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## Nuove veicolazioni farmacologiche e piattaforme tecnologiche per la diagnosi e terapia di tumori e malattie cronico-degenerative

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## *Lectures*

## *Comunicazioni orali selezionate*

## *Poster*

**New technologies in medical oncology: farmacogenomics, proteomics and drug delivery.**

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At the beginning of the new century, medical oncology is facing a novel and somewhat contraddictory scenario. Although major advances have led to a clear improvement in the management of important diseases, with long term survival and even cure achieved both on regional and metastatic disease, the prognosis of most solid tumours in advanced stages remains dismissal and all efforts have led to only minor benefits with a survival gain of few months or even weeks produced by expensive new compounds. The novel molecular technologies have clearly led to enhanced understanding of cancer biopathology allowing the reclassification of tumors on the basis of gene expression and at a lesser extent of proteomic profiling, producing a new rational approach for the definition of tumor heterogeneity with major clues in terms of target identification or individualized treatment. At the same time, accumulating evidence in the field of genomic variants by the use of polimorphic variant at single nucleotide level (SNP) has led to high and low throughput technologies for individualizing treatment by prediction of specific toxicities. These latter technologies have a potential major role for the identification of individuals at risk of cancer, for istance for high carcinogen activation capability or for "low penetrance" genetic lesions, in the fascinating field of molecular epidemiology of cancer. Novel strategies in the field of drug delivery are now taking benefit from the availability of carriers like monoclonal antibodies, liposomes and nanovectors, which can couple selective targeting and capacity of reaching a favourable bioavailability in the specific tumor microenvironment.

We will try to rapidly describe the status of these fascinating areas of research and, at the same time, we will define the major concerns in terms of validation in the clinical setting, in order to be translated in the every-day practice, focusing on:

A) molecular profiling of human tumors, gene expression and proteomic profiling, prognostic and predictive implications, development of prognostic platforms;
B) pharmacogenetics and pharmacogenomics of human cancer;
C) new delivery technologies, nanovectors.

**Molecular profiling of human tumors, gene expression and proteomic profiling, prognostic and predictive implications, development of platform for clinical decision-making**

The "omics" revolution, by the development of novel technologies for the analysis of whole cell gene expression (transcriptome) or the whole cell proteome have offered a new framework of human cancer, allowing reclassification of human tumors on the bases of molecular phenotypes. A classical scenario, under this point of view, is offered by breast cancer, where the pioneering research activity by Sørlie T, Perou CM (PNAS 2001) have offered a molecular classification which appears to retain important clinical implications as differential sensitivity to anticancer drugs (Clin Cancer Res 2005).

Molecular profiling technologies have demonstrated a powerful capacity of prognostication or prediction of sensitivity to antitumour treatment. Adjuvant treatment of early breast cancer appears a provocative and challenging area of development for these novel technologies. The Oncotype Dx tm (Clinican chem 2007) and Mammaprint® platform (BMC genomics 2007 ) appear promising tools which have been recently made available. A crucial point is the validation of these platforms in prospectively designed clinical trials as the TAILORx and the MINDACT (Cancer Genomics Proteomics 2007). As a general idea, the clinical research in this specific field needs a specific methodology to be further developed in order to translate this specific technological tools in biomarker useful for decision making in the clinical practice. The American Society of Clinical

Oncology has recently updated the reccommendation for Biomarker use in the management of breast tumors. (J Clin Oncol 2007)

**Pharmacogenetics and pharmacogenomics of human cancer**

The emerging technologies, as well as the understanding of molecular mechanisms underlying the antitumor activity as well as the toxicity of the novel or conventional drugs has recently led to a major improvement of pharmacogenetics and pharmacogenomics of human cancer (Ann Oncol 2007). The dream of patient-tailored treatment is becoming a reality. However it has to be considered that a major effort is to perspectively validate pharmacogenetic and pharmacogenomic tools. It appears clear that molecular profiling technologies must be intended as "fishing technologies" for the search of new markers (mRNA, proteins) which should be routinely analyzed in less expensive and higlhy quantitative assays (Real time-PCR, ELISA). An additional important point is that validation trials should be designed in order to compare the outcome of patients wher treatment has been "biologically" selected against patients where treatment planning is not based on these criteria. Lung cancer appears an important area of development for customized treatment on the basis of molecular factors (Lung Cancer 2007).

