Material and Methods: We have compared the secretomes from senescent versus young fibroblasts using secretome proteomics, combined with Western-blotting, ELISA, RT-PCR and zymographies. A fraction of NHEK growth culture medium was replaced by fibroblast (either young or senescent)-conditioned medium to investigate the secretome impact on neoplastic initiation of NHEKs.
Results: Four major groups of proteins were modulated in senescent vs young fibroblast secretomes. The expressions of extracellular matrix collagens, SPARC, and decorin were strongly affected in senescent fibroblasts secretome; expression and activation of numerous metalloproteinases were promoted, while the expression of their inhibitors was reduced; growth factors (among which HGF/SF) and cytokines were overexpressed in association with the loss of anti-angiogenic molecules. Senescent fibroblasts could then relevantly contribute to a tumour-promoting environment. Hence, the replacement of a fraction of primary NEHKs' growth culture medium by conditioned medium from normal primary senescent fibroblasts induced a strong promotion of the neoplastic initiation from primary NHEKs emerging from senescence in our culture model. It led to the acquisition of enhanced migratory and scattering capacities, and the development of small clones in soft Agar.
Conclusions: These results point to the microenvironment of normal aging fibroblasts as a factor promoting initial changes in normal human keratinocytes emerging from replicative senescence in vitro that result in cancerous phenotype.

867 Long-term GLI1 expression induces mammary gland tumour formation in nulliparous transgenic mice

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Background: The main effectors of the Hedgehog (Hh) signalling pathway are the zinc finger transcription factors of the GLI family. In human breast cancer, up regulation of GLI1 expression correlates with unfavourable overall survival. We have previously shown that multiparous conditional transgenic mice expressing GLI1 develop hyperplastic lesions and tumours. Furthermore, the Hh pathway is thought to be involved in the regulation and maintenance of CD44 positive breast cancer stem cells. Skin stem cells with active Hh signalling pathway as well as intestinal stem cells express the orphan G protein coupled receptor LGR5. The expression pattern and role of LGR5 in mammary gland tissue and cancer is not known.
Material and Methods: GLI1 expression was induced, up to 108 weeks, in female transgenic mice (MMTVrtTA;TREGLI1 and MMTVrtTA;TREGLI1;Lgr5-LacZ). The mice were monitored for the occurrence of tumours. Palpable tumours and hyperplastic lesions developed in the mice with induced GLI1 expression. Normal and tumour tissue were analysed by immunohistochemistry.
Results: Hyperplastic lesions and palpable mammary gland tumours, including solid and acinar adenocarcinomas, developed in the nulliparous mice after long-term low level GLI1 expression. Both cytokeratin 5 (K5) and cytokeratin 6 (K6) positive tumour cells were detected. Only few tumours also harboured some cytokeratin 18 (K18) positive cells. The expression of the stem cell marker CD44 was increased in the mammary ducts and tumours in the GLI1 positive mice. Lgr5 was expressed in the basal cell layer of the large mammary ducts as well as in the GLI1 induced tumours.
Conclusions: Mammary gland specific, long-term expression of GLI1 induces formation of different types of K5 and K6 positive tumours with basal character in transgenic mice. Induction of various types of tumours and expression of Lgr5 in the tumours as well as increased expression of the stem cell marker CD44 indicate that the expression of GLI1 affects mammary stem cells.

868 Genotoxicity/clastogenicity of ptaquiloside, the bracken (Pteridium aquilinum) carcinogen, towards human peripheral blood lymphocytes

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Background: Ptaquiloside (PTA), a bracken toxin, is a known carcinogen for animals but its implications on public health remain controversial (Yamada *et al*., 2007). This work addresses PTA's genotoxicity for human peripheral blood lymphocytes.
Material and Methods: PTA was isolated from bracken shoots collected in Ponte da Barca, Portugal, following methods by Ojika *et al*. (1985). Nuclear magnetic resonance techniques were used to confirm the compound's identity. The alkaline comet assay was performed according to Costa *et al.* (2008) on cells from 10 healthy donors exposed to 5 μg/ml PTA (or DMSO, negative control) in RPMI at 37ºC, for 5, 10, 20, 30, 40 or 50 min. Electrophoresis took place at 30 V for 20 min. Comet Assay IV (Perspective Instruments) software was used for slide analysis and for calculating tail intensity (TI). For chromosomal aberrations (CA) (5 donors) and sister-chromatid exchanges (SCE) tests (2 donors) cells were cultivated on supplemented RPMI. PTA was added at 24h (5, 10 or 20 μg/ml final dilutions). Bromodeoxyuridine was added to replicate cultures for SCE. Cells were then incubated for 48h and harvested after colcemid arrest. CA and SCE were counted on 100 and on 25 metaphases for each donor, respectively.
Results: The TI values for control/exposed cells at 5, 10, 20, 30, 40 and 50 min were 4.85/6.17, 5.11/5.75, 3.61/22.60, 4.77/28.53, 1.76/12.76, 1.62/10.52, respectively. Cytogenetic results were expressed, for controls and for each PTA dilution (5, 10 or 20 μg/ml) as the mean percentage of aneuploid cells (3, 15.3, 22.7, 46.4 respectively) and cells with chromosome/chromatid gaps and breaks (0.2, 2.4, 7.2, 14.5), mean number of gaps and breaks per 100 cells (0.2, 2.4, 7.8, 16.4), and the mean number of SCE per cell (9.4, 14.2, 18.4, 25.7).
Conclusions: The comet assay demonstrated that even PTA doses as low as 5 μg/ml are enough to induce DNA damage in a human *in vitro* model. Maximum damage was observed at 20−30 min, diminishing at 40−50 min, presumably due to DNA repair mechanisms. The cytogenetic tests show that at 48 h, despite such mechanisms, PTA originates structural and numeric CA and increased SCE in a dose-dependent manner. This suggests that PTA exerts its genotoxicity through multiple mechanisms and further support the hypothesis that PTA represents a significant threat to public health.

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869 The lichen compound usnic acid disturbs mitochondrial function and induces autophagy in cancer cells

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Background: The lichen compound usnic acid (UA) is a component of the fat-burner Lipokinetix and has been shown to reduce ATP production in liver cell mitochondria. The effect on mitochondria can be directly related to the property of UA to shuttle protons across membranes. Autophagy is a process that can aid cell survival during nutrient shortage. UA inhibits the growth and proliferation of cancer cells but does not induce apoptosis.
Aims and Methods: To test for changes in inner membrane mitochondrial potential using JC-1 staining and measure levels of cellular ATP in UA-treated breast and pancreatic cancer cells. Also, to test if cells treated with UA showed signs of autophagy, using electron microscopy and immunostaining for the autophagosomal marker LC-3 and Western blotting for the autophagosomal cargo p62.
Results: A drop in inner membrane mitochondrial potential was demonstrated and reduced levels of ATP were observed in breast and pancreatic cancer cells treated with 5 μg/mL and 10 μg/mL of UA for 24 hours. Clear signs of autophagy were seen after treatment with UA, but results indicate that degradation of p62 does not occur. Therefore, in ongoing experiments we are testing for autophagosomal-lysosomal fusion and acidification using a tandem-tagged mRFP-GFP-LC3 fusion construct.
Conclusion: UA treatment of cancer cells causes a drop in mitochondrial membrane potential leading to reduced ATP production. This stimulates autophagy but apparently without degradation of autophagosomal content.

870 The lichen compound protolichesterinic acid affects lipid metabolism and induces ER stress in cancer cells

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Background: The lichen metabolite protolichesterinic acid (PA) is a potent inhibitor of 5- and 12-lipoxygenase and has anti-proliferative effects on several types of cancer cells, as well as inducing apoptosis in multiple myeloma cells. Fatty acid synthase (FAS) is highly expressed in human carcinomas and appears to be required for their survival. The chemical structure of PA is very similar to known FAS inhibitors. Aims and methods: To test if PA inhibited FAS by measuring uptake of ^{14}C-acetate into cells and to test for ER-stress, which is a known consequence of FAS inhibition, using Western blotting for phosphorylated-eIF2α. Signalling through major stimulatory pathways was tested by measuring activation of ERK1/2 and STAT3.
Results: Uptake of ^{14}C-acetate into breast cancer cells was reduced in a dose-depedent manner by PA reaching 33% at 10 μg/mL. The same concentration

of the known inhibitor cerulenin caused 40% inhibition. PA treatment led to increased expression of phosphorylated-eIF2α after treatment for 6 hours with 2.5 and 5.0 μg/mL. Constitutive activation of STAT3 in U266 multiple myeloma cells was not affected by PA and activation of ERK1/2 in RPMI-8226 multiple myeloma cells was only partially inhibited.
Conclusion: The results suggest that the anti-proliferative and pro-apoptotic effects of PA are not primarily mediated through inhibition of signalling from growth factor receptors but may be the consequence of ER-stress possibly related to disturbed lipid metabolism.

871 Deficiency of the WWOX Fragile Gene Impairs DNA Damage Response

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Common chromosomal fragile sites are preferential targets of replication stress in preneoplastic lesions, resulting in deletions involving archetypal fragile genes encoded at these conserved chromosome regions, such as *FHIT* and *WWOX*. The *WWOX* (WW domain-containing oxidoreductase) gene encompasses the second most active chromosomal fragile site, FRA16D; a region involved in loss of heterozygosity and homozygous deletions in cancers and cancer-derived cell lines, in chromosome translocations in multiple myeloma, and its promoter region is frequently hypermethylated in cancers. Indeed, Wwox expression is reduced or absent in most common human cancers and its restoration in Wwox-negative cells suppresses tumourigenicity both *in vitro* and *in vivo*. Targeted ablation of the *Wwox* gene in mice demonstrated *bona fide* tumour suppressor function. Recently, it has been suggested that damage to fragile sites, with lost function of genes located at these sites, is coincident with activation of DNA damage response (DDR) checkpoint proteins suggesting that fragile sites might function as DNA damage warning sensors. Nevertheless, role of the *WWOX* fragile gene and the mechanism it might play in DDR are largely elusive. Here, we demonstrate that *Wwox*-deficient murine fibroblasts (MEF) display increased number of total chromosomal breakage as compared to wild type counterparts following treatment with aphidicolin, a mild DNA replication inhibitor. Overexpression of Wwox in *Wwox*-deficient MEF rescued this phenotype. Moreover, our findings show that this genomic instability in murine fibroblast is associated with delayed gH2AX foci formation. Furthermore, manipulation of Wwox expression in human cancer cell lines is associated with altered DDR checkpoint activation and DNA repair. Our data suggest that loss of the *WWOX* fragile gene product impairs DDR thus contributing to genomic instability. These findings present a fresh prospective on the role of Wwox as a tumour suppressor, which is inactivated early in pre-neoplastic cells, and how its loss may provide a selective advantage for clonal expansion of neoplastic cells.

872 Promoter hypermethylation in Bulgarian patients with glial and laryngeal cancer

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Background: Promoter hypermethylation is one of the major mechanisms in the transcriptional inactivation of certain carcinoma-associated genes. O6-methylguanine-DNA methyltransferase (MGMT) repairs the cytotoxic and mutagenic O6-alkylguanine produced by alkylating agents such as chemotherapeutic agents and mutagens. *hMGMT* expression is inversely linked to hypermethylation of the CpG island in the promoter region. Methylation in the promoter region of the DNA mismatch repair gene *hMLH1* is responsible for its inactivation and is associated with increased mutations in simple repeats in genomic DNA and microsatellite instability. The methylation analysis of these DNA repair genes may provide important information about laryngeal and glial carcinogenesis.
Materials and Methods: Genomic DNA was extracted from 50 tumour tissue samples (30 glial and 20 primary laryngeal tumours) and bisulfite conversion was performed. All samples were analyzed for promoter hypermethylation of *MGMT* gene by using a methylation-specific polymerase chain reaction (MSP) assay. The other DNA repair gene *hMLH1* was analyzed by MSP in 20 primary laryngeal carcinomas.
Results: MSP analysis demonstrated hypermethylation of *hMGMT* gene in 9 patients (30%) with glioma and 6 patients (30%) with laryngeal cancer. Promoter hypermethylation of *hMLH1* was observed in 11 (55%) of the cases with laryngeal cancer, whereas promoter hypermethylation of both *hMLH1* and *hMGMT* was found only in 3 cases (15%). The epigenetic inactivation of *hMLH1* and *hMGMT* in Bulgarian patients was detected in similar frequencies to relative studies of both cancers.
Conclusions: Our results indicate that methylation modifications in *hMLH1* and *hMGMT* genes are implicated in a significant proportion of cases with glial and laryngeal cancer.

873 Association study of polymorphic variants in chromosome locus 8q24 linked with prostate cancer in Bulgarian patients

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Background: In developed countries, prostate cancer (PC) is the most common noncutaneous malignancy in men. The molecular pathology of PC is not clear yet. Twin studies and epidemiologic observations have suggested a substantial genetic contribution to the disease risk. Linkage, admixture mapping and genome-wide studies have identified variants with moderate effects on PC risk at multiple loci in 8q24. Three distinct regions within this hot spot locus in the genome have been associated with PC risk. The locus itself is a 1.2-Mb region devoided of genes, delimited by the genes *FAM84B* and *MYC*. It is not yet known how 8q24 variants influence PC development.
Material and Methods: We have performed a case control study of the polymorphic variants rs1447295, rs16901979, and rs10505477 on locus 8q24 for association with PC. One hundred and ten PC samples and 195 controls were genotyped by using TaqMan® method.
Results: The three polymorphic variants did not show association with increased PC risk after comparison of all samples and controls. Significant association was found for rs6983267 and rs10505477 when we compared genotype and allele frequencies of patients with Gleason score above seven with the controls samples. The A/A genotype of rs10505477 (OR = 3.29, 95% CI=1.38–7.83, p = 0.007) and G/G genotype of rs6983267 (OR = 3.04, 95% CI=1.28–7.24, p = 0.011) showed association with PC in patients with Gleason score above 7. The results for the A allele (OR = 2.06, 95% CI=1.10–3.89, p = 0.016) and the G allele (OR = 1.94, 95% CI=1.03–3.65, p = 0.027) of the same variants are analogous and show statistical significance.
Conclusions: Although rs1447295 is not associated with the total PC risk or with grade and stage of the carcinoma, rs6983267 and rs10505477 demonstrated association with PC in Bulgarian patients with high Gleason score. These two polymorphisms lead to three fold increased risk for development of aggressive form of the disease.

874 Large genomic aberrations in MSH2 and MLH1 genes in Bulgarian colorectal cancer patients

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Background: Hereditary nonpolyposis colorectal cancer is caused by inactivating mutations in the genes of the DNA mismatch repair (MMR) system. Previous studies have shown that large-fragment aberrations in MMR genes are responsible for a considerable proportion of hereditary colorectal cancer (CRC) in different populations.
Material and Methods: In the present study we performed ligation-dependent probe amplification analysis (MLPA) of large genomic *MLH1*/*MSH2* alterations in 38 Bulgarian patients with CRC, in which neither epigenetic changes nor mutations were found by traditional screening methods.
Results: The frequency of the large genomic *MLH1*/*MSH2* alterations was 13.2%, which was in consistency with previous studies in other populations. One deletion was found in *MLH1* (2.6%): **del MLH1 ex 7** in a patient from family with Lynch syndrome. The observed genomic alterations in *MSH2* were four (10.5%). Two patients from HNPCC families possessed **dup MSH2 ex 9** and **del MSH2 ex 4**, respectively. The **del MSH2 ex 1** and **del MSH2 ex 3** were found in two patients with sporadic CRC and early onset, correspondingly. All cases with deletions/duplications correlated with high microsatellite instability.
Discussion: Our results indicate that genomic large-fragment deletions and duplications in *MLH1* and *MSH2* genes play a role in the pathogenesis of Bulgarian patients with both familial and sporadic CRC, as reported in other populations.