently, we are evaluating the effect of DNA degradation on methylation content. In summary, these preliminary data suggest that archival bone marrow smears can be used for epigenetic research.

Epi-genotoxic effect of TiO_2-np exposure in 16-HBE cells

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With the increase in industrial and clinical use of titanium dioxide nanoparticles (TiO_2-np), a better understanding of their safety is important. In the present study the effects of TiO_2-np were studied using a battery of cyto-genotoxicity and DNA (de-)methylation assays in 16-HBE (human bronchial epithelial) cells. Cytotoxic response was observed at a concentration of

25µg/ml. Results of comet assays revealed significant genotoxic effect of the particle at 12.5µg/ml and higher. Increase in number micronucleus was observed as a function of dose and time. Flow cytometry analysis revealed arrest of cells in the S-phase of cell cycle. Effect on global DNA methylation and hydroxymethylation levels were studied using LC-MS/MS analysis. Though no changes were observed for 3h treatment schedule, significant hypomethylation was observed at 24h. An inverse correlation between levels of 5-Methyl-2′-deoxycytidine and 2'-Deoxy-5-(hydroxymethyl)-cytidine confirmed demethylation induced by TiO_2-np. In conclusion, apart from cyto-genotoxic response TiO_2-np induced DNA methylation changes in 16-HBE cells. Hence, it can be recommended that epigenetic studies should be performed along with conventional toxicity testing methods for complete characterization of nanoparticle toxicity.

Hypomethylation of LINE-1 in placental tissue of preeclampsia-complicated pregnancies

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Preeclampsia (PE) is a common pregnancy complication that occurs in 2-5% of all pregnancies. However, the pathogenesis is largely unknown. Recent studies suggest involvement of DNA methylation as underlying mechanism. We hypothesize that global DNA methylation is lower in placental tissue of PE complicated pregnancies compared to control pregnancies. *Material and methods* Tissue (1 cm2) was obtained from the fetal side of the placenta from two cohorts. The discovery cohort consisted of 16 PE patients and 19 controls. The replication cohort consisted of 23 PE patients and 25 controls. DNA was isolated from placental tissue and treated with bisulfite. Global DNA methylation was quantified by LINE-1 by Sequenom Epityper. Logistic regression was performed to assess whether LINE-1 methylation was lower in tissue from PE-complicated pregnancies compared to tissue from controls pregnancies. Analysis was corrected for small for gestational age and cohort differences. *Results* Within the LINE-1 amplicon 4 CpG sites were quantified (i.e. CpG sites 1,2,3 and 5), of which CpG site 1 and 5 tended to be lower in placental tissue of PE complicated pregnancies of the discovery cohort ($P<0.20$). In the replication cohort LINE-1 CpG site 2 tended to be lower ($P=0.13$). Combining both cohorts to increase statistical power showed significantly lower LINE-1 methylation of CpG sites 2 and 5 in placental tissue of PE complicated pregnancies (compared to control pregnancies ($P<0.05$). CpG3 tended to be lower in the combined cohort as well ($P=0.12$). *Conclusion* This study shows that LINE-1 DNA is hypomethylated in placental tissue of PE patients, which underlines LINE-1 DNA methylation status as future promising new biomarker.

Embryonic Stem Cell (ES)-Specific Enhancers Specify the Expression Potential of ES Genes in Cancer

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Cancers often display gene expression profiles resembling those of undifferentiated cells. The mechanisms controlling these expression programs have yet to be identified. Exploring transcriptional enhancers throughout hematopoietic cell development and derived cancers, we found that ES-specific enhancers (ESSEs) are essentially prone to DNA methylation modifications, indicative of their chromatin activity states. Strikingly, ESSE methylation predicts gene transcriptional activity in cancer: methylated ESSEs are hypermethylated in cancer relative to normal somatic cells and associated with silenced genes, whereas unmethylated ESSEs are hypomethylated in cancer and associated with reactivated genes. Constant, or hematopoietic stem cell-specific enhancers do not show these trends, suggesting selective reactivation of ESSEs in cancer. Further analyses of a hypomethylated ESSE downstream to the VEGFA gene revealed a novel regulatory circuit implying VEGFA transcript levels across cancers and patients. We suggest that ESSEs provide a framework for reactivation of ES genes in cancer.

Transcriptome pro-oncogenic signature in human mammary epithelial cells infected with human cytomegalovirus.

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Breast cancer is responsible for the majority of the cancer associated death among women in the world. Several chemicals and biological entities have been postulated to trigger cancer including viruses. Human cytomegalovirus (HCMV) is a member of Herpesviridae family, subfamily Betaherpesvirinae. Although the presence of HCMV in human is often unnoticed however severe complications have been observed in immunocompromised and immunosuppressed patients. In the last decade, the presence of HCMV has been reported in several kinds of cancers including breast cancer. Human mammary epithelial cells (HMECs) are present in the mammary gland ducts and could be targeted by HCMV present in the milk. In this preliminary study we investigated the potential of HCMV in triggering abnormal epigenetic and genetic signatures that could potentially lead to cancer. We found the presence of the viral ULb' region responsible for governing viral latency and cell tropism in the HCMV-DB clinical isolate, but not in laboratory strains tested such as AD169 and TB40F. We infected HMECs individually with HCMV-DB, AD169 and TB40F and 24 h post infection we conducted a PCR array to determine the gene profile altered by HCMV in HMECs and compared them with uninfected HMECs and breast cancer cell lines. Our results indicate that HCMV, especially HCMV-DB, was able to alter the cellular transcriptome with significant similarity to the transcriptome profile of breast cancer cell lines. We will further study the role of ULb' region in inducing such changes in infected HMECs. In addition, epigenetics signatures altered by HCMV in HMECs will be further assessed.

Epigenome Deregulation in Cancer: Searching for Drivers and Passengers on the Road to Malignancy

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The field of epigenetics has witnessed a recent explosion in our knowledge regarding the importance of epigenetic mechanisms

in normal cellular processes and abnormal events associated with cancer development. The challenge posed by major international sequencing efforts, is to identify changes in the genome and epigenome that precede and promote tumour development, and to differentiate functionally important ("drivers") from non-functional *"passenger"* events. In addition, there is little understanding about whether epigenetic changes in cancer and surrogate tissues can be used as biomarkers for risk stratification, exposure assessment, early detection and an intermediate biomarker for different health outcomes. The spectacular advances in epigenomics and the emergence of powerful technologies that allow the analysis of the epigenome with unprecedented resolution in both high throughput and genome-wide settings have dramatically accelerated investigations in this area. I will discuss how these advances opens the exciting possibility of identifying changes affecting the (epi)genome in not only cancer but also in surrogate tissues thus improving the knowledge of epigenetic mechanisms in cancer development, providing clues to causation (etiology), and identifying new generation of epigenetics-based biomarkers.

Nucleosomics®- translating epigenetic bio markers into clinical diagnostics

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Background: Nucleosomics® combines cutting edge epigenetic profiling with a simple low cost immunoassay technology to improve clinical diagnosis of cancer. Immunohistochemistry studies show genome-wide epigenetic changes in cancer tissue and have identified histo-oncoproteins - histone modifications linked to cancer. In addition, nucleosomes -147 base pair DNA sequences wrapped around four pairs of histone proteins - are released as chromatin fragments on cell death. Total levels of cell free nucleosomes can be elevated by inflammation, infectious disease and cancer limiting diagnostic utility. However, circulating cell-free, nucleosome bound DNA fragments contain mutations found in cancer tissue from the same patients suggesting a tumor chromatin origin for, at least some, circulating nucleosomes. Profiling of global levels of epigenetic modifications in nucleosomes can provide disease specific diagnostic information. We have developed global (as opposed to gene specific) epigenetic profiling of cell free nucleosomes as a potentially powerful clinical platform for accessible, affordable and accurate diagnosis of a range of cancers. We present preliminary data from a major validation trial in colorectal cancer together with a series of pilot studies in prostate, pancreatic and lung cancer patients vs. benign disease and healthy controls. Methodology: Specific nucleosome associated histone modifications, histone variants and DNA modification levels in circulating nucleosomes were evaluated in patient and control serum by ELISA (NuQ®). Assay combinations with optimal discriminations were identified using a linear discrimination algorithm. Results: Data from the first phase of a large scale clinical trial have demonstrated test performance that exceeds both traditional stool testing and blood based methylated Septin 9 in detection of colorectal cancer (84% sensitivity at 78% specificity) as well as polyps (60% sensitivity). This was achieved with just 3 individual NuQ® assays. Pilot results show equivalent levels of performance for sensitivity and specificity in Prostate cancer (80%,70%), Lung cancer (77%/92%) and Pancreatic cancer (84/%/92%).

Urinary miRNAs for early detection of prostate cancer

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Prostate cancer (PCa) is the most prevalent male cancer and the third leading cause of cancer related deaths in European men with especially high mortality rates observed in Lithuania. There is an urgent need for new diagnostic biomarkers with a potential to predict disease progression at early stages. In present study, diagnostic and prognostic potential of urinary miRNAs was evaluated in urine specimens collected from 238 PCa patients and benign controls (BPH). Initial miRNA profiling with TaqMan Low Density Array (TLDA) miRNA cards was performed on 42 PCas and 12 non-cancerous prostate tissues (NPTs). Over 100 miRNAs were found deregulated in PCa as compared to NPT. Expression of 43 miRNAs differentiated the cases with biochemical diseases progression (BCR) from the non-progressing cases. Also, the miRNA profile of *TMPRSS2-ERG* fusion-positive PCas was identified. Based on the levels of expression and the most significant associations with clinicopathological variables, 18 miRNAs were selected for further validation on custom-designed array. After validation step, the expression of 8 selected miRNAs was further evaluated in urine from two independent PCa cohorts. Deregulated expression ($p<0.05$) of urinary miR-148a, -19b, -21, -365, -375 and -429 was detected at least in one cohort after comparing PCas to BPHs. ROC analysis revealed high sensitivity and specificity of urinary miR-148 and miR-375, and the diagnostic value of combined analysis of these urinary miRNAs (AUC=0.92) markedly exceeded the same value of the PSA test (AUC=0.51). In addition, miR-375 was over-expressed in urine of *TMPRSS2-ERG* fusion-positive cases ($p=0.038$) of one cohort, but none of analysed miRNAs was predictive for BCR. In conclusion, urinary miRNAs can serve as a sensitive and specific diagnostic biomarker of PCa, while the prognostic significance these biomarkers needs further investigations.

Functional effects of somatic tumor mutations in epigenetic enzymes

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The rapid development of DNA sequencing techniques has provided many important new insights into cancer biology, among them the identification of many somatic mutations in cancer tissues. Interestingly, many of the affected genes mediate epigenetic modifications either directly or indirectly, like IHD, Dnmt3a, TET2 or EZH2 which are hotspots of somatic cancer mutations. Many of the somatic cancer mutations in DNA Methyltransferases are likely to have a gain-of-function effect, because they occur heterozygously and the mutational spectrum shows many missense mutations but only few insertion, deletions or nonsense mutations. Gain-of-function mutations in Dnmts could activate the enzymatic activity, change its flanking sequence preferences, sub-nuclear localization or compromise its regulation and by one of these mechanisms cause hypermethylation of select loci in cancer cells. I will report on our new data on the mechanism of the Dnmt3a R882 mutations which are very prev-

alent in cancer samples. Moreover, I will present our data on the effect of somatic cancer mutations in different histone lysine methyltransferases including MLL3 and MLL1, which also show gain of function effects.

Epigenetic Drug Discovery

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In this talk a short overview over recent entries of epigenetic inhibitors into clinical trials will be given. Then as a case study from our research new inhibitors of histone acetyltransferases with anticancer activity in a mouse xenograft is presented. We have previously described novel histone acetyltransferase (HAT) inhibitors that block neuroblastoma cell growth in vitro. Here we show that two selected pyridoisothiazolone inhibitors, PU139 and PU141, induce cellular histone hypoacetylation and inhibit growth of several neoplastic cell lines originating from different tissues (e.g. colon, leukemia). Broader in vitro selectivity profiling shows that PU139 blocks the HATs Gcn5, PCAF, CBP and p300 while PU141 is selective towards CBP and p300. The pan-inhibitor triggers caspase-independent cell death in cell culture. Both inhibitors block growth of SK-N-SH neuroblastoma xenografts in mice and the pan-inhibitor PU139 was shown to synergize with doxorubicin in vivo. This inhibitor also reduces histone lysine acetylation at concentrations similar to those inhibiting neoplastic xenograft growth. This is one of the very few reports on hypoacetylating agents with in vivo anticancer activity.

Epigenetic inactivation of the novel candidate tumor suppressor gene *ITIH5* in colon cancer predicts unfavorable overall survival in the CpG island methylator phenotype

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Background: Inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5) is supposed to be involved in extracellular matrix stability and thus may play a key role in the inhibition of tumor progression. The current study is the first to analyze in depth ITIH5 expression as well as its potential clinical and functional impact in colon cancer. Methods: Based on 30 tumor and 30 adjacent normal tissues we examined *ITIH5* mRNA expression and promoter methylation whose significance was further validated by independent data sets from The Cancer Genome Atlas (TCGA) platform. In addition, ITIH5 protein expression was evaluated using immunohistochemistry. Results: *ITIH5* mRNA expression loss was significantly associated (P<0.001) with hypermethylation of the *ITIH5* promoter in primary colon tumors. Furthermore, treatment of tumor cell lines with demethylating (DAC) and histone acetylating (TSA) agents induced *ITIH5* expression. In line independent TCGA data revealed a significant expression loss of *ITIH5* particularly in the MSI-high and CIMP-positive phenotype concordant with an increased *ITIH5* hypermethylation in CIMP-positive colon tumors (P<0.001). In proximal, i.e. right-sided tumors, abundant *ITIH5* expression was associated with longer overall survival (OS, P=0.049) and the CIMP-positive (P=0.032) subgroup. Functionally, ITIH5 re-expression mediated a reduced proliferation in HCT116 and CaCo2 cells. Conclusions: Our results indicate that *ITIH5* is a novel putative tumor suppressor gene in colon cancer with a potential impact in the CIMP-related pathway. *ITIH5* may serve as a novel epigenetic-based diagnostic biomarker with further clinical impact for risk stratification of CIMP-positive colon cancer patients.