**New delivery technologies, nanovectors**

The astonishing development of "omics" technologies has occurred together to major advancements in the targeting of anticancer agents in the tumor tissue. The old paradigma of magic bullets, which has led to the exciting results of tumor cell targeting by monoclonal antibodies is now paralleled by new technologies of drug delivery which take benefits from emerging fields like nanotechnology. Pegylated liposomal doxorubicin (Caelyx) has now a widespread use replacing conventional doxorubicin in a variety of different clinical conditions. Nano delivery of anticancer agents using nanovectors and hybrid nanoparticles is an important area of investigation (Brit J Cancer 2007). ABI-007(Abraxane) is an albumin-bound, 130-nm particle formulation of paclitaxel which has demonstrated a favourable activity and toxicity profile if compared to standard paclitaxel in women with advanced breast cancer (J Clin Oncol 2005) and has gained approval by FDA.

In order to exploit their enormous potential, all the above mentioned diagnostic and therapeutical tools need an integrated view of human malignancies. It appears evident that the information from genomics- SNP profiling, whole gene expression profiling, proteomics and so on must be interpretated in the light of sound integration with clinical data. Phenomics is new a science in its infancy. Cancer phenomics can be described as a systematic effort to collect phenotypic data at clinical, molecular and cellular level (Nat Rev Cancer 2006) [ Fig. 1 ] for an "operational" under standing of the whole story.

It is now clear that all these technological tools and the rapid increase of basic knowledge on the biology of tumor cell growth as well of the factors which regulate response to therapeutic agents require a redesign of clinical strategies for validation and use of these resources and define a novel and unpredictable scenario for modern clinical oncology.

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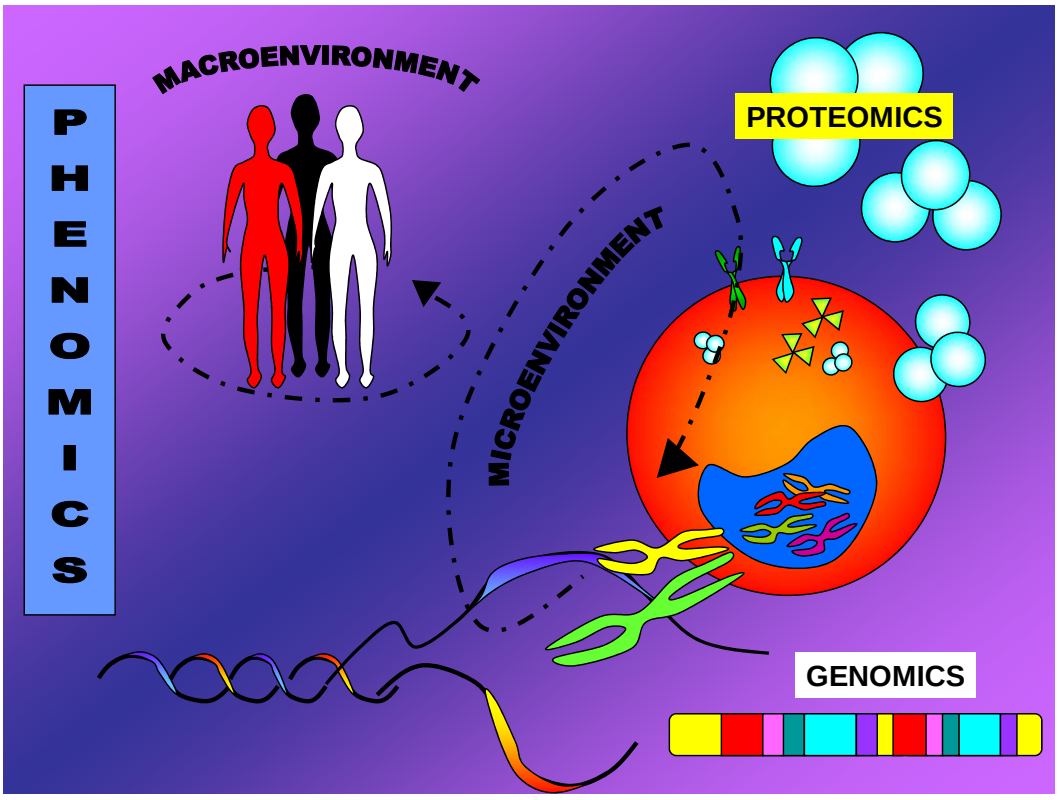


**Fig 1 Cancer phenomics**

**Sunitinib: the multi-target therapy for Present and Future Cancer Treatment**

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**ABSTRACT**

Tirosine kinase receptors (RTK's) are an eterogeneous group of transmembrane proteins involved in signal transduction. The expression of these receptors on many different cells is variable and these are involved in regulation of cellular growth, differentiation and angiogenesis. The structural alteration and\or overexpression of different RTK's classes are generally associated to cancer, and when RTK's mediated signal transduction pathways are abnormally activated, generate cancer growth, angiogenesis and metastatization. Therapeutic intervention targeting RTK's concern antagonist drugs as little molecules or monoclonal antibodies.

Sunitinib malate, a little molecule that block intracellular Tirosine-Kinase Domain of different RTK's, and with direct anti-cancer and anti-angiogenetic activity, that targets selectively VEGF, KIT, Flt-3 and PDGF receptors and the receptor encoded by the ret proto-oncogene.

This drug is indicated in the treatment of GIST resistant to Imatinib and metastatic Renal Cell Carcinoma.

In our review we report the most recent data relative to phase III trials of Sunitinib in the treatment of GIST and Renal-Cell Carcinoma, and we try to plane what will be future applications of Sunitinib in other types of cancer, even in association to chemotherapy, radiotherapy and/or monoclonal antibodies.

Tirosine kinase receptors (RTK's) are an eterogeneous group of transmembrane proteins involved in signal transduction. More than 20 different classes of RTK's have been identified. These receptors have similar structure: a ligand-binding extracellular domain, a transmembrane portion and an intracellular tyrosine kinase domain.

These receptors are expressed in many different cells and regulate cellular growth, differentiation and angiogenesis. By the ligand-mediated activation RTK's activate different intracellular pathways determining a great variety of cellular responses as differentiation, proliferation, cell migration, angiogenesis and cell survival.

structural Alteration and\or overexpression of different RTK's classes are generally associated to cancer, and when RTK's mediated signal transduction pathways are abnormally activated, generate cancer growth, angiogenesis and metastatization.

Plateled Derived Growth Factor (PDGF) stimulate the growth of fibrocytes and PDGFR hyperexpression generate different diseases caracterized by accelerated cellular growth, as fibrotic disorders and cancer.

Epidermal growth factor is overexpressed and abnormally activated in Non Small Cell Lung Cancer (NSCLC), Breast, Prostate and Colon Cancer.

Vascular endothelial Growth Factor (VEGF) has an important rule in cancer-induced angiogenesis, and in other pathologic conditions.

The co-expression of KIT receptor and of it's ligand, Stem Cell Factor, is present in about 70% of Small Cell Lung Cancer Cells, and about 80-85% of patients with GIST have activating-mutations of KIT[1,2].

FLT3 is the most frequently mutated gene in Acute Myeloid Leukemia (AML), and more frequently, mutation is an internal tandem duplication (Flt3-ITD).

**Preferential Pathways**

Activated RTK's use the pathway Ras/Raf/Mytogen activated Protein (MAP) proteins, directly involved in the RTK's depending signal, as the catalytic subunit of Phosphoinositide 3-Kinase, can

work as oncogenes, determinating mutations in gene transcription, cytoskeletal structure, cell mobility and in the apoptotic and anti-apoptotyc signals. Even the interaction (cross-talk) between RTK's and the oncostatin's receptor can influence oncogenesis.
Therapeutic intervention targeting RTK's concern antagonist drugs as little molecules or monoclonal antibodies[1,2].
Sunitinib malate is a little molecule able to block intracellular Tirosine-Kinase Domain of different RTK's: this drug has both direct anti-cancer and anti-angiogenetic activity.
Sunitinib targets selectively EGFR, VEGFR 1and2, PDGFRβ, PDGFRα, KIT, CSF-1R, RET and Flt-3 receptors. In a ATP site-dependent binding assay, Sunitinib showed its high binding-affinity for the primary targets (higher than Sorafenib, which have a similar panel of targets) and the activity to bind 73 additional kinases, even serine-threonine kinases[3,4](Figure).
Sunitinib is indicated in the treatment of GIST resistant to Imatinib and metastatic Renal Cell Carcinoma, but many clinical ongoing trials are testing this drug in different types of cancer.

**GIST**

GIST are rare tumors which have constitutively activated KIT receptor in 90% of the cases or PDGFR in about 5%.
Imatinib, a selective inhibitor of these receptors, is effective in the treatment of metastatic or non resecable GISTs. About 12-14% of patients is resistant to Imatinib, 40% develops resistance to the drug in 2 years and 5% has intolerance to Imatinib[5]. This explain the need of new effective drugs for the treatment of these patients. Generally resistance to Imatinib is secondary to new mutations of KIT or PDGFRα, and Sunitinib can bypass this resistance for the antiangiogenetic effects and for the activity on structural variants of mutated tyrosine kinases or on alternative pathways[,6,7].
This ipothesis was proved in a phase I/II trial enrolling 97 patients with metastatic GISTs resistant to Imatinib. On 92 evaluable patients, 88 progressed during therapy with Imatinib and 4 had intolerance to the drug.
Partial Response, according to RECIST Criteria, was obtained in 7 (8%) and Stable Disease was obtained in 53 patients (58%), with Clinical Benefit of 65%.
The Response of 39 patients was examined by FDG-PET, evidentiating a PR in 28 patients (72%). This trial showed that Sunitinib is effective in patients with primary and secondary resistance to Imatinib, and that FDG-PET is a method to have an early indication of response[8].
The encouraging results of this trial, induced to start a randomized phase III trial of Sunitinib versus placebo in patients with GIST resistant or intolerant to Imatinib[6]. Patients were randomized in double blind 1:1 to receive Sunitinib or Placebo. The interim analysis of the first 13 weeks from randomization, showed significative improve in Time to Progression (TTP – primary objective of the trial), in Sunitinib arm. Because of this results, all patients of the placebo arm were crossed over to Sunitinib. TTP in placebo arm was 6.4 weeks, versus 27.3 weeks in Sunitinib arm ($p<0.00001$).
Partial Response was obtained in 7% and Stable Disease in 58%, with Clinical Benefit (SD+PR) in 65%. Even if, apparently low, 7% response rate was significantly higher than placebo (0%).
In this trial were enrolled 13 patients with intolerance to Imatinib, and 9 were in Sunitinib arm. Between these 9 patients, 4 obtained PR and 4 SD[5].
59 patients were crossed over to Sunitinib, obtaining PR in 10% and 31%. Even after only 13 weeks, 59% of the patients passed from placebo to Sunitinib.
Is important to consider the relationship between cancer-mutations and efficacy to Sunitinib, examined in a group of patients enrolled in a phase I/II trial. Clinical Benefit, defined as PR and SD > 6 months, was observed in all the more important molecular subtypes of GIST, with Sunitinib. PR rate in GIST with primary mutations of exon 9 was 37% versus 5% in GIST with mutations of exon 11. TTP and OS rate were significantly higher in patients with KIT mutations on exon 9 or with native KIT/PDGFRα genes than in patients with KIT mutations on exon 11. Secondary KIT mutations were observed more frequently in GIST with primary mutations on exon 11 (62%) than GIST with primary mutations on exon 9 (16%). Secondary KIT mutations on exon 13 and 14 resulted sensible to Sunitinib, much more than mutations on exon 17 and 18. As Imatinib is more

effective in GIST with KIT mutations on exon 11, Sunitinib seem to be more active with mutations on exon 9, observable in 13% of patients[7].

**Renal Cell Carcinoma**

Progresses of molecular biology opened new therapeutic prospectives for Renal Cell Carcinoma (RCC) treatment. There are different histological types of RCC, caused by different genes and with different prognosis. The most frequent is Clear Cell Carcinoma (75%). The hereditary and the sporadic form of RCC are both related to mutations of VHL gene (von Hippel Lindau), an oncosuppressor gene, which activation causes von Hippel Lindau syndrome. Hereditary RCC has an incidence of 40% in patients with von Hippel Lindau syndrome, for a germinal mutation of the VHL gene followed by a second somatic mutation, according to the Knudson hypothesys.

In patients with Sporadic RCC, a biallelic inactivation for somatic mutations of VHL gene is detectable in 75%, while absence of gene expression for hyper methylation is detectable in about 20%.

The molecular therapy for RCC born after the explaining of working-mechanism of VHL gