Materials and Methods: We have tested a CRC biomarker DNA chip (*RanplexCRC®*, Randox Laboratories) which assays 28 mutations in 4 CRC-related genes (*K-ras, APC, BRAF, TP53*). These represent the most genetic markers ever to be tested simultaneously in a CRC screening study. Our test series include (1) DNA from the matched tumour and normal tissue of German CRC patients (N = 55) and (2) An Irish sample cohort (N = 250) from participants who have undergone colonoscopy following positive iFOBT results, comprising samples of stool and tissue from 100 patients with polyps and advanced adenomas, 50 with CRC, and stool from 100 individuals normal after colonoscopy. We use spin column extraction (using sample specific *Qiagen* kits) for DNA isolation.
Results: From the German CRCs, mutations were observed in 71% of the cancerous tissues (39/55) and 25% of the matched 'normal' tissue (13/55). In a majority of cases mutations were from *Kras* and *p53* (30 of the 46 mutations detected), suggesting a signature for advanced stages as the German CRCs tested were stage 2 and 3. The majority of the mutated tumours had only one mutation (25 out of 39). To test assay utility in screening for colorectal neoplasms we are currently examining in our Irish iFOBT +ve cohort how the mutation profile in the disease and normal matched tissue is reflected in DNA isolated from the stool of our patients. Mutation detection is possible in 1000-fold excess of wild-type DNA. The initial advanced adenoma results (8 mutated out of 16) indicate a maximum detection rate of 50% for these highly screen relevant growths, which if confirmed in the stool screening would be superior to FOBT. None of the matched normal adenoma tissues were mutated.
Conclusions: These data confirm the high detection rate of DNA mutations in CRCs and show that mutations associated with CRC carcinogenesis can be detected from advanced precancerous colorectal lesions using the *Ranplex* method. Genetic assays have great potential to achieve highly specific and sensitive detection of colorectal neoplasias, especially for eminently treatable early stage cancers and advanced adenomas. In addition the types of mutations detected, such as in *Kras*, will also provide important molecular prognostic information separate to detection alone.

129 Molecular diagnosis of pancreatic cancer using a combination of genetic and epigenetic markers

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Background: *KRAS* mutations have proved useful as a diagnostic marker in FNA of pancreatic masses. Methylation markers have shown promise in the early diagnosis of pancreatic carcinoma. The aim of this study was to assess the diagnostic utility of hypermethylation status of candidate genes in combination with *KRAS* mutation detection in the evaluation of pancreatic masses.
Experimental design: As a first step the methylation status of *HRH2*, *EN1*, *SPARC, CDH13, APC, RASSF1A, TMS1, CRBP1, INHBB, CPLX* and *RARB2*, was tested in paired tumoural and non tumoural tissue samples of 11 primary pancreatic adenocarcinoma by melting curve analysis (MCA) optimized to have an analytical sensitivity of 5%. Sixty-one Fine Needle Aspirates (FNA) of pancreatic masses (43 pancreatic adenocarcinomas and 18 chronic pancreatitis) were studied; their methylation status was analyzed using Melting Curve Analysis after DNA bisulfite treatment. *KRAS* mutations were detected by a Restriction Fragment Length Polymorphism/Polymerase Chain Reaction (RFLP/PCR) method.
Results: Five genes where promoter hypermethylation was detected in more than 50% of analyzed tumours were selected: *HRH2*, *EN1*, *SPARC*, *CDH13* and *APC*. The methylation panel had a sensitivity of 73% (27 of 37) and a specificity of 100% whenever two or more promoters were found hypermethylated. *KRAS* mutations showed a sensitivity of 77% (33 of 43) and a specificity of 100%. When combined, genetic and epigenetic analyses, resulted in a sensitivity of 84% maintaining the 100% specificity.
Conclusion: Both genetic and epigenetic molecular analyses offer an excellent diagnostic yield in this setting allowing cancer diagnosis in an overwhelming majority of the cases. The good clinical performance is more relevant at a time where EUS-guided FNA is increasingly used in the preoperative evaluation of small pancreatic masses.

130 GINS proteins as candidate markers for urine-based cancer screening

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Background: A urine-based screening strategy for urogenital tract malignancies would offer an affordable non-invasive alternative to current approaches. The GINS complex (a heterotetramer of Sld5, Psf1, Psf2 and Psf3) is a highly conserved DNA replication factor. GINS interacts with the MCM complex in eukaryotes and archaea. Given the value of MCM proteins as specific and sensitive markers for cancer screening, we investigated whether GINS subunits might also be of potential diagnostic value in applications where the sensitivity and/or specificity of MCM proteins is low.
Materials and Methods: We investigated the expression of GINS proteins in human cell lines by immunoblotting, and in tissue sections by immunohistochemistry. We also tested the stability of GINS proteins by incubating intact tissue culture cells or cell lysates in urine. Finally, we have set up an ongoing clinical study of Sld5 protein as a potential biomarker in urine samples from prostate and bladder cancer patients. In this study, we have screened for Sld5 and Mcm2 by multichannel immunofluorescence microscopy in the cellular fraction and measured Sld5 protein levels in the urine supernatant by ELISA.
Results: Consistent with the published findings for MCM, GINS proteins are expressed in all cycle phases of cultured proliferating cells, but are down-regulated in cells undergoing quiescence. Examination of histological sections indicates that Sld5 and Psf3 expression is restricted to the proliferative compartment in normal tissue, but spreads to the majority of cells in a wide range of dysplastic and malignant tissues, including cervix, colon and skin. Our urine spiking experiments suggest that Sld5 protein is more stable than Mcm2 in harsh extracellular environments. In the pilot clinical study of over 50 prostate and bladder cancer patients, Sld5 was readily and specifically detectable in the cellular fraction of the samples from cancer patients. We also present an evaluation of Sld5 protein levels in the supernatant portion of those same urine samples as an easy-to-screen diagnostic/prognostic marker for male urogenital cancers.
Conclusions: Owing to their stability, GINS proteins hold promise as independent or complementary markers to the MCM proteins for cancer screening in harsh extracellular environments such as urine. Work is ongoing to identify further applications of GINS in cancer screening and prognosis.

131 Relevance of copy number alterations in Ewing Sarcoma: gain of 1q defines a subset of patients with worse outcome, probably caused by DTL/CDT2 overexpression and subsequent cell cycle deregulation

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Background: Despite extensive characterization of the molecular implications of EWS-ETS, little is known about secondary genetic alterations in Ewing Sarcoma (ES), the only ones that could shape molecular subtypes of the disease with prognostic significance. Research in this field is crucial since the prognosis is poor in patients with primary disseminated disease or after relapse, with a survival rate of nearly 20%.
Material and Methods: *Patient and samples*: tumour samples from a total of 105 ES patients were included in the study along with 16 ES cell lines.
BAC-microarrays: Sanger 1Mb BAC collection was amplified and spotted in triplicate onto Codelink slides (GE). Cy5/Cy3-dCTP-labelled DNA from tumour samples and healthy donors were co-hybridized with the BAC-microarrays.
Expression microarrays and SNP-microarrays: Total RNA from *DTL*-silenced cell lines was extracted with Trizol (Invitrogen) and hybridized with the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix) after quality assessment using an Agilent 2100 Bioanalyzer (Agilent).
Lentiviral shRNA transduction of ES cell lines for functional validations: The MISSION shRNA collection of 5 pLKO.1-shRNA constructions (SIGMA Aldrich) was selected for assays aimed at reducing *DTL* overexpression.
Results: We assessed copy number alterations (CNA) in **67 ES tumours**, finding 1q gain (31% of tumours) markedly associated with relapse and poor