The effects of long-term daily folic acid and vitamin B12 supplementation on genome-wide DNA methylation in elderly subjects

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Folate, and its synthetic form folic acid, function as donor of one-carbon units and have been, together with other B-vitamins, implicated in regulation of DNA methylation. The aim of this project was to identify effects of long-term supplementation with folic acid and vitamin B12 on genome-wide DNA methylation in elderly subjects. This project was part of a randomized, placebo-controlled trial on effects of supplemental intake of folic acid/vitamin B12 on fracture incidence among 2919 elderly subjects (B-PROOF study). Participants with mildly elevated homocysteine levels, aged 65-75 years, were randomly assigned to take 400 µg folic acid and 500 µg vitamin B12 per day or a placebo during an intervention period of two years. All participants received 15 µg vitamin D. Blood was collected at baseline and after two years of intervention and DNA was isolated from buffy coats. Genome-wide DNA methylation at baseline and after two years of intervention was determined in 87 participants (n=44 folic acid/vitamin B12, n=43 placebo) using the Infinium HumanMethylation450 BeadChip. After intervention with folic acid/vitamin B12, 106 (versus 9 in the placebo group) of the 431,312 probes were differentially methylated as compared to baseline (Benjamini-Hocherg adjusted p-value<0.05). Overall, identification of differentially methylated regions consisting of multiple probes revealed that 8 regions (consisting of 2-12 consecutive probes) differed between the intervention and the placebo group with most pronounced changes for regions in the *DIRAS3* and *ALX1* gene. For probes in the differentially methylated regions, the average changes in DNA methylation after intervention with folic acid/vitamin B12 were small and ranged from <1%-2.5%. Furthermore, a number of probes were identified for which DNA methylation after intervention tend to be associated with changes in serum folate levels of the participants. In conclusion, long-term supplementation with folic acid/vitamin B12 resulted in significant effects on DNA methylation in elderly subjects.

Influence of PRMT6 on hematopoiesis and evaluation as a therapeutic target

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The interplay between transcription factors and epigenetic cofactors is important for hematopoietic gene expression control and decisive for lineage specification. We have identified the protein arginine methyl transferase 6 (PRMT6) as an important corepressor, which is associated with the central hematopoietic transcription factor RUNX1. Furthermore, our data suggest that PRMT6 is involved in differentiation at the megakaryocytic/erythroid bifurcation and in growth control. In this project, we are studying the function of PRMT6 in hematopoietic differentiation and its molecular function at target genes. Moreover, we are evaluating PRMT6 as a molecular target for cancer therapy. To determine if PRMT6 contributes to leukemia or influences differentiation, we have established a colony formation assay using primary human CD34+ cells and a PRMT6 transplantation mouse model. First results hint towards a function of PRMT6 in megakaryocytic/erythroid differentiation. We also found that knock down of PRMT6 inhibits cell proliferation in vitro and tumor growth in a Nod/Scid xenograft-model. In order to connect these studies to human leukemia we are currently analyzing the status of PRMT6 protein expression in patient samples by immunostaining. Here, we are investigating if PRMT6 expression is altered in acute myeloid leukemia. Additionally, genome-wide target gene identification of PRMT6 will be performed using a combination of ChIP-seq, expression-arrays and RNA-seq experiments. Because knock down of PRMT6 inhibits cell growth, inhibition of PRMT6 represents a potential strategy for an epigenetic therapy, similar to HDAC inhibitor treatment. Thus, we are currently testing if novel small molecule inhibitors of PRMT6 influence cell growth, gene expression and epigenetics.

DNA methylation at nonCpG sites and outside promoter regions are associated with clinical features of neuroblastoma

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Neuroblastoma, the most frequently occurring solid pediatric tumor, accounts for 15% of cancerrelated deaths in childhood. Probability of cure varies greatly according to patient's age, extent of disease and tumor biology. However, the etiology of this developmental tumor is unknown, and long-term survival for high-risk patients is still less than 40%. Given the low mutation rates reported for neuroblastoma, it has become clear that understanding the underlying epigenetic mechanisms will be essential to understand the molecular pathogenesis of this tumor and improve patient outcome. Historically, DNA methylation changes in neuroblastoma have been described greatly in CG islands and promoter regions. We have analyzed the DNA methylome of neuroblastoma using high –density microarrays. Our results reveal that DNA methylation changes in ne uroblastoma affect not only promoters but also intragenic and intergenic regions at both CpG and non -CpG sites . These epigenetic changes showed a non-random distribution relative to functional chromatin domains, and targeted development and cancer -related genes. Furthermore, methylation changes at these epigenetic marks were associated with clinicopathological features of this pediatric tumor, thus suggesting an important role for non-CpG and non-promoter methylation. Our study provides new insights into t he molecular basis of neuroblastoma and reveals DNA methylation changes that may have functional and clinical implications in the pathogenesis of this tumor and, potentially, of other developmental cancers. Thereby contributing to the identification of specific targets against which new therapeutic strategies can be designed.

The CpG island methylator phenotype in breast cancer is associated with the lobular subtype

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Background: Aberrations in DNA methylation patterns are well-described in human malignancies. The concordant hypermethylation of many genomic regions, termed CpG island methylator phenotype (CIMP), was associated for various malignancies with tumor subgroups harboring distinct clinical and genetic characteristics. Aim: However, the existence of CIMP as well as its relation to clinical, morphological, and genetic features is still controversial, especially in breast cancer. Therefore, this study investigated genome-wide DNA methylation aberrations in breast cancer subtypes. Material and Methods: Illumina´s HumanMethylation 450K BeadChip was used to analyze genome-wide DNA methylation patterns within various breast cancer subgroups and normal breast tissues. The study cohort comprised invasive lobular (n=10), sporadic invasive ductal (n=10), familial invasive ductal (n=8) breast carcinomas and four normal breast tissues. Chromosomal abnormalities were determined by array-based CGH. Results: Invasive lobular breast carcinomas exhibit the highest number of differentially methylated CpG

sites with the highest level of DNA methylation, whereas all familial invasive ductal carcinomas are most similar to normal breast tissue with regard to their DNA methylation profile. In relation to genetic alterations, a strong inverse correlation of aberrant DNA hypermethylation and copy number alterations was observed. Employing four analytical algorithms nine differentially methylated regions within seven genes (*DNM3*, *mir129-2*, *PGLYRP2*, *PRKCB*, *RGS7*, *SHF*, *TACC1)* discriminating the investigated subgroups were identified and validated in an independent validation cohort (n=117) by bisulfite pyrosequencing. In case of *DNM3* and *TACC1* elevated levels of DNA methylation could be correlated to diminished gene expression. Survival analysis showed better relapse free survival in patients expressing *DNM3*, *PRKCB*, *RGS7*, and *TACC1* as compared to patients not expressing any of these genes. Conclusion: These results depict a clear difference between primarily genetically and mainly epigenetically unstable breast carcinomas indicating different ways of tumor progression and/or initiation and providing new options for detection and therapy.

Diagnostic implications of DNA hydroxymethylation marker 5-hydroxymethylcytositne in melanoma

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Melanoma, an environmentally-induced cancer, is one of the few human malignancies with steadily increasing incidence worldwide, with more than 232,000 new cases and 55,000 deaths in 2012. Despite the deployment of therapies directed at specific genomic mutations in melanoma, the incidence and mortality rates from this deadly disease continue to increase - faster than that of any other potentially preventable cancer. Convincing data demonstrate that epigenetic events play an important role in melanoma pathogenesis. We have identified in human melanoma a global epigenetic defect, "loss of 5-hmC", a melanoma biomarker mediated by Ten-eleven translocase (TET) genes that (1) correlates with clinical melanoma virulence, (2) regulates melanomagenesis in pre-clinical models, and (3) upon reconstitution via TET2 pathway, inhibits melanoma growth (*Cell* 2012). Most importantly, we and other groups have shown that increasing morphologic atypia in dysplastic melanocytic nevi correlates with the progressive loss of 5-hmC levels, as detected by immunohistochemistry (*Modern Pathology,* 2014). We further assess the potential clinical applications of this epigenetic biomarker by immunohistochemistry to enhance diagnostic and microstaging accuracy in the histopathologic evaluation of pseudomaturing primary cutaneous melanoma and refine prognostic evaluations by enabling the distinction of metastatic melanoma from its diagnostic mimic, nodal nevus, in sentinel lymph node biopsies (*Modern Pathology,* 2015). Furthermore, our GWAS study identified a melanoma susceptibility locus and somatic mutation in TET2 (*Carcinogenesis*, 2014). In addition, clinically-applicable next-generation sequencing platforms enable us to confirm TET2 mutation and identify novel epigenetic regulator gene mutations in melanoma.

Aberrant DNA damage response-, epigenetic- and growth factor receptor signaling in lung cancer tumor initiating cells

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Increasing evidence suggest that tumor initiating cells (TICs), also called cancer stem cells (CSC), are partly responsible for resistance to DNA-damaging treatment. Using a model of sphere-forming non-small cell lung cancer (NSCLC) cells we previously showed that NSCLC TICs are refractory to ionizing radiation (IR) and DNA-damaging treatment e.g. cisplatin. Our profiling revealed that apoptotic signaling, cell cycle distribution and DNA double strand break (DSB) repair was impaired and/or altered in NSCLC TICs. By analyzing basal and IR-induced activation of 28 receptor tyrosine kinases (RTK) and 11 important signaling nodes in bulk cells and TICs we found that TICs displayed decreased basal phosphorylation of insulin-like growth factor 1 receptor (IGF-1R) and signal transducer and activator of transcription 1 (STAT1) (Tyr701). Moreover, phospho-ERK was increased in TICs and MEK inhibition decreased clonogenicity upon IR, suggesting that MEK and downstream signaling impart on TIC radiation response. It has been postulated that a heterochromatin structure may prevent formation of DNA-damage and constitute a barrier to DNA DSB repair. Here we set out to analyze heterochromatin structure in lung cancer bulk cells and TICs. Our results show that both NSCLC and small cell lung cancer (SCLC) TICs have elevated levels of the heterochromatin markers heterochromatin protein 1γ (HP1γ) and trimethylated lysine 9 of histone 3 (H3K9Me3). Accordingly, histone deacetylase inhibitors vorinostat, panobinostat and trichostatin A sensitized TICs to DNA damage inflicted by cisplatin in NSCLC TICs. In conclusion, we demonstrate that NSCLC TICs have altered DNA damage signaling response, different RTK activation and changed epigenetic signaling, which may cause their resistance to DNA-damaging treatment. These pathways may be targeted in combination with IR to increase responsiveness.

SERPINB5 as a prognostic and diagnostic marker for pancreatic ductal adenocarcinoma

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Background: Diagnosis and management of pancreatic ductal adenocarcinoma (PDAC) are challenging, especially in the setting of pancreatitis. SERPINB5 is abnormally expressed in pancreatic ductal adenocarcinoma. We investigated the gene's promoter methylation as a diagnostic marker and its protein expression as a prognostic marker for the disease. Methods: Patient tissue and blood samples were investigated for SERPINB5 promoter methylation by Methylation specific PCR (MSP): PDAC primary tumor (n=26), PDAC lymph node metastasis (n=20), pancreatitis (n=19). Further tissue samples of PDAC UICC stage II (n=91) and benign, non-inflammatory pancreatic disease (n=13) were investiged by tissue array. Kaplan-Meyer, Fisher's Exact test and Chi² test were used where appropriate. $P<0.05$ was considered significant. Results: In patient tissue samples, presence of an unmethylated SERPINB5 promoter differentiated pancreatitis from PDAC with a sensitivity of 57% and a spec-

ificity of 95% (P<0.001). Unmethylated SERPINB5 was not detected in blood samples. PDAC UICC Stage II patients with low tumor grading and adjuvant gemcitabine treatment had greater median survival (P=0.0001). SERPINB5 was significantly overexpressed in PDAC vs. benign tissue (P=0.026). In univariate analysis, low SERPINB5 expression was correlated to greater overall survival (P=0.0377) and recurrence free survival (P=0.0014). Conclusions: SERPINB5, initially known as a tumor suppressor, appears to assume an oncogenic role in PDAC. Its expression was linked to PDAC and low SERPINB5 expression was correlated to greater survival. In clinical samples, detection of unmethylated SERPINB5 was a specific marker for PDAC even in the context of pancreatitis and may - with improved detection methods - provide the basis for a liquid biopsy option to detect PDAC.

Identification of Genes Susceptible to Epigenetic Change in Response to Maternal Folate Supply in Acute Lymphoblastic Leukemia

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Altered folate metabolism and inadequate maternal folate intake may be associated with increased childhood acute lymphoblastic leukaemia (ALL) risk. Folate provides methyl groups for DNA methylation, the patterns of which are dramatically disrupted in ALL. Differences in maternal folate intake during pregnancy and/or altered folate metabolism may therefore affect DNA methylation, consequently influencing ALL risk. We investigated the potential aetiological role of maternal folate intake during pregnancy on ALL risk by identifying genes in which methylation changes occur both in response to folate levels and in ALL. We used previously generated DNA methylation array data from a mouse model of *in utero* folate depletion to identify genes in which methylation is altered in response to inadequate maternal folate intake: in total 591 genes showed altered DNA methylation. From the literature, we identified 2615 differentially methylated genes in ALL. We selected target genes to investigate DNA methylation from the overlap of these two gene lists. For 20 ALL patient samples, we quantified DNA methylation by pyrosequencing for 5 target genes (*ASCL2, HTRA1, KCNA1, SH3GL3, SRD5A2*), all of which were highly methylated in ALL samples. Methylation was then assessed for these 5 genes in a nested cohort of 148 cord blood samples from the North Cumbria Community Genetics Project and analysed in relation to maternal and infant red blood cell folate and vitamin B_{12} concentrations. Preliminary analysis suggests DNA methylation of some target genes appears to be related to maternal folate and B_{12} levels. These findings demonstrate that folate responsive changes in DNA methylation identified in animal studies can be used to determine relevant gene targets in human studies of diseases for which folate intake is an associated risk factor, and that DNA methylation may be one mechanism by which maternal folate intake (and related pathways) may influence ALL risk.