survival. The 1q gain was also related to a profile of cell cycle deregulation and to overexpression of *DTL* in an independent **38-tumour set**. DTL proved to be a potent regulator of the cell cycle by abrogating p53, p21 and p27 and to be a major contributor to the 1q gain-expression profile. Moreover, tumours with this alteration showed higher proliferation rates as assessed by Ki-67 immunohistochemistry.
Other relevant findings from the CNA study were: the percentage of genome affected by CNA per sample proved a tight and progressive correlation with overall survival. The gain of chromosome 8 was associated with metastasis location in lungs. Several smallest regions of overlap were defined, containing relevant candidate genes.
Conclusions: CNA have a marked impact on ES outcome. The gain of 1q molecularly defines a substantial subset of ES patients with worse outcome who could benefit from new prognostic biomarkers (1q gain/*DTL* overexpression) and from specific targeted therapies.

132 POLQ up-regulation is associated with poor survival in breast cancer, perturbs DNA replication and promotes genetic instability

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Background: "Replicative stress", one of the main factors underlying neoplasia from its early stages, can arise from a deficiency in DNA replication. Genes involved in DNA synthesis may therefore represent an under-explored source of prognostic markers with therapeutic potential in cancer.
Material and Methods: Gene expression profiles were generated here from two independent cohorts (France and the UK; n = 206 and n = 117, respectively) of previously untreated primary breast cancers. We also generated human cells that mimicked the observed genotype.
Results: We report here that among the 13 human nuclear DNA polymerase genes, the Polq gene (or *POLQ*) is the only one significantly up-regulated in breast cancers compared with normal breast tissues. Importantly, *POLQ* up-regulation significantly correlates with poor clinical outcome, with a 3.1-fold increased risk of death. *POLQ* expressionwas independent of Cyclin E expression, which is also correlated with a poor prognosis in breast cancer. In addition, we show that POLQ expression can discriminate the survival outcome of patients with a high number of positive lymph nodes, considered to date as a negative marker for breast cancer.
POLQ is a specialized DNA polymerase believed to function primarily in the replicative bypass of endogenous DNA damage. Aiming to decipher the molecular consequences of *POLQ* up-regulation, we generated human cells stably over-expressing this polymerase. Our data shows that a high level of *POLQ* gene expression was directly associated with defective DNA replication fork progression and double strand break induction, resulting in cancer-associated chromosomal aberrations.
Conclusions: We propose that *POLQ* over-expression may facilitate tumour selection and progression through DNA replicative stress. In addition, it is a new promising prognostic marker with therapeutic potential.

133 mTOR is a druggable molecule in Malignant Pleural Mesothelioma targeted therapy: antiproliferative effect of sorafenib and everolimus in preclinical models

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Background and Rationale: Malignant Pleural Mesothelioma (MPM) is an aggressive tumour, with no effective therapies and an increasing incidence as a result of widespread exposure to asbestos. The identification of molecular targets for novel therapeutical strategies is mandatory. With this work, we aim at (i) indagate activated oncogenic pathways in MPM focusing on PI3K–AKT–mTOR and the BRAF/KRAS/MAPK pathways and (ii) explore the preclinical efficacy of the multikinase inhibitor sorafenib (SOR) and the mTOR specific inhibitor, everolimus (EV).
Methods: FFPE sections were obtained from patients diagnosed and followed at IRCCs San Matteo of Pavia. Phospho-mTOR expression was checked by immunohistochemistry. Genomic DNA was then extracted from MPM and corresponding surrounding non tumoural tissues. The mutational status of oncogenes in PI3K and MAPK pathways was assessed by PCR and sequencing. 3 human MPM cell lines established from the pleural effusion were treated with scalar doses of SOR, EV and their combination for 72 hours. The IC50 values and the combinatorial index (CI) were calculated with Calcusyn.
Results: The mTOR kinase was activated in all the MPM samples and in the adjacent hyperplastic mesothelium but not in normal not-transformed mesothelium. No mutations were found in "hot spot" coding regions in EGFR (exons 18−21), KRAS (exon 2), BRAF (exon 15) PIK3CA (exons 9−20) genes. In vitro assays demonstrated that SOR and EV have synergistic effect in the inhibition of MPM cell line proliferation: SOR showed a dose dependent inhibition effect (IC50 = 1.72 μM). EV alone is able to affect no more than 35% cell line proliferation. In combination they displayed synergistic effects (the IC50 of SOR was reduced to 0.91 μM and the IC50 of EV was calculated (45.54 nM) in combinatorial schedule.
Conclusions: Our results suggest that mTOR kinase is highly activated in MPM onset. This status is not consequent to the occurrence of activating mutations affecting the PI3K and MAPK signaling. SOR is a promising therapeutic molecule in MPM mostly in combinatorial schedule with EV. Molecular studies will further elucidate the cross-talk between pathways.

134 Dissecting the mTOR pathway in osteosarcoma

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Background: Osteosarcoma (OS) is the most common bone tumour of the paediatric age. Despite improved prognosis, recurrent or metastatic forms are still fatal. A better understanding of molecular mechanisms involved in OS onset, progression and metastatization is a clear priority both in the assessment of targeted agents and to identify and select patients that are likely to achieve a clinical benefit. Our work is focused on the identification of OS activated oncogenic pathways and we specifically are studying the PI3K/AKT/mTOR pathway. To this goal we planned to use a triple approach (i) analysis of immunophenotype and mutational profile on 30 OS samples (ii) *in vitro* studies with molecular targeted drugs commercially available such as the multikinase inhibitor sorafenib (SOR) and the mTOR specific inhibitor, everolimus (EV) (iii) *in vivo* experiments on OS xenografts.
Material and Methods: The activation of mTOR pathway was assessed by immunohistochemistry in 30 samples from patients diagnosed at Istituti Ortopedici Rizzoli of Bologna. Primary antibodies against phospho-mTOR (Abnova) and PTEN (C-term, Millipore) were used. Mutational analysis by PCR and Sanger sequencing was performed starting from DNA extracted from OS and surrounding non tumoural tissues when present. Effects of EV (from 500 to 15.125 nM) and SOR (from 10 to 0.3125 μM) were tested on cell proliferation (Cell Titre GLO assay), cell cycle (flow cytometry) and apoptosis (Annexin V/PI). Synergism (SOR+EV) was calculated through normalized isobologram and combination index (CI). 10^6 OS cells were injected in SCID mice. After tumour establishment, mice were orally treated for 6 wks with SOR (5 and 1 mg/kg/day) or EV (1 and 0.1 mg/kg/day) and their combination.
Results: Phospho-mTOR is overexpressed in only 5/30 samples. PTEN expression is lost in the majority of samples (28 out of 30). No "hotspot" mutations were found in KRAS (exons 1−2), PIK3CA (exons 9−20) and Akt (exon 1). *In vitro* assays demonstrated that EV alone is able to inhibit no more than 40% of cell proliferation but it synergistically potentiates the antiproliferative effect of SOR after 72 hours of treatment. The activity of the 2 drugs as single agent or in combination orally administered to OS-bearing NOD/SCID mice will be presented.
Conclusions: This work shows the in vitro and in vivo antiproliferative effect of SOR, EV and their combination. This pharmacological approach warrants to be tested in OS clinical setting.

135 Epigenetic control of pi3k/akt activity in prostate cancer during hormone manipulation

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In advanced stages of prostate cancer, the phosphatidylinositol-3′ kinase (PI3K)/Akt signaling cascade, one of the major survival pathways in the cell, is frequently and constitutively activated due to increased loss of the tumour suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and/or increased expression/activity of growth factor receptors.
Here we asked whether the increase in Akt phosphorylation may contribute to the development of androgen independence.
To mimic the clinical situation and to test the role of androgen manipulation in prostate cancer progression we cultured androgen receptor positive 22rv1