Potentials and Limitations of miRNomes in the Cancer Context

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Small non-coding RNAs play a key role in many physiological and pathological processes. Since 2004, miRNA sequences are catalogued in miRBase, which is currently in its 21st version that contains over 28,000 entries including over 2,500 different mature miRNAs for *Homo sapiens*. Others and we have analyzed the potential of blood-borne miRNAs as non-invasive disease biomarkers. In our previous studies we have shown diseases-specific miRNA expression profiles in whole peripheral blood from patients with various cancers including Wilms tumor, lung cancer, brain tumors, and others. Towards the use of miRNAs as biomarkers it is essential to understand the biological meaning of such miRNA signatures. As a first step it is essential to determine the type of blood cells that is significantly involved in cancer-specific miRNA expression patterns of whole blood samples. We analyzed the miRNA expression patterns of different blood cell subsets, including eosinophilic and neutrophilic granulocytes, monocytes, B-cells, T-cells, and natural killer cells and compared the respective expression patterns between cancer patients and healthy controls. To use miRNAs as biomarkers it is also essential to determine the influence of confounding factors that can be related to the patient (age /gender) or to the sample processing (storage conditions, platforms for data generation). We will discuss the relevance of these factors for disease specific signatures of circulating miRNAs.

New approaches for defining prognostic factors in acute myeloblastic leukemia: methylome profile of T inflitrating lymphocytes (TIL).

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There is now more and more evidence that the immune microenvironment plays a fundamental role in the outcome of leukemia. However, few studies have concentrated yet on a thorough investigation immune cellular environment as a potential independent prognostic factor in acute leukemia. In our work, we focus on the main aspects of epigenetics, which control the profile of T inflitrating lymphocytes **(**TIL) in acute myeloblastic leukemia (AML), mainly DNA methylation alterations. The comparison of methylation profile of T lymphocytes from AML patients at diagnosis to the healthy donors showed a significant differentially methylated CpG in both bone marrow and peripheral blood. Interestingly, our results analysis of the repartition of differentially methylated CpG depending to their relative position, to the CpG islands showed that approximately 80% of methylated CpG are concentrated inside of the promoter of different genes, suggesting that the polarization of T response to the disease is controlled by epigenetic mechanisms implicating the DNA methylation. Among these genes, some seem to play an important role in the response of T lymphocyte, such as, IL1RAPL2, IL21R, IL2RG, CD7, CD3e, CD28, CD40LG. These results showed that DMCpG localized also in the coding regions for some miRNAs such as, mir16, mir19, mir105, mir223, mir450, mir 500. The classification of patients on the basis of differentially methylated CpG to healthy donors showed the

existence of two groups of AML patients both in the bone marrow and blood. One of these groups presents a profile that is closer to the donor than the second group of patients. The comparison between the two groups of patients showed a distinct differentially methylated bone marrow T lymphocytes profile, suggesting the possibility of the existence of distinct polarisation of TILs in response to AML disease regulated by DNA methylation.

The Epigenetics of Mood Disorders

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Mood disorders are a heterogeneous group of disorders, varying from anxiety to severe major depressive disorder (MDD). A major focus of my research is dissecting the molecular aetiology of MDD and associated endophenotypes (e.g. suicide and psychosis). An estimated 350 million people are affected by MDD worldwide, representing a major social and economic health burden. MDD is associated with a reduced life expectancy and constitutes a major risk factor for suicide, a leading cause of mortality among young people in developed countries. Moreover, MDD predicts the incidence and progression of diseases of aging with an inflammatory aetiology including cardiovascular disease and many autoimmune disorders. Despite this associated burden the molecular pathology of depression remains poorly understood. Recently, it has been hypothesized that epigenetics plays a significant role in the pathology of both MDD and related psychiatric illnesses. I will present findings from two ongoing psychiatric epigenetic studies **i)** a study examining differential DNA methylation profiles in the brains of depressed suicide completers (n=20) compared to non-psychiatric, sudden-death controls (n=20) in two brain regions (Brodmann Area 11 (BA11) and Brodmann Area 25 (BA25)) and **ii)** a monozygotic (MZ) twin study to explore whether differences in DNA methylation in early childhood (measured at ages 5 and 10) are associated with MZ twin discordance for psychotic symptoms.

Genomic Imprinting and Epigenetic Mechanisms of Disease

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Several human diseases have both a genetic and an epigenetic component manifesting in complex phenotypes. Genomic imprinting is an epigenetic phenomenon that occurs during embryogenesis and results in parent-of-origin allele- specific silencing of a subset of genes required for fetal growth and development. Aberrant imprinting is the cause of a number of congenital syndromes and is a feature of adult onset diseases such as cancer. The study of genomic imprinting has uncovered numerous mechanisms of epigenetic regulation of gene expression that serve as paradigms widely applicable to almost all genes. We now know that the interaction of key epigenetic components for maintaining gene silencing include DNA methylation states, post translational histone modifications, higher order chromatin looping structures and long noncoding RNAs. Loss of imprinting in congenital imprinted diseases is confined to epimutations in one or more imprinted genes. However it is also feasible that when imprinted genes acquire epimutations in cancer – their subsequent silencing can spread to neighbouring genes. In this talk I shall give an overview of the epigenetic mechanisms of maintaining an imprinted chromatin domain and how disruption of an imprinted domain can lead to long range epigenetic silencing (LRES) of the wider locus. LRES occurs in many cancers to silence several contiguous genes on a chromosome. Lessons from genomic imprinting may help our understanding of pathological gene silencing such as LRES in cancer.

Early stage induction of chromatin remodeler mutations during development of esophageal squamous cell carcinoma

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Chromatin remodeling factors (chromatin remodelers), such as the SWI/SNF complex, are involved in the regulation of transcription by modulating chromatin structures. Genes encoding chromatin remodelers are frequently inactivated by somatic mutations in various types of cancers. However, their mutations in esophageal squamous cell carcinoma (ESCC) are still not fully analyzed. In this study, we aimed to clarify, in ESCC, 1) the frequency of mutations of chromatin remodelers, and 2) the timing of their occurrence. Ninety-four primary ESCC samples and their paired non-cancerous tissue samples were collected from patients who underwent endoscopy. Somatic mutations of 18 genes encoding chromatin remodelers were analyzed by amplicon sequencing using a bench top next generation sequencer (average reading depth = 1369). It was revealed that 6 of 94 ESCCs (6.4%) had 9 somatic mutations of 5 genes, *ARID1A*, *PBRM1*, *SMARCA4*, *SMARCAL1* and *SMARCC1*. *SMARCA4* (2 mutations in 2 ESCCs) and *PBRM1* (4 mutations in 2 ESCCs) were relatively mutated. *SMARCA4* mutations were detected in helicase (85Ser>Leu) and SANT domains (882Glu>Lys). *PBRM1* mutations were detected in a bromodomain (80Asn>Ser) and an HMG-box domain (1377Glu>Lys). The cancer cell content was then assessed by a profile of cancer-specific methylation, and the association between the fraction and mutant allele frequency was analyzed in individual ESCCs. The mutant allele frequency was close to the cancer cell content in half of the samples. Genetic alteration of chromatin remodelers was detected in ESCCs, and the mutations were suggested to have been induced at an early stage of ESCC development.

Quantitative MS-based analysis of histone modification patterns as markers for response to HDAC inhibitors in breast cancer

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Abnormalities in histone post-translational modification (hPTM) patterns are frequently implicated in the development of can-

cers and could represent biomarkers for drug response and patient stratification. Histone deacetylases (HDACs) are a class of enzyme that deacetylate histone lysine residues and have emerged as attractive targets for the therapy of various diseases, including breast cancer. Starting from our observation that primary breast cancer cells in culture display different sensitivities to HDAC inhibitors we sought to identify the epigenetic biomarkers that determine cellular responses to inhibition of HDAC in breast cancer by using mass spectrometry approaches. Methods: We performed proliferation assays to identify groups of breast cancer cell lines that are either sensitive or resistant to HDAC inhibitors and employed a novel analytical platform that combines ultra-high pressure liquid chromatography with high resolution mass spectrometry analysis on a Q Exactive instrument to carry out a comprehensive analysis of their hPTMs patterns. This approach was combined with stable isotope labeling with amino acids in cell culture (SILAC), using a super-SILAC set up where a mix of heavy-labelled breast cancer cells served as spike-in reference for comparative analysis with unlabelled cells. Results: We profiled 24 distinct modifications at 14 different sites on histone H3 and H4, covering well-characterized histone marks, in addition to identifying novel or poorly characterized modifications. Comparison of breast cancer cells that are sensitive or resistant to HDAC inhibitors in the presence and in the absence of the compounds revealed hPTM patterns that could be associated with the cellular response to the drugs and pinpointed the modifications affected by HDAC inhibitors with different specificities. Conclusions: The robust method that we have developed for the identification of hPTM allowed the profiling of modification patterns linked to drug sensitivity in breast cancer cells. Currently, we are applying this strategy to the analysis of clinical samples, such as fresh-frozen and formalin-fixed-paraffin embedded tissues, for which we have developed *ad hoc* histone extraction protocols.

Epigenetic and genetic alterations in 16HBE and THP1 cell lines exposed to different sizes of gold nanoparticles.

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Gold nanoparticles (AuNPs) can be used in applications such as cancer diagnostics and in therapies such as DNA/gene delivery. Their unique physic-chemical properties drive both their beneficial and hazardous potential. Although, evidence suggests the uptake of AuNPs by different cells, data are conflicting regarding the cyto- and genotoxicity. Till date, epigenetic alterations have not been extensively studied. Here, we investigated genotoxic effects (DNA damage and clastogenic effects) and epigenetic alterations (DNA methylation and hydroxyl-methylation), induced by three different sizes of AuNPs (5, 60 and 250 nm). THP1 cells (human monocytic cells) and 16HBE cells (human bronchial epithelial cells) were exposed to AuNPs for 3h and 24h prior to the experiments. We observed low but size dependent cytotoxicity, 5 nm being the most toxic after 24h of exposure in higher concentrations. Significant DNA damage (DNA tail % and tail moment) was observed in cells exposed to AuNPs after 24 h. No significant induction of Micronuclei was observed after 3 or 24 h exposure in both cell lines. However, we observed an increase in binucleated cells (incubated without cytochalasin B) in both cell lines after 24 h of exposure, which is an indication of failed cytokinesis. No changes in global (full genome) DNA methylation was observed in the cells exposed to AuNPs. In conclusion, AuNPs induced low cyto- and genotoxicity and possibly failure in cytokinesis. More investigations regarding the epigenetics are required to assess complete result of AuNPs toxicity.

Antiherpetic action of adenosine ointment: an epigenetic mechanism?

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Adenosine ointment has been found to be effective against *Herpes simplex* (HSV1) infection of the skin in mice using a model of delayed type hypersensitivity. In humans suffering from cutaneous efflorescences in the nasolabial area due recurrent HSV infection adenosine ointment improved significantly the healing process in 23 patients (placebo controlled trial). In order to analyze the involved mechanisms we focused on intracellular adenosine (Ado) metabolism and its relation to S-adenosyl-homocysteine (SAH) hydrolase. This enzyme catalyzes the reversible hydrolysis of SAH to Ado and homocysteine. Since SAH generated from S-adenosyl-methionine (SAM) acts as a potent product inhibitor of SAM-dependent methyl-transferases, intracellular accumulation of SAH may interfere with methylation of substrates such as histones, mRNA, DNA which are relevant for control of recrudescent HSV infections. Using purified SAH hydrolase we demonstrated that Ado inhibited the enzyme in the direction of hydrolysis with a Ki of 3 µM. Next we analyzed Ado binding to SAH hydrolase and identified two binding sites, depending on the NAD+/NADH ratio of the enzyme, with a low affinity (Kd 4.9 µM) for the NAD+ form and a high affinity (Kd 48.3 nM) for the NADH form of the enzyme. In order to show whether increased intracellular SAH concentrations are the result of SAH hydrolase inhibition, we incubated HepG2 cells with the SAH hydrolase inhibitor adenosine-2'3'-dialdehyde (Ado-2'3'dial). This inhibition of the enzyme resulted in an increase of intracellular SAH concentrations by more than 50 fold leading to a highly significant decrease in overall mRNA methylation. Our data are compatible with the assumption that adenosine might be involved in epigenetic processes of inflammation due to recurrent HSV infection.

Maintenance of the methylation pattern on fresh and cultured Chorionic Villi (CV) in normal and Beckwith Wiedemann Syndrome (BWS)-suspected pregnancies

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BWS is an imprinting-related disorder that can be prenatally suspected following established clinical guidelines. Molecular confirmation is commonly performed on amniocytes and the possibility to use fresh (CVF) and cultured (CVC) CV should be proved since embryonic and extra-embryonic compartments usually have different methylation profiles. To verify whether

CVF and CVC are eligible sources of DNA, we tested by pyrosequencing in normal pregnancies the methylation percentage at: ICR1, ICR2, *H19* promoter (imprinted locus 11p15.5), PWS/AS-ICR (imprinted locus 15q11-13) and *MGMT*, *RASSF1A* (not imprinted gene). We highlighted stable methylation levels at the imprinting-driving regions ICR1 (CVF: 45.38% ± 1.77; CVC: 45.04% ± 1.81), ICR2 (CVF: 44.32% ± 1.84; CVC: 43.67% ± 2.10) and PWS/AS-ICR (CVF: 43.70% ± 5.60; CVC, 43.15% ± 3.41). Conversely, *H19* promoter was severely hypomethylated at both CVF (11.33% ± 1.92) and CVC (19.30% ± 4.30), and showed a significantly increased methylation after culture. In two unrelated and biallelic genes, the methylation remained stable at *MGMT* promoter (CVF: 2.06% ± 0.48; CVC: 2.16% ± 0.53) and changed at *RASSF1A* (CVF: 52.50% ± 7.33; CVC: 28.00 ± 11.10). As second step, we investigated ICR1 and ICR2 methylation level on both fresh (CVF) and cultured (CVC) chorionic villi of two BWS-suspected fetuses (P1 and P2). P1 showed hypomethylation at ICR2 both in CVF and CVC (CVF: 17.63% ± 0.88; CVC: 16,13% ± 0.18); P2 showed normal methylation profiles. Taken together these findings suggest that: i) ICR1 and ICR2, but not H19, are reliable targets for BWS prenatal methylation test in CV also after culture; ii) similarly, PWS/AS-ICR is steadily hemimethylated in CV from healthy pregnancies, independently from culture. Thus, methylation analysis of these regions represents a very useful tool for prenatal diagnosis of imprinting related syndromes.

Real-time monitoring efficiency and toxicity of chemotherapy in patients with advanced lung cancer

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Nowadays, clinicians often respectively adopt RECIST guideline and CTCAE criteria to assess the efficiency and toxicity in advanced lung cancer patients. But there's no real-time and synchronous indicators which can evaluate chemotherapy outcomes. We want to evaluate tumor response and toxicity in advanced lung cancer chemotherapy by using a novel, real-time, synchronous and rapid strategy. Two hundred and sixteen advanced lung cancer patients treated with cisplatin-based therapy were enrolled and followed for 3 years. Pre-chemotherapy and 24h post-chemotherapy plasma of every cycle were obtained to perform quantitative assay of the APC/RASSF1A genes methylation and total plasma DNA to evaluate tumor response and toxicity, respectively. When use "methylation level of 24h after chemotherapy >methylation level before chemotherapy (methylation$_{24h}$>methylation$_{0h}$)" of at least one gene to predict tumor response, the correct prediction rate was 82.4%. Patients with this methylation alteration had higher efficient response rate (75.6%) and longer survival time (25 months) than those without this methylation change (8.6% and 6 months, resp.). Additionally, in 132 cases with "total plasma DNA concentration 24h after medication≤2 folds of total plasma DNA concentration before medication ($DNA_{24h} \leq 2 \cdot DNA_{0h}$)", most toxicities belonged to Grade 1. While in 84 cases with "$DNA_{24h} > 2 \cdot DNA_{0h}$", most toxicities belonged to Grade 2. Therefore, "methylation$_{24h}$>methylation$_{0h}$" and "$DNA_{24h} \leq 2 \cdot DNA_{0h}$" was defined as the criteria for better tumor response and fewer adverse events with high correct prediction rate (88.43%). Quantitative analysis and combination of total plasma DNA and plasma APC/RASSF1A methylation may be a real-time, synchronous and rapid monitoring indicator to assess the therapeutic outcomes. The new strategy will be a reference or supplementary material in the evaluation of chemotherapy efficiency.

Co-localisation of two active epigenetic histone marks H4K12ac and H3K9ac in promoters of spermatozoal DNA from fertile and subfertile patients.

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In human spermatozoa, 85% histones are replaced by protamines. 15% of the remaining histones carry epigenetic marks for the establishment of epigenetic information in the offspring. This study aimed to analyse the genome wide binding pattern of acetylated histone H4 at lysine 12 (H4K12ac) in promoters and acetylated histone H3 at lysine 9 (H3H9ac) in whole gene sequences (ENCODE -encyclopaedia of coding elements) in order to study the binding patterns of active chromatin marks between fertile and infertile patients. Chromatin immunoprecipitation with anti-H4K12ac and anti-H3K9ac was performed with ejaculated sperm DNA from subfertile patients with impaired sperm chromatin condensation as assessed by aniline blue staining and fertile donors as controls. H4K12ac immunoprecipitates were analysed by hybrydisation on HG18 human promoter array (NimbleGen) and H3K9ac were placed on ENCODE array containing promoters and intergenic sequences of 375 genes. Peak finding algorithms and data overlapping were performed by using the R software (http://www.r-project.org). Co-localisation of binding sites for H4K12ac and H3K9ac has been detected by 38 genes promoters in fertile donors. Among them 10 genes are involved in reproductive process and embryonic development e.g: AFF4 (AF4/FMR2 family, member 4), AXIN1, NCOA6 (nuclear receptor coactivator 6), OR52A1 (olfactory receptor, family 52, subfamily A, member 1). A global depletion of H4K12ac was detected in a group of subfertile patient (496 from fertile donors vs. 146 peaks from infertile). H3K9ac are bound to 84 promoters in fertile sperm. We detected only 15 promoters interacting with H3K9ac in subfertile patients. Data analysis showed no co-localisation of H4K12ac and H3K9ac with any of investigated promoters. Based on our study, we may suggest that aberrant acetylation of H4K12ac and H3K9ac within developmentally important promoters in infertile men might reflect insufficient sperm chromatin compaction and in consequence may lead to inappropriate transfer of epigenetic information to the zygote.

HDAC5-mediated attenuation of BDNF expression in the brain as novel mechanism associated with cognitive aging in response to transient obesity during childhood/adolescence

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Obesity's immediate clinical impacts have been extensively studied; however, current clinical evidence underscores the long-term implications. We explored the impacts of brief childhood/adolescent obesity and insulin resistance on cognitive function in later life. In order to mimic childhood/adolescent obesity and insulin resistance, we exposed 9-week old C57BL/6J mice to a high-fat diet for 15 weeks, after which the mice exhibited diet-induced obesity and insulin resistance. We then put these mice back on a normal low-fat diet, after which the mice exhibited normal body weight and glucose tolerance. However, a spatial memory test in the forms of the Morris water maze (MWM) and contextual fear conditioning at 85 weeks of age showed that these mice had severe deficits in learning and long-term memory consolidation. Mechanistic investigations identified increased an expression of histone deacetylases 5 (HDAC 5), accompanied by reduced expression of brain-derived neurotrophic factor (BDNF), in the brains 61 weeks after the mice were off the high-fat diet. These findings are consistent with previous evidence that HDAC 5 regulates BDNF, which is important for synaptic function. Electrophysiology studies showed that hippocampal slices isolated from these mice are more susceptible to synaptic impairments compared to slices isolated from the control mice. Collectively, we demonstrated that a 15-week occurrence of obesity and insulin resistance during childhood/adolescence induces irreversible epigenetic modifications in the brain that persist following restoration of normal metabolic homeostasis, leading to brain synaptic dysfunction during aging. Our study provides experimental evidence that limited early life exposure to obesity and insulin resistance may have long-term deleterious consequences in the brain, i.e., leads to histone deacetylase modifications in the brain, contributing to the onset/progression of cognitive dysfunction during aging. Future studies on HDAC 5 regulation in animals will help to clarify the epigenetic role of deacetylases in cognitive function.

Epigenetic mechanisms and lipid raft signaling in human colon cancers

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Background: The nexus between signaling pathways and DNA and histone modifications is emerging. Signaling pathway(s) linking epigenetic modifications and chromatin dynamics has not been explored adequately. Results: Lipid raft signaling works via RAS/ERK axis to modulate gene expression by differentially altering active and repressive histone 3 marks; H3K4Me3, H3K9Me3 and H3K9AcS10P. We have deciphered that lipid raft/RAS/MAPK signaling pathway is involved in modification of various types of histone H3 codes, which control gene expression globally and gene specifically (for example, CAV1 gene) in HCT-15 colon cancer cell line. Our work reveals that there is global fluctuation in both expressive and repressive histone 3 codes after lipid raft disruption. CAV1 gene expression is upregulated by differential enrichment of H3K4me2, H3K4me3 and H3K9AcS10p, depending on which component of the lipid raft/RAS/ERK axis is blocked. The enhancement of transcription of the CAV1 gene is associated with very high occupancy of histone 3 H3K4me3 and H3K9AcS10p and very low H3K9me3 marks in its promoter nucleosomes. Conclusion: Lipid raft orchestrated RAS/MEK-signaling regulate histone modifications and chromatin dynamics. In accordance with the global and gene specific modulations of the three histone H3 codes after lipid raft destabilization, the expression of respective chromatin histone H3 modifying enzymes were found to be upregulated. These active histone H3K4me3 and H3K9AcS10p and repressive histone H3K9me3 marks are recruited or deleted differentially by RAS/RAF/MEK/ERK signaling axis originated from membrane lipid raft.

Global DNA methylation and hydroxymethylation during pregnancy: the effect of pre-pregnancy BMI and gestational weight gain

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Introduction: Epigenetic modifications, e.g. DNA methylation, have the ability to change the susceptibility to metabolic diseases like obesity. DNA methylation can change during a life course due to environmental exposures like hormones, diet,.. In this rapidly evolving field, other epigenetic modifications, such as hydroxymethylation, have been discovered recently. Its biological function is not yet clear, but it is thought to be an intermediate in the DNA de-methylation pathway. Aim: In this preliminary study, we want to investigate whether maternal global DNA methylation and hydroxymethylation changes during pregnancy. The possible influence of pre-pregnancy BMI and gestational weight gain was studied. Methods: 13 women (age range: 25-34 years, BMI range: 18.7-32.8 kg/m²) were recruited pre-conceptionally. Peripheral fasted blood samples of the mother were taken before pregnancy, during each trimester, at delivery and 6 weeks postpartum. Both global DNA methylation and hydroxymethylation were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results: No significant changes in global DNA methylation ($p=0.34$) and hydroxymethylation ($p=0.54$) across pregnancy could be observed. Despite the small sample size, we observed a significant decrease in global methylation between the third trimester and time of delivery (-0.17%, $p=0.03$). Pre-pregnancy BMI was negatively correlated with hydroxymethylation in the first trimester ($r= -0.69$, $p= 0.009$). Gestational weight gain was positively correlated with hydroxymethylation in the third trimester ($r=0.71$, $p=0.007$). Conclusion: This first pilot study gives an indication that total DNA methylation is rather stable over pregnancy. However, these preliminary results also show a small decrease of DNA methylation by the end of pregnancy. This will be further studied in a larger group of women together with the analysis of specific DNA methylation changes.

Assessment of methyl-group donor intake during the course of pregnancy using a validated food-frequency questionnaire: a preliminary study

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Introduction: Our diet plays a central role in the one-carbon metabolism and therefore in the methylation of DNA. Dietary intake of methyl-group donors (methionine, choline, betaine, and food folate) can influence this pathway and may affect DNA methylation. A novel food-frequency questionnaire (FFQ) was developed and validated to assess the daily intake of these methyl-group donors among Belgian women of reproductive age. The validation of this FFQ was done by the comparison with a diet record and blood biomarkers, since biomarkers of nutritional intake might provide a more objective measure. Aim: The MANOE study (Maternal Nutrition and Offspring's Epigenome) was set up to investigate the relationship between the intake of maternal methyl-group donors during the course of pregnancy and offspring DNA methylation. Methods: The novel FFQ was used to assess the daily intake of methyl-group donors in 28 women (age range: 25-41years). We also assessed the intake of folic acid (FA) supplements and calculated total folate intake. Each participant filled out 3 FFQ's during pregnancy (12, 20, and 30 weeks of pregnancy). A one-way ANOVA with repeated measures or Friedman test was conducted in order to compare the intake of methyl-group donors during pregnancy. Results: No significant changes in daily intake of methionine (p=0.16), choline (p=0.09), betaine (p=0.09), the sum of the 4 methyl-group donors (p=0.19), and FA supplement intake (p=0.34) was observed during pregnancy. For food folate and total folate, a significant decrease was found between 12 weeks and respectively 20 weeks (p=0.03,p=0.006) and 30 weeks (p=0.04,p=0.045) of pregnancy. No seasonal variation was found for food folate and total folate intake. Conclusion: This preliminary study gives a first indication that the intake of methyl-group donors during the course of pregnancy is stable, except for a decrease in food folate and total folate intake in the second and third trimester.

TSC2 epigenetic defects in primary lymphangiomyolipomas and angiomyolipomas cells

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TSC2 gene encodes for tuberin that, together with hamartin (*TSC1* gene), forms the TSC complex, a key negative regulator of the PI3K-AKT-mTOR pathway. Constitutive inactivating mutations in TSC genes cause tuberous sclerosis (TSC), an autosomal dominant genetic disease. TSC is a multisystemic syndrome characterized by hamartoma benign tumors in several tissues, such as renal angiomyolipomas (AML), neurologic disorders and abdominal lymphangiomyolipomas. Lymphangioleiomyomatosis (LAM), a lung disease affecting almost exclusively women, is caused by proliferation of cancer-like LAM cells with mutations in *TSC1* or *TSC2* genes. The TSC lesions are evident the two-hit model for tumor suppressors. The second somatic event inactivating the wild-type allele has been attributed either to genetic or epigenetic alterations in TSC genes. Recently, we demonstrated that heterogeneous epigenetic defects of the *TSC2* promoter leads to loss of tuberin in smooth muscle-like cells from AML of a TSC2 patient and LAM cells from LAM/TSC patients. Intriguingly, tuberin could be expressed after treatment with trichostatin A and 5-azacytidine. Beside methylation, other factors are involved in the regulation of gene expression, such as histone modifications and other dynamic alterations in chromatin conformation. Here, analyzed the chromatin structure and accessibility on the basis of the sensibility to DNase digestion of the region spanning from 5' to 3' *TSC2* locus, to deeply assess the *TSC2* epigenetic defects occurring in LAM and TSC cells. The chromatin accessibility was tested before and after treatment with 5-azacytidine and was correlated to the mRNA expression to define the level of gene silencing. The results we achieved could be crucial to shed light on the undisclosed mechanisms of *TSC2* epigenetic regulation.

Cyclin D1 Integrates G9a-Mediated Histone Methylation and Nuclear Lamina Association With Lamina-Associated Domains

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Histone methylation is dynamically regulated by histone methyltransferases (HKMTs) and histone lysine demethylases (HKDMs). Both histone and non-histone substrates have been reported for HKMTs. The key HKMTs include G9a/KMT1C, which methylates histone H1 and H3 (K9 and K27) *in vitro*. The Su(var)3-9-Enhancer of *zeste*-Trithorax (SET) domain of Suv39h1/KMT1a encodes the catalytic domain for lysine methylation. Both Suv39h1 and G9a catalyze mono-, di-, and trimethylation reactions on H3K9. Histone H3 lysine 9 dimethylation (H3K9me2) is enriched in silent regions on euchromatin and correlates with gene silencing. In mouse and human, di-methylation of H3K9 is catalyzed by the G9a/GLP enzymatic complex, where G9a is essential for the catalytic function and stability of the complex. G9a associates with heterochromatin protein 1 (HP1) to regulate chromatin binding and association with methylated histones. Nuclear lamina (NL) interact with genomic regions known as lamina-associated domains (LADs) and G9a plays a critical role in nuclear lamina associated large chromatin domain interactions. The positioning of chromosomes within the nuclear three-dimensional space involves interac-

tions between nuclear lamina (NL) and the lamina-associated domains (LAD). Recent evidence suggests the contact of nuclear lamina with LADs is linked to H3K9 dimethylation conducted by G9a. Contact of individual LAD with the NL is linked to transcriptional repression and is dependent upon H3K9me2 by G9a. In this manner, G9a contributes to dynamic spatial architectural changes of chromosomes in relation to gene regulation. The *cyclin D1* gene encodes a labile regulatory subunit of the holoenzyme that phosphorylates and inactivates the retinoblastoma (pRb) and NRF1 proteins thereby regulating both the DNA synthetic phase of the cell cycle and mitochondrial biogenesis. Cyclin D1 also promotes chromosomal instability (CIN). Several recent studies have implicated the cell cycle protein cyclin D1 in regulating gene transcription in a kinase-independent manner. Cyclin D1 conveys transcriptional repression via a repressor domain (amino acids 179-241). Herein, endogenous cyclin D1 enhanced H3K9me2 through forming a complex with G9a. Cyclin D1 association with G9a involved the cyclin D1 transcriptional repressor domain and the G9a Cys domain. Herein, endogenous cyclin D1 was required for LAD-NL interaction. The augmentation of NL/LAD interactions by cyclin D1 suggests a potential direct line between the cell cycle and the plasticity of chromosome folding.

Optimization of the Quantitative Methylation-Specific PCR Method

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Cancer is the result of the accumulation of genetic and epigenetic aberrations, including promoter DNA hypermethylation. Several of these aberrations are promising cancer biomarkers, including for early non-invasive diagnostics, prognostication and monitoring. DNA promoter methylation is intensively studied in an increasing number of scientific articles and quantitative methylation specific PCR (qMSP; also called MethyLight) is commonly used to estimate the relative amounts of methylation at a specific locus. However, diverging qMSP results are frequently being reported in the literature for the same gene promoter in the same cancer type, underscoring the need for standardization of the individual steps of the qMSP protocol. By testing the most likely sources of variability, such as the choice of normalization reference (also called the endogenous control), the amount of template, type of bisulfite conversion kit, sample storage time and temperature, etc., we are currently identifying the major pitfalls of qMSP. After completing the last experiments, we aim at suggesting a standardized and optimized qMSP pipeline that hopefully can contribute to reduce the current variability of PMR (percent of methylated reference) values generated within and between labs. More than 150 rounds of qMSP have been analyzed so far in this project. As expected, the choice of normalization reference is crucial. Furthermore, careful control of the DNA input amount is essential, especially in the qMSP, but also in the bisulfite conversion reaction. Importantly, up to 20% variation in PMR values should be expected when performing practically identical qMSP analyses, and this should be taken into account when presenting qMSP results.

The epigenome of birthweight discordant monozygotic twins and its link to disease

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Monozygotic twins are genetically and epigenetically nearly identical. However differences in the epigenetic profile between the twins increase with age due to environmental influences. Nevertheless they provide an unequivocal source to study disease related changes, as they are perfect matched biological controls. Recent epigenome-wide association studies of disease-discordant twins revealed an association between the phenotypes and differentially methylated regions for several traits. In the presenting study we investigated the methylome of 24 monozygotic twin pairs (n=48) using Illumina's Infinium HumanMethylation450 BeadChips. Illumina's Bead Chip interrogates 485577 single CpG's distributed over the whole genome and returns the methylation state of each individual investigated cytosine in terms of percentage. Statistical analysis was conducted to identify differentially methylated regions between the twin pairs with respect to different phenotypes like discordances in birth weight, birth height or blood pressure. The top loci showing differential methylation further underwent validation experiments using targeted deep amplicon sequencing on an Ion Torrent. Statistical evaluation of both the BeadChip data as well as the Ion Torrent data revealed the presence of unique methylation patterns and differentially methylated loci between the different investigated phenotypes. Although differential methylation between the twins was highly significant, it was also shown that the difference in methylation intensities was for many CpGs below 10%. Consequently, the conducted epigenome-wide association study confirmed differential methylation in phenotype-discordant monozygotic twins. In the future longitudinal studies would be needed to reveal the role of the identified epigenetic characteristics in disease.

Impact of epigenetic reprogramming on hepatic tumorigenic properties

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Background and Aims: Reversal of DNA hypermethylation and associated gene silencing is an emerging cancer therapy approach. Here we addressed the impact of epigenetic alterations and cellular context on functional and transcriptional reprogramming of HCC cells. Methods: Established and primary human HCC-derived cell lines were plated in 2D culture at various cell densities and exposed to a transient dose of a DNMT1-inhibitor Zebularine (ZEB). After a 3-day treatment, cells were cultured in 3D non-adherent condition. Differences in self-renewal, gene expression, tumorigenicity and metastatic potential of spheres at generations G1-G5 were examined. Results: Transient ZEB exposure produced differential cell density-dependent responses. In cells grown at low density, ZEB caused a remarkable increase in self-renewal and tumorigenicity associated with long-lasting gene expression changes and a stable overexpression of cancer stem cell-related and key epithelial-mesenchymal

transition genes. These effects persisted after restoration of DNMT1 expression. In contrast at high cell density, ZEB caused a gradual decrease in self-renewal and tumorigenicty, and up-regulation of apoptosis-related genes. A permanent reduction of the DNMT1 protein using shRNA-mediated DNMT1 silencing rendered HCC cells insensitive both to cell density and ZEB effects. Similarly, hepatoblastoma cells expressing low basal levels of DNMT1 also possessed a high self-renewal irrespective of cell density or ZEB exposure. Spheres formed by low density cells treated with ZEB or shDNMT1A displayed a high molecular similarity which was sustained through consecutive generations. Conclusion: These results identify DNA methylation as a key epigenetic regulatory mechanism determining pool of cancer stem cells in liver cancer and possibly other solid tumors.

Epigenetic regulation of *RASSF1A* and *APC* during normal placentation and its related abnormalities.

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Background: Abnormal expression of tumor suppressor genes associated with epigenetic deregulation is a hallmark of cancer development. Further, the similarity between cancer development and normal placentation is well documented. Hypothesizing some similar epigenetic mechanisms in transcriptional regulation of two important tumor suppressor genes in placentation, we aimed this study to analyze the role of DNA methylation and H3K9/27me3 at promoter regions of *RASSF1A* and *APC* in normal pregnancy and their deregulation in the development of placental disorders: preeclampsia and gestational trophoblastic diseases. Results: Normal advancing gestation was observed to be associated with decreased expression of these tumor suppressor genes, decreasing by 2.2- and 3.5- fold in case of *RASSF1A* and 2- and 6.2-folds in case of *APC* in second ($p<0.01$) and third ($p<0.001$) trimester respectively relative to first trimester placental villi. The decrease in expression of *RASSF1A* and *APC* seemed to be regulated by their increased promoter region DNA methylation (raised by 20%, $p<0.001$) and H3K9/27me3 levels in third trimester placental villi. Development of gestational trophoblastic diseases was observed to be associated with a generalized decrease in mRNA expressions of both *RASSF1A* and *APC* which were observed to be associated with increased methylation at *RASSF1A* and *APC* promoter regions in JEG-3 cells (choriocarcinoma cell line) raised by 32.7% and 34.8% ($p<0.001$) respectively and increased H3K27me3 level at *RASSF1A* promoter region in molar villi ($p<0.05$) relative to first trimester villi. Development of preeclampsia was associated with decreased expression of *RASSF1A* which might be associated with raised H3K27me3 level at *RASSF1A* promoter in preeclamptic villi. Conclusion: *RASSF1A* and *APC* might have some regulatory roles during normal pregnancy and were observed to be strongly regulated by DNA methylation and histone modifications. Epigenetic deregulations of *RASSF1A* and *APC* might be contributing factors for development of preeclampsia and gestational trophoblastic diseases.

Molecular mechanisms behind concentration dependent effect of folate supplementation on invasion of placental trophoblasts

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Background: Placental trophoblasts exhibit higher invasive potential. Further, there is an increased folic acid requirement during pregnancy due to rapid cell Div. and growth. Therefore, we aimed to study the effect of folic acid supplementation at different concentrations on the invasive potential of different trophoblasts and to delineate the molecular and epigenetic mechanisms regulating the altered invasiveness, using two placental derived cell lines JEG-3 (choriocarcinoma derived cell line) and HTR-8/SVneo (transformed first trimester extravillous trophoblasts). These were treated with folic acid at 10^{-7}M (physiological concentration) and 10^{-4}M (higher than physiological range) followed by analysis of invasion potential, mRNA expression, gene specific DNA methylation (*MMP -2* & *-9*, *TIMP -1* & *-2*, *c-jun*, *c-myc* & *APC*) and global methylation by matrigel invasion assay, quantitative real time PCR, Methylation-Specific High Resolution Melting (MS-HRM) and ELISA based kit respectively. Results: Folic acid was observed to induce dose dependent change in invasiveness of both cell lines with increased invasion in JEG-3 (2.2 fold, $p<0.01$) and HTR-8/SVneo cells (2.4 fold, $p<0.001$) at lower 10^{-7} M folate concentration and 2 fold ($p<0.01$) reduced invasiveness at higher folate concentration of 10^{-4} M relative to untreated control cells. The altered invasion was associated with dose dependent decrease in global methylation by 3.7-4.9% and 4.3-5.7% ($p<0.001$) in JEG-3 and HTR-8/SVneo cells at 10-7 and 10-4 M folic acid supplementation respectively. At the molecular level, folic acid supplementation significantly enhanced the mRNA expression of *MMP-2*, *-9*, *c-jun* and *c-myc* while reduced the mRNA expression of *TIMP -1*, *-2* and *APC* in the cell lines at both folic acid concentrations. Analysis of the effect of folic acid supplementation on promoter DNA methylation revealed significantly decreased methylation in *c-jun* and *c-myc* while increased methylation in *APC*. However, there was no change in promoter region methylation of *MMPs* and *TIMPs*. Conclusion: Folate supplementation enhances the invasive potential of placental trophoblasts at physiological concentration which is mediated by the reduced global methylation, enhanced expression of invasion promoting genes and reduced expression of invasion inhibiting genes. The expression of *c-myc*, *c-jun* and *APC* was found to be regulated by promoter region DNA methylation.

Epigenetic adaptation to regular exercise in human

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Exercise, even in small doses, may change the expression of our innate DNA through epigenetic mechanisms. The aim of this presentation is to describe the genome-wide DNA methylation pattern in human adipose tissue and skeletal muscle, and the alterations observed in response to regular exercise. Furthermore, epigenetic changes in response to exercise will be discussed in the light of metabolic diseases, including genes important for the pathogenesis of obesity and type 2 diabetes. We also pre-

sent evidence for an epigenetic regulatory role on the level of mRNA expression for some, but not all, genes or regions. Thereby, epigenetic changes may contribute to altered gene expression and improved metabolism and may subsequently be one explanation for how exercise improves health. In conclusion, epigenetic mechanisms may link external stimuli, like physical exercise, to altered genome function. However, we still do not know to what extent the phenotypical variance that arises from environmental signals is actually mediated by epigenetic alterations.

Micro-RNAs and Long non-coding RNAs de-regulation in Rhabdomyosarcoma.

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Rhabdomyosarcoma (RMS) is an aggressive pediatric soft tissue sarcoma of myogenic orgigin. Conversely to normal myoblasts, RMS cells are highly invasive and unable to differentiate, thus they proliferate indefinitely. MicroRNAs (miRNAs) are fundamental regulators of myogenesis and have been involved in RMS pathogenesis. We evaluate here the expression and regulation of miRNAs and of the long-noncoding RNA HOTAIR, which can work with the Polycomb group Protein EZH2 in RMS patients. HOTAIR, located within the Homeobox C (HOXC) gene cluster on chromosome 12q13 region, was up-regulated in the embryonal RMS sub-type (ERMS) while being down-regulated in the alveolar PAX3-FOXO1 (P3F) RMS (ARMS) compared to normal muscle tissues. RMS cell lines shared a similar behaviour as compared to skeletal myoblasts cultured in vitro. The microRNA miR-196a followed a similar trend. The mature miR-196a is expressed by both pri-miR-196a-2, co-linear with HOTAIR, and pri-miR-196a-1, located on the 17q21 region. Both pri-miRNAs were up-regulated in ERMS and down-regulated in ARMS. Interestingly, HOTAIR and miR-196a expression were also associated to those of HOXC10 and HOXC11. HOXB13, collinear with miR-196a-1, was up-regulated in both ERMS and P3F-positive ARMS cell lines suggesting that this gene locus is not involved in the down-regulation of miR-196a seen in the latter cells. Further, human myoblast shared Myogenin increase when cultured in differentiation medium, which was associated to sustained induction of HOTAIR, HOXC9 and HOXC10 suggesting that these genes could be regulated during myogenesis. Experiments are in course to understand the impact of HOTAIR modulation in RMS cells and in myoblasts. Altogether, these results indicate that the expression of genes on chromosome 12 region surrounding HOTAIR and miR-196a could be de-regulated in RMS. They also suggest a different regulation of the involved genes in the two RMS subtypes.

Epigenetic Editing: An Innovative Anti-Fibrosis Approach?

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Plod2 expression initiates pyridinoline (pyr) cross-linking of fibrilar collagen. These cross-links are increased in fibrotic pathologies and result in enhanced stability and presence of collagen molecules. To date, no successful therapeutics have been developed to reduce pyr cross-linking in fibrotic disease. Artificial Transcription Factors (ATFs) can be engineered to repress the expression of any gene at will. However, ATFs have no catalytic activity and this approach is presumed to result in transient effects. Therefore, we aimed to induce sustained gene repression by introducing repressive epigenetic marks at our locus of interest (*Epigenetic Editing*)[1]. We engineered several DNA binding zinc finger proteins (ZFPs) to target the *plod2* promoter. These ZFPs were fused to various effector domains to repress gene activity. Stable inducible primary human dermal fibroblasts (HDFs) were constructed to control the expression of ZF-fusions. Without induction, the pro-fibrotic cytokine TGFβ1 effectively induced the expression of *plod2* in these HDF cells. Upon expression of ZFPs fused to the supposedly transient transcriptional repressor SKD for 2 days, the TGFβ1 induced LH2 expression was effectively prevented. Similar targeting of DNA methyltransferases to *plod2* introduced DNA methylation, but only prevented induction of LH2 expression by 50%. To address sustainability of the repressive effect, cells were allowed to recover from the ZF-treatment for 10 additional days in the presence of TGFβ1. The induced repressive effects of either the presumed transient repressor or a DNA methylator were maintained during the 10 days. To further improve the effect of targeted DNA methylation, we currently investigate the ATF-induced epigenetic reprogramming to identify potent epigenetic writers[2] or erasers[3] for Epigenetic Editing. In conclusion, our data show that targeting transcriptional effector domains to a gene of interest by fusion to ZFPs is a promising new strategy to reduce gene expression, even in a pro-fibrotic environment.

Influences of the culture condition and chromatin remodeling agents on the epigenetics of normal and tumor lung organotypic cultures.

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Alterations of epigenetic pathways are frequently observed in tumor cells, together with genetic/genomic variations. Malignant cells show changes in histone modifications and DNA methylation, that are of increasing relevance to clinical practice, because they are important druggable targets for therapy using chromatin remodeling agents (CRAs). The main "epigenetic-drug" classes, histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors. New evidences highlight the relevance of microenvironment on the epigenetics and the need to use culture models that preserve the tissue morphology, to better under-

stand the mode of action of CRAs. We studied the epigenetic response induced by culture condition and treatment with CRAs, in a preclinical model based on organotypic culture, from normal and neoplastic lung specimens, that preserves *ex vivo* the tissue microenvironment and morphology. We assessed different epigenetic signatures, in particular the expression pattern of HDACs, the methylation profile of long interspersed nuclear elements (LINE1) and of a panel of tumor suppressor genes. In addition, we evaluate the global levels of 5-methylcytosine and 5-hydroxymethylcytosine, an intermediate of active DNA demethylation. We found a different behavior of the organotypic culture respect to that reported for other *ex vivo* models. Culture induced an overall increase of LINE1s methylation, while the use of CRAs caused a reduction of LINE1s methylation. Otherwise, culture and CRAs produced opposite effects on the analyzed genes. Each sample had an individual response, showing demethylation for some promoters and increased methylation for others. In addition, CRAs treatment induced an increase of 5-hydroxymethylcytosine suggesting the triggering of active demethylation pathway. Finally, we observed that the culture induced profound alterations in the HDAC expression. Our data highlight the importance of maintaining the cells in their original organ architecture to better study the action of the CRAs, and suggest that CRAs do not work only in a non-specific way, as previously thought.

Targeting active demethylation through epigenetic drugs - a possible mechanism in HCC therapy

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Introduction: Global deregulation of the methylation status is a crucial cause of HCC. It has been reported that the anti-cancer drug 5-Azacytidine (5-Aza) mediates the activation of tumor suppressor genes through passive demethylation by inhibiting DNMT1. Our aim was to ascertain if 5-Aza also induces an active demethylation by increasing the expression of 5hmC, which is lost in various types of cancers. A validation of our hypothesis may lead to new approaches in cancer therapy. Methods: HCC cells (Huh-7, HLE, HLF) and tissue sections from healthy and HCC patient cohorts (55 patients) were analyzed by immunofluorescence (IF) staining and immunohistochemistry staining for 5hmC and 5mC, and via Real-time PCR for TETs. Next, the HCC cells were stimulated with different concentrations of 5-Aza (0-20 µM). Viability and toxicity were measured after 24 and 48 hours via Resazurin conversion and LDH release. The expression of TETs, 5hmC-5mC, and PCNA (as a proliferation marker) were measured via Real-time PCR, IF staining, and Western blotting, respectively. Results: The expression of 5-hmC was lower in HCC tissue than in healthy tissue samples. This finding was confirmed by comparing HCC cells to hHeps. The expression of TET2 and TET3 was lower in HCCs than in non-tumor liver tissues and in hHeps. After 48 hours, 5-Aza inhibited the proliferation of HCC without any significant LDH release, no increase of the expression of TET2 or TET3 on mRNA level, or of 5hmC. Discussion / Conclusion: Our data exhibit a decrease of 5-hmC and an increase of 5-mC in HCCs through down-regulation of the TETs. However, the expression levels of 5-hmC and the TETs can be re-induced by 5-Aza through active demethylation, which is a novel function of this drug. We suggest that the loss of the expression of 5-hmC is an additional marker for HCC diagnostics.

Increased expression of the DNA demethylation enzymes TET1, TET2 and TET3 in monocytes and TET2 in T cells in patients with early rheumatoid arthritis

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Recently, the enzymes involved in active DNA demethylation have been identified as TET1, TET2 and TET3. We have assessed the relative expression of these enzymes in three blood subpopulations of rheumatoid arthritis (RA) patients (T and B cells and monocytes), detecting an increased relative expression of TET enzymes in RA patients regards to healthy controls in monocytes for TET1 ($P = 0.037$), TET 2 ($P = 0.019$) and TET3 ($P = 0.0014$); and in T cells for TET2 ($P = 0.045$). These changes coincided with global DNA hypomethylation in monocytes and T cells. However, one month after initiating treatment with methotrexate (MTX) the DNA methylation level was normalized but the increased expression of TET enzymes was not reverted, at least in monocytes. In fact, the differences in monocytes between the healthy controls and the RA patients one month after MTX treatment were equivalent to those observed before the treatment (TET1, $P = 0.039$, TET2, $P = 0.013$ and TET3, $P = 0.0033$). Therefore, in this work we detected an increased relative expression of several TET enzymes in monocytes and T cells in RA patients. These findings are in agreement with DNA hypomethylation in these blood subpopulations; however do not match with the reversion of the low levels of methylation after MTX treatment. Therefore, its causal implication is questionable and other mechanisms should be envisaged (as, for instance, the decreased level of DNMT1 previously described by our research group). Funding: The present work was supported by Fondo de Investigación Sanitaria of the Instituto de Salud Carlos III (Spain), grants PI11/01048, PI12/01909 and RD12/0009/0008 that are partially financed by the European Regional Development Fund of the European Union. Conflicts of interest: The authors do not have conflicts of interest to declare.

Early results from a phase 2 study of RRx-001, a novel epigenetic modulator, show resensitization to irinotecan in colorectal cancer

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Background: Poor patient survival in advanced colon cancer is linked to acquired resistance to multiple lines of standard therapy. Therapies that modify the epigenetic profile of tumors could favorably impact chemoresistance allowing effective treatment

with previously failed therapies. The ROS-mediated epigenetic modifier, RRx-001 may resensitize third line colorectal cancer patients to irinotecan-based therapies leading to increases in OS vs regorafenib as standard third line therapy. Methods: The Phase 2 ROCKET trial studies the sequential rechallenge to irinotecan-based therapies post RRx-001 or regorafenib. Eligible patients have an ECOG PS 0-1 with irinotecan-refractory metastatic colorectal cancer and have progressed on oxaliplatin-, and irinotecan-based regimens with or without bevacizumab, cetuximab or panitumumab. Patients are randomized 2:1 to receive RRx-001 16.5 mg/m2 IV 1x/week or regorafenib 160 mg orally 21 of 28 days until progression or unacceptable toxicity then treated with irinotecan-based therapies. Primary end point is overall survival with secondary endpoints to investigate resensitization. Recruitment is ongoing with 190 patients planned. Study Status and Results: To date, 28 patients have been randomized with 10 patients evaluable for irinotecan-based therapies. Post RRx-001 patients demonstrated marked decreases in CEA in 6/7 patients compared to 4 post regorafenib patients who were too systemically unwell to continue to subsequent treatment. Conclusions: Early results suggest that resensitization to irinotecan-based therapies by RRx-001, a systemically non-toxic novel epigenetic mediator, may be general. Although these results are early, they are intriguing and may predict for increased overall survival, adding an additional regimen after disease progression.

Impact of TETs in accomplishment of sperm chromatin: a crucial point towards production of potent spermatozoa and in safeguarding of male fertility

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Among all cell types spermatozoa are known to have the lowest genome-wide 5C-methylation. Ten eleven translocation enzymes (TETs) catalyze in somatic cells and in early embryo the 5C-hydroxymethylation (5hmC) towards DNA-demethylation. Here, we asked the function of TETs in spermatogenesis and their role in production of fertile sperm. Material and Methods: We examined TET2- and TET3-expression at mRNA- and protein level in testis tissue samples exhibiting normal spermatogenesis by in-situ-hybridization and immunohistochemistry (IHC), respectively. 5hmC was analyzed by immunofluorescence (IF). Sperm cells were analyzed by westernblot and IHC regarding TET2/3-proteins, and by RTqPCR - regarding TET2/-mRNA. Further, we compared TET-mRNA-amount in spermatozoa of fertile donors (n=51) and sub-fertile men (n=44), who underwent ART (assisted reproductive technology), and correlated the data to different fertility parameters (spermiogram data according to WHO criteria and ART-outcome). Statistics were done using SPSS-20. Results: TET2-mRNA and –protein expression could be found in cytoplasm of pachytene-spermatocytes (stage I) up to elongated spermatids (stage II). TET3-mRNAexpression was present in cytoplasm of pachytene-spermatocytes (stage III) up to elongated spermatids (stage II), whereas TET3-protein-expression was examined in nucleus of round spermatids (stage I to IV). 5-hmC was detected in elongated spermatids (stages V and VI). Analyzing mature spermatozoa we could reveal non-degraded TET2- and TET3-protein as well as a verifiable amount of non-degraded TET2- and TET3-mRNA, respectively. Comparing fertile donors with sub-fertile patients we observed that low level of TET2- and TET3-mRNA in spermatozoa is significantly associated with low sperm-concentration and - motility, with oligo- and asthenozoospermia, and moreover, with low fertilization and pregnancy rates. Conclusion: The final step of spermatogenesis, which is characterized by intense genome-wide chromatin-hypercondensation, is accompanied by 5hmC-process. The latter is supported by TET2 and TET3, whose amount seems to be crucial for production of "feature-complete" sperm cells with the ability to fertilize the oocyte.

Identification of 4-hydroxybenzoic acid derivatives as HDAC6-specific inhibitors modulating microtubule structure and HSP90α chaperone activity in prostate cancer cells

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Histone deacetylases (HDACs) by targeting histones represent a diverse family of transcriptional regulatory enzymes that play a key role in numerous biological processes and diseases. HDAC isoenzymes modulate also a broad panel of non-histone protein functions implicated in cancer-related regulatory processes including cell proliferation, migration, metastasis and angiogenesis. Therefore, HDACs are considered as promising targets for anti-cancer therapy and various HDAC inhibitors (HDACi) belonging to different structural classes and with various inhibitory profiles have been developed. Most agents are pan-HDACi; however, isoenzyme-selective HDACi may appear as a successful alternative over pan-HDACi in terms of efficacy or toxicity in chemotherapy. HDAC6 is structurally and functionally unique among the zinc-dependent non-sirtuin HDACs and therefore may represent a promising anti-cancer target. We identified two 4-hydroxybenzoic acid (4-HBA)-based derivatives displaying potent and selective *in vitro* inhibitory activity against HDAC6. In human prostate cancer (LNCaP and PC-3) cell lines these 4-HBA inhibit cell growth and induce cell death. Accordingly, these compounds modulate microtubule architecture through increased α-tubulin acetylation leading to cell cycle perturbation and reduced migration potential. Furthermore, 4-HBA compounds decrease androgen receptor levels in correlation with increased HSP90α acetylation in LNCaP cells. In conclusion, our results reveal that 4-hydroxybenzoic acid-based derivatives represent promising lead compounds for further drug development or pharmacological tools of selective HDAC6 inhibition with potential for basic research and anti-cancer therapeutic applications.

DNA methylation signature in peripheral blood reveals distinct characteristics of human X-chromosome numerical aberrations

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Abnormal sex chromosome numbers in human are observed in Turner (45,X) and Klinefelter (47,XXY) syndromes. Both syndromes are associated with several clinical phenotypes, whose molecular mechanism are obscure, and show a range of inter-individual penetrance. In order to understand the effect of abnormal numbers of X-chromosome on the methylome and its correlation to the variable clinical phenotype we performed a genome wide methylation analysis using both MEDIP and Illumina Infinium assay on individuals with 4 karyotypes: 45,X, 46,XY, 46,XX and 47,XXY. DNA methylation changes were widespread on all autosomal chromosomes in both 45,X and 47,XXY individuals, with Turner individuals having 5 fold more affected loci. Differentially methylated CpGs in most cases have intermediate methylation levels and tend to occur outside CpG islands, especially in individuals with Turner's syndrome. We verified several loci by pyrosequencing in a large number of individuals and observed only weak inter-loci correlations between the verified regions. This suggests a certain stochastic/random contribution to the methylation of each locus. Interestingly, methylation patterns on some PAR2 loci differ between males and Turners, and between Klinefelters and females, which could have contributed to this distinguished, and unique autosomal methylation patterns in Turners and Klinefelters.

Systematic fine-scale DNA methylation analysis at 5´UTR region of human LINE-1 retrotransposon in common tumors

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Long interspersed nuclear elements [LINE-1 (L1 retrotransposon)] constitute a substantial portion of the human genome, and LINE-1 methylation correlates with global DNA methylation status. Over recent years, several approaches and global assays targeted 5´ regions of LINE-1 and studied its correlation with various human disease states. However, the analyses performed by the existing methods are restricted to two or three CpG sites resulting in underrepresentation of detailed LINE-1 methylation pattern. For better refinement and to assess all CpG sites in the promoter region, we performed the fine-scale systematic DNA methylation analysis at 5´UTR region of human LINE-1 retrotransposon. To achieve this we designed, highly quantitative and reproducible SNuPE based high performance liquid chromatography (SIRPH) assay. Our analysis highlights about previously uncharacterised LINE-1 CpG sites embedded into the LINE-1 promoter whose hypo/hyper methylation patterns are highly tissue specific, particularly colon tissue shows unique methylation patterns. LINE-1 methylation levels were able to distinguish tumor from healthy tissue particularly in bladder and prostate but to less extent in stomach and pancreas. In addition, LINE-1 methylation measurement performed in peripheral blood proved to be associated with gender specific differences, but with non-consistent direction among different CpGs. Additionally, we provide further evidence and we confirm the presence of specific CpG sites that are prone to hypomethylation in both healthy and tumor tissues, which might act as 'seeds' for spreading of hypomethylation in tumors. Overall, our analysis by degenerate assay not only pointed towards tissue and sex specific DNA methylation signature but also provided a useful way to discriminate tumor from healthy tissues.

Comprehensive & Quantitative Multilocus Methylation Analysis in Beckwith-Wiedemann Syndr. and Hepatoblastoma

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Expression of imprinted genes is regulated by DNA methylation of differentially methylated regions (DMRs). There are two kinds of DMRs: maternally methylated DMRs (matDMRs) and paternally methylated DMRs (patDMRs). In addition, there is another classification, into gametic DMRs and somatic DMRs, based on the timing of the establishment of differential methylation. Beckwith–Wiedemann Syndrome (BWS) is an imprinting disorder caused by epimutations of DMRs at 11p15.5. To date, multilocus methylation defects (MMDs) have been reported in BWS patients with epimutations; however, limited numbers of DMRs have been analyzed. The susceptibility of DMRs to aberrant methylation, alteration of gene expression due to aberrant methylation, and causative factors for MMD remain undetermined. To clarify these issues, comprehensive methylation analysis with two quantitative methods, MALDI-TOF mass spectrometry and bisulfite-pyrosequencing, was conducted over 29 DMRs in 54 BWS patients with epimutations (KvDMR1-LOM: 44, H19DMR-GOM: 10). 34% of KvDMR1-LOM patients and 30% of H19DMR-GOM patients showed MMDs. MatDMRs were susceptible to aberrant hypomethylation in KvDMR1-LOM patients, suggesting that matDMRs might be vulnerable to DNA demethylation during the pre-implantation stage when hypomethylation of KvDMR1 occurred. Biallelic expression of the genes was associated with aberrant methylation. *Cis*-acting pathological variations were not found in any aberrantly methylated DMR. In addition, we also analyzed 33 DMRs in 12 hepatoblastomas because hepatoblastoma was one of the embryonal tumors associated with BWS. Hypomethylation was observed at certain DMRs not only in tumors but also in a small number of adjacent histologically normal liver tissues, whereas hypermethylation was observed only in tumor samples. These results suggested the possibility of hypomethylation prior to tumor development. Since 11p15.5 and 20q13.3 loci showed the frequent occurrence of both genetic and epigenetic alterations, these alterations would play an important role in tumorigenesis of hepatoblastoma.

Impact of the antineoplastic lanthanum compound KP772 on *ABCB1* promoter methylation in multidrug resistant epidermal carcinoma cells

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Simultaneous resistance to structurally and mechanistically unrelated drugs, so-called multidrug resistance (MDR), is a significant impediment to successful chemotherapy. MDR is frequently caused by overexpression of ATP-binding cassette (ABC) transporters which act as ATP-dependent drug efflux pumps. Thus, inhibition of ABC transporters is considered a promising strategy to reverse resistance to chemotherapeutic agents. The lanthanum compound KP772, [tris(1,10-phenanthroline)lanthanum(III)] trithiocyanate, was found to show anticancer activity *in vitro* and *in vivo* by inducing cell cycle arrest and/or apoptosis. KBC-1 cells, *ABCB1* overexpressing epidermal carcinoma cells serving as MDR cell model, are particularly sensitive against KP772. Long-term subtoxic treatment of KBC-1 cells with KP772 even resulted in loss of *ABCB1* expression and consequently restoration of the sensitivity to ABCB1 substrate drugs. In the present study we investigated if the loss of *ABCB1* expression in long-term KP772-treated KBC-1 cells is associated with hypermethylation of the promoter region of *ABCB1*. Genomic DNA was extracted from untreated and long-term KP772-treated KBC-1 cells. Subsequently, the extracted DNA was bisulfite converted, amplified by polymerase chain reaction (PCR) and subjected to pyrosequencing. The pyrosequencing method developed in the present study allows to determine the methylation status of 7 CpG dinucleotides in the promoter region of *ABCB1*. In long-term KP772-treated KBC-1 cells, the 7 CpG dinucleotides showed a higher methylation status than those in untreated KBC-1 cells. Our data suggest that the loss of *ABCB1* expression in KBC-1 cells is associated with hypermethylation of the promoter region of the *ABCB1* gene.

High Resolution Melting Analysis versus Pyrosequencing - Determination of the DNA Methylation Status of *ESR2* in Tumor Tissue from Breast Cancer Patients

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Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death in women worldwide. Aberrant DNA methylation patterns are considered as potential biomarkers for diagnosing breast cancer and/or for predicting the efficacy of breast cancer therapy. The present study focused on determining the DNA methylation status in the promoter region of estrogen receptor beta (*ESR2*) in tumor tissue from breast cancer patients. Two different methods were used: methylation sensitive high resolution melting (MS-HRM) analysis and pyrosequencing (PSQ). MS-HRM has a low limit of detection and is rather inexpensive. It can, however, not be applied to determine the methylation status of heterogeneously methylated templates. In addition, one only obtains the average methylation status of all CpG dinucleotides contained in the amplicon. PSQ is rather costly but it can be applied to heterogeneously methylated DNA and makes it possible to determine the methylation status of every single CpG dinucleotide in the amplicon. We therefore applied MS-HRM as a screening tool to select those amplicons requiring more detailed quantitative investigation and used PSQ to accurately determine the DNA methylation status of single CpGs. We found out that biotinylation of one of the primers, which is necessary for pyrosequencing, has no effect on the melting behavior of the DNA. Thus, PCR amplicons subjected to MS-HRM analysis can be directly used for quantitative DNA methylation analysis by pyrosequencing. Furthermore, we could show that by applying an advanced data processing procedure the average methylation status obtained by MS-HRM analysis is in accordance with the mean calculated from the methylation level obtained for single CpG dinucleotides by PSQ.

Promoter methylation status of *RASSF1A, HIN-1* and *MGMT* as indicator for field cancerization in breast cancer

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Aberrant DNA methylation is an early event in carcinogenesis, suggesting that DNA methylation alterations may precede classical transforming events such as gene mutations. In cancer cells, the promoter region of a variety of tumor suppressor genes is frequently hypermethylated, leading to transcriptional silencing of these genes and loss of normal cellular functions. Recent studies have shown that epigenetic modifications, in particular alterations in the DNA methylation status, occur not only in the tumor tissue itself but also in tissue that is adjacent to the tumor and appears histologically normal. The occurrence of aberrant epigenetic (and genetic) signatures in tissues that appear histologically normal has been discussed under the term "field cancerization". Field cancerization is of clinical significance because DNA methylation alterations might drive some of the cells towards the malignant phenotype. Field cancerization is assumed to play a role in local recurrence of cancer. If the field showing these molecular abnormalities is not removed completely by surgery, aberrant DNA methylation patterns might lead to neoplasms and subsequent transformation to a tumor. Thus, the detection of field cancerization could help reduce local tumor recurrences. In the present study we investigated the applicability of the promoter methylation status of six tumor suppressor genes as biomarker for detecting field cancerization in breast cancer. Biopsy samples were taken from tumor, tumor-adjacent and tumor-distant tissue from 17 breast cancer patients. The methylation status of the promoters of *CCND2, DAPK1, GSTP1, HIN-1, MGMT* and *RASSF1A* was determined by methylation-sensitive high resolution melting (MS-HRM) analysis. 94%, 82% and 65% of the tumors showed methylation of *RASSF1A*, *HIN-1* and *MGMT* promoters, respectively. The methylation status of these promoters was significantly lower in tumor-distant tissues than in tumor tissues. Tumor-adjacent tissues showed higher methylation status of *RASSF1A*, *HIN-1* and *MGMT* promoters than tumor-distant tissues, indicating field cancerization.

Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions

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Cervical cancer is caused by a persistent infection with high-risk human papillomavirus (hrHPV), though only affects a minority of infected women and takes 10-30 years. Cervical cancer develops through so-called transforming precancerous lesions (high-grade cervical intraepithelial neoplasia; hgCIN). In hgCIN the normal viral life cycle is aborted and the viral oncogenes E6 and E7 are overexpressed in proliferating cells. This results in the induction of (epi)genetic changes that drive progression to cancer. We demonstrated that the level of molecular aberrations (i.e. DNA copy number changes and DNA hypermethylation) in hgCIN marks the short-term progression risk to invasive cancer. Therefore, these aberrations may serve as molecular disease markers to determine the risk of cancer development in population-based cervical screening programs. Particularly, with the introduction of HPV-testing in cervical screening, there is an urgent need for markers enabling risk stratification of hrHPV-positive women. Towards this goal we assessed the value of several methylated genes as triage marker for hrHPV-positive women. Analysis of cervical scrapes from a population-based screening trial showed that methylation analysis of the genes *CADM1/MAL* or *FAM19A4* was equally discriminatory for hgCIN and cancer as cytology (w/w-o HPV16/18 genotyping) in hrHPV-positive women. Importantly, high methylation levels were shown to reflect the presence of advanced hgCIN lesions and cancer. Studies on self-collected cervico-vaginal specimens of screening non-attendees revealed that methylation marker analysis is also suitable for triage testing on these samples. Clinical validation in a prospective clinical trial showed that the marker panels *MAL/miR-124* and *FAM19A4/miR-124 are* attractive triage assays that allow the detection of hgCIN3 and cancer in HPV-positive self-samples. Other methylation markers are currently under evaluation. In conclusion, methylation analysis is an attractive triage tool for hrHPV-positive women, which is particularly useful for self-collected specimens. This brings full molecular cervical screening to a reality.

DNA methylation as a mediator of early life exposures on risk of childhood acute lymphoblastic leukaemia (ALL)

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Childhood ALL arises from genetic abnormalities that can occur in utero. Reported frequencies of certain genetic abnormalities at birth are significantly higher than the number of associated ALL cases arising, suggesting secondary 'hits' are required for disease development. Evidence suggests several environmental exposures influence childhood ALL risk. DNA methylation is modifiable by environment and altered in childhood ALL, and therefore may plausibly act as a second 'hit' mediating environment and disease. We identified environmental factors associated with childhood ALL (i.e. smoking, maternal alcohol, folic acid, iron, caffeine, sweetened soft drinks, herbicides, pesticides, household chemicals and paints) via literature searches using the NCBI and ScienceDirect databases (1987-2014). The Illumina Infinium HumanMethylation450K platform was used to analyse genome-wide DNA methylation at birth using cord blood from a subset of children (n=836) from the Avon Longitudinal Study of Parents and Children cohort (ALSPAC). ALSPAC also hold environmental data on mothers throughout pregnancy. The effect of environmental exposures associated with childhood ALL risk on DNA methylation at individual sites were analysed using linear regression. DNA methylation was modelled as a continuous variable, in a multivariate regression model accounting for potential confounders (sex, parity, gestation, and batch). Using a stringent FDR of 0.05 for significance, altered gene methylation was associated with maternal smoking (n=83 genes), maternal alcohol intake (n=18), sweetened soft drinks (n=4), and caffeine (n=3). To be more inclusive the criteria was relaxed ($P \leq 0.9 \times 10^{-5}$) and DNA methylation changes were found in association with all of environmental risk factors analysed (n=958 genes combined for all risk factors). Of these, aberrant methylation has been reported for 255 genes in childhood ALL. Environmental influences associated with childhood ALL risk were associated with altered DNA methylation of several genes with reported aberrant methylation in disease. DNA methylation may mediate environmental risks associated with disease development. The authors acknowledge funding from Newcastle University Inst. of Health & Society, North of England Children's Cancer Research Fund and The Newcastle upon Tyne Hospitals Special Trustees NHS Foundation Trust.

Epigenetic Control of Hypothalamic-Pituitary-Axis and Immune Phenotypes

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Environmental regulation of the HPA axis is an important stress adaptation mechanism that is mediated by the differential use of the multiple, epigenetically sensitive, independent, glucocorticoid receptor promoters. These promoter regions have been shown to be activated in a specific manner, producing unique GR transcript profiles for each different cell type. By combining 5' mRNA cap labelling and massively parallel sequencing, we determined the exact GR transcription start sites (TSS). Transcription was not, however, initiated at a fixed position. The GR was found to possess ~30 times more TSSs than previously thought with frequencies from 6.74 x 10-4 % to 38.5% of the total 5' m^7G cap labelled reads. A total of 358 microvariable TSSs in clusters of four to ten adjacent nucleotides, termed loci, were observed. These TSS patterns were cell- and stimuli-specific. This microvariability also has significant consequences for the regulation of the GR by DNA methylation. Complete de-methylation with AZA induced 12 AZA specific TSSs and another 115 that were common to other stimuli or cell type, although after AZA treatment, no new loci were identified. The vast increase in the overall number coupled with only a very small number of demethylation specific functional TSSs, raises doubts over the importance of single nucleotide methylation for the regulation of GR expression. With a ~30 fold increase in GR TSSs, methylation of single CpG dinucleotides within the GR promoter will probably disrupt a small number of TSSs within a nearby locus. As we have previously reported a strong distance dependent correlation in methylation levels with CpGs in clusters of ~125bp, we suggesting that methylation or de-

methylation of such CpG clusters may be necessary to influence total GR levels, by silencing one or more transcriptional locus. Using a mouse neonatal infection model we have also demonstrated that not only can the glucocorticoid receptor and the HPA axis be programmed, but that this programming can be transmitted through several generations. Early-life LPS exposure increased HPA axis activity and reactivity, as well as significantly down-regulating the pro-inflammatory cytokine response (TNFα, IL-6, IL-1β) to a subsequent LPS challenge in adulthood (F0 generation). This was accompanied by a significant increase of global methylation levels in splenic lymphocytes. Only the male (F1) offspring exhibited a programmed cytokine and HPA axis response to an adult LPS challenge, suggesting a male bias and potentially paternal transmission. The F1 generation showed an overcompensation to the F0 programming, with inversely, a decreased HPA axis activity and reactivity, but increased cytokine secretion. The male F2 offspring showed a medium programming, again inversed and similar to the F0. These alternating cytokine and HPA axis responses across the different generations suggests a compensatory hysteresis adjusting the programming, eventually returning to the equilibrium that existed prior to the initial programming. Overall, the stress response is known to be epigenetically sensitive, and we are now starting to understand the underlying molecular mechanisms. We suggest that methylation of single CpG nucleotides does not play a role, rather favouring methylation over broader regions. In our mouse model, early life exposure to LPS provides an inter-generationally transmitted immune and HPA axis programming that appears preliminarily to be transmitted through the paternal line.

Epigenetic mechanisms underlying arsenic-associated lung carcinogenesis

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Arsenic is an established human carcinogen, but the mechanisms through which it contributes to for instance lung cancer development are still unclear. As arsenic is methylated during its metabolism, it may interfere with the DNA methylation process, and is therefore considered to be an epigenetic carcinogen. In the present study, we hypothesize that arsenic is able to induce DNA methylation changes, which lead to changes in specific gene expression, in pathways associated with lung cancer promotion and progression. A549 human adenocarcinoma lung cells were exposed to a low (0.08 µM), intermediate (0.4 µM) and high (2 µM) concentration of sodium arsenite for 1, 2 and 8 weeks. DNA was isolated for whole genome DNA methylation analyses using NimbleGen 2.1M deluxe promoter arrays. In addition, RNA was isolated for whole genome transcriptomic analysis using Affymetrix microarrays. Using a systems biology approach, extensive software applications were used in order to investigate methylation differences between different conditions and additional integrative analyses with transcriptomics data. Arsenic modulated DNA methylation and expression levels of hundreds of genes in a dose- and time-dependent manner. By performing cross-omics analyses, in which whole genome DNA methylation and gene expression data with possibly involved transcription factors were combined, a large molecular interaction network was created based on transcription factor-target gene pairs, consisting of 216 genes. A tumor protein p53 (*TP53*) subnetwork was identified, showing the interactions of *TP53* with other genes affected by arsenic. Furthermore, multiple other new genes were discovered showing altered DNA methylation and gene expression. In particular, arsenic modulated genes which function as transcription factor, thereby affecting target genes which are known to play a role in lung cancer promotion and progression.

The effect of mitochondrial ROS on nuclear epigenetics.

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Mitochondria are often referred to as the powerhouses of the cell, as they produce the majority of the body's energy by oxidative phosphorylation (OXPHOS). As a byproduct of OXPHOS, mitochondria generate reactive oxygen species (ROS). Too much ROS production can damage the mitochondrial DNA (mtDNA), resulting in mitochondrial dysfunction, and vice-versa. As most co-factors required for the activity of epigenetic enzymes are produced during (mitochondrial) metabolism, it is hypothesized that mitochondrial dysfunction would affect nuclear epigenetics. Furthermore, when mitochondrial ROS (mtROS) diffuses to the cytoplasm/nucleus, it might directly react with epigenetic enzymes or nuclear DNA, and as such influence nuclear epigenetics. To study the role of mtROS on nuclear epigenetics, we generated a mtDNA-targeted engineered DNA binding domain (zinc finger protein) fused to a photosensitizing fluorescent protein. This fluorescent protein generates ROS upon exposure to light of a certain wavelength. Mitochondrial localization of the fusion protein was confirmed by confocal microscopy. Further validation of the construct is ongoing: Optimization of dose-response will be monitored by measuring (mt)ROS production, cell viability and site-specific mtDNA damage. After validation, this fusion protein will be used to determine the role of mtROS on nuclear epigenetics. Results of this study are expected to give us a better understanding of how mitochondria can communicate with the nucleus. These insights might help us to better understand the role of mitochondria and oxidative stress in many physiological and pathological processes, including ageing and cancer.

Epigenetic Mechanisms of Aging

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Replicative senescence of cells in culture and aging of the organism are two related processes that are both associated with highly reproducible DNA methylation (DNAm) changes – but there are significant differences in senescence- and age-associated patterns at specific sites in the genome. So far, there is little evidence how DNAm is regulated and whether these modifications are cause or consequence of the aging process. I will demonstrate that specific epigenetic modifications can be

used as biomarker for replicative senescence: an Epigenetic-Senescence-Signature based on six CpGs can be used to track the number of population doublings for quality control of cell preparations. Hypermethylated regions are enriched in H3K27me3, H3K4me3, and H3K4me1 histone marks, whereas hypomethylation is rather associated with H3K9me3 and lamina-associated domains (LADs). Furthermore, an Epigenetic-Aging-Signature based on three CpGs can be used to estimate donor-age of blood samples. Upon allogeneic hematopoietic stem cell transplantation the epigenetic age does not correspond to the patient any more, but to the age of the donor - indicating that the microenvironment of elderly patients has little impact on the epigenetic aging process. Notably, senescence-associated as well as age-associated DNAm changes are reversed in induced pluripotent stem cells (iPSCs) and this may play a central role for their escape from both – replicative senescence and aging. Upon re-differentiation of iPSCs towards mesenchymal stem cells the senescence-associated modifications are reacquired, whereas age-associated patterns remain rejuvenated. Overall, these results demonstrate that replicative senescence and aging are associated with tightly regulated epigenetic modifications.

The Effects of Folate on Transcription of *FOLR1* in Human Colon Adenocarcinoma Cells

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Folate is an essential micronutrient of one-carbon metabolism, is involved in DNA synthesis, repair and methylation. Folate and choline are interrelated and intersecting at the point where homocysteine is converted to methionine**.** The concentrations of these methyl chemicals may affect the function of folate receptor genes via the mechanisms of promoter modification by the methylation. The research aimed to understand whether different reduced folate affects the translation of folate receptor α gene (*FOLR1*) at the constant level of choline(CL). The modified RPMI-1640 with the combinations of 12 μmol/L of CL and both the most oxidative or reduced folate(folic acid, FA; 5-methyltetrahydrofolate,5-MeTHF respectively) at the concentrations of 0, 15, 30 and 120 nmol/L was set as the intervening medium. Human colon adenocarcinoma cell line COLO205 and COLO320 were intervening cultured for 20d and the media were changed every 3 d. The transcription levels of *FOLR1* were analyzed by real-time fluorescence quantitative PCR. The results showed that the transcription levels of *FOLR1*were negative correlated significantly with the concentrations of FA and 5-MeTHF both in two tested cells ($p<0.01$). 5-MeTHF deficiency (15 and 30 nmol/L) was more effective on *FOLR1* expression upregulating than same concentrations of FA in COLO205 ($p<0.01\sim0.05$). We concluded that folate deficiency up-regulates the transcription of *FOLR1*, probably by changing the methylation status of promotor, it may compensate the need of folate of tested cells and reduce the damage induced by folate deficiency.

DNA-methylation- and autoantibody- biomarker development strategies for minimal invasive diagnostics

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It is well accepted that early cancer diagnosis can improve survival, thus there is a great need to identify novel biomarkers for cancer diagnosis at the earliest possible stage, which can ideally be integrated in minimal-invasive diagnostic assays. DNA methylation changes are a hallmark of cancer and these epigenetic changes in tumors can be used as markers for detection of circulating tumor DNA in serum/plasma and saliva samples. Moreover cancer onset and progression produces mutated or aberrantly expressed proteins termed as tumor associated antigens which are able to act as antigens and evoke an immune response which results in the production of autoantibodies. These autoantibodies and the early DNA methylation changes during neoplastic transformation are able to be detected months or years before the clinical diagnosis of cancer and can therefore be used as biomarkers for early cancer diagnostics. We have setup genome-/immunome-wide discovery technologies for elucidation of novel biomarkers. From microarray based discovery-studies we have defined cancer-specific multivariate classifiers with very good diagnostic performance, obtaining AUC-values of 0.9- 1 for the big 4 cancer entities. Efficient targeted multiplexed technologies were established for validation of findings. Using methylation sensitive restriction digestion based high throughput qPCR we can detect 0,1-1% of tumor-derived methylated DNA in the very limited amounts (10ng) of cell free DNA in plasma. As an example data we have tested 680 plasma samples, and could obtain AUC values of 0,84-0,91 for the 4 different lung cancer subtypes using a multiplexed methylation test. In addition we have tested these patients using autoantibody-profiles. Current methodologies and tools in the field of protein-, peptide-based analyses will be presented. Based on findings form different cancer-studies, both DNA methylation and autoantibody based strategies outperform the current clinical diagnostic methods and would be of high value for improving cancer diagnostics and patient management.

The effect of valproic acid on 5-methylcytosine pattern changes in the nuclear and mitochondrial DNA of primary human hepatocytes by using the Methylated DNA Immunoprecipitation Sequencing method

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Valproic acid (VPA) is one of the most widely prescribed antiepileptic drugs worldwide. Despite its pharmacological importance, it may cause liver-toxicity. VPA is a simple fatty acid and induces hepatic steatosis. Furthermore, it has been shown that exposure to VPA resulted in decreased 5-hydroxymethylcytosine (5hmC) but not in the 5-methylcytosine (5mC) contents of mitochondrial-DNA (mtDNA). The aim of this study is to investigate VPA-induced changes in patterns of methylation in nuclear-DNA (nDNA) and mtDNA, and to relate this to mechanisms of liver-toxicity. Furthermore, the persistence of the VPA-induced DNA-methylation changes upon terminating the VPA-exposure will be investigated. Primary human hepatocytes

(PHH) were exposed to 15 mM VPA for 5 days daily followed by 3 days washout. DNA was isolated and used for identification of methylated DNA-regions by using the 'Methylated DNA Immuno-Precipitation -sequencing' (MeDIP-seq) method. Data analysis included: quality control; mapping from reads against the human genome 19 with Bowtie2; selecting significant differentially-methylated-regions (DMRs); annotation of DMRs using HOMER; and merging of consecutive frames/windows. The number of hypermethylated-DMRs and hypomethylated-DMRs of nDNA were roughly the same after VPA-exposure (classified in liver-toxicity related GO-Terms), while after 3 days washout the number of hypomethylated-DMRs of nDNA slightly prevailed over the number of hypermethylated-DMRs. Furthermore, after 3 days washout, the induced changes on DNA-methylation level at day 5 of VPA-exposure remained persistent for some DMRs of nDNA. Finally, we observed hypomethylated-DMRs of mtDNA after VPA-exposure which were not persistently changed after 3 days washout. In general we conclude that repetitive exposure of PHH to VPA leads to significant changes in patterns of 5mC in nDNA and mtDNA. After the washout, some methylation changes in DMRs of nDNA remained persistent while DMRs of mtDNA were not persistent. Finally, we speculate that there is a crosstalk between the nDNA and mtDNA methylation changes after VPA-exposure.

Epigenetic changes in prostate cancer metastasis: the role of microRNAs

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Distortions of the epigenome, including changes in DNA methylation, altered histone modifications and deregulated microRNA (miRNA) expression, are functionally associated with prostate cancer initiation and progression. miRNAs are endogenous, small non-coding RNA molecules, acting as key regulators of gene expression, mainly at a post-transcriptional level. In addition, miRNAs can affect gene expression by targeting effectors of the epigenetic machinery. Growing evidence supports the involvement of miRNAs in the most salient phases of the multistep cascade fostering a prostate cancer cell to leave the primary tumor and acquire the ability to form secondary lesions at distant sites. In my presentation, after a survey of specific miRNAs proved to be involved in *i)* the regulation of epithelial-to-mesenchymal transition (EMT) and cell migration, *ii)* the interplay between cancer cells and the surrounding stroma, *iii)* the control of angiogenesis, and *iv)* the regulation of anoikis, I will focus on miR-205, which we found to affect multiple aspects of the metastatic process in prostate cancer. Such miRNA was consistently found down-regulated in prostate cancers compared with matched normal prostate tissues in different studies, with a particularly pronounced reduction in carcinomas from patients with disseminated disease. We also demonstrated that miR-205 participates in a network involving ΔNp63α, which is essential for maintenance of the basement membrane in prostate epithelium. The relevance of miR-205 in the control of prostate cancer metastasis relies on the observation that its reconstitution in prostate cancer cells *i)* exerts a tumor-suppressive effect by counteracting EMT and reducing cell migration/invasion, at least in part through the down-regulation of its target PKCε, and *ii)* counteracts cancer-associated fibroblast (CAF)-induced EMT, thus impairing enhancement of tumor cell invasion, acquisition of stem cell traits, tumorigenicity, and metastatic dissemination. In addition, miR-205 blocks tumor-driven activation of surrounding fibroblasts by reducing secretion of the pro-inflammatory cytokine IL-6, suggesting miR-205 as a brake against PCa metastasis by blocking both the afferent and efferent arms of the circuit between tumor cells and CAFs, thus interrupting the pro-oxidant and pro-inflammatory circuitries engaged by reactive stroma. Finally, the possibility to manipulate metastasis-related miRNA functions as a novel therapeutic strategy for prostate cancer will be discussed.

Is Mercury a carcinogen? What is its mechanism of action? A hypothesis beyond genotoxic effects, beyond inhibition of intercellular communication, through epigenetic effects.

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There are several theories of carcinogenesis, because the whole scientific community doesn't agree with its causes and earlier phases. Paul Vineis et al., reviewing the main five current carcinogenesis models, proposed that they could be accommodated in two models thereby facilitating our understanding of how the cancer disease develops. Accordingly, (i) biological changes in the epithelium alone lead to malignancy and (ii) changes in stroma/extracellular matrix are necessary (along with changes in epithelium) for malignancy. Our experiences on mercury cytotoxicity, investigated through the study of its effect on the intercellular communication via Gap Junctions might explain the carcinogen effect of this metal. Accordingly, it is well---known that a promoter effect could occur by the inhibition of intercellular communication. However, this mechanism alone does not enable to classify mercury as carcinogen. Among many theories that try to explain this complex disease, lastly the theory of the microenvironment has gained many supporters. Bissell et al highlighted that the emergence of cancer is not a cell-autonomous phenomenon but is heavily dependent on microenvironmental cues derived from the surrounding tissue. Laconi et al affirmed that the resulting altered tissue architecture translates into the emergence of a unique tumour micro-environment inside these lesions, associated with altered blood vessels and/or blood supply that in turn can trigger biochemical and metabolic changes fuelling tumour progression. Summing up the main outcomes of our studies we propose that the carcinogenic effect of mercury might ensue from: 1) inhibition of gap junctions-mediated intercellular communication linked to increased production of reactive oxygen species; 2) reduction of the pro-inflammatory interleukins IL1 Beta and TNF alpha causing alteration of the earlier immune response. These two mercury-induced key events might act synergically to transform a group of proliferating cells in a war machine highly capable of invading the host tissues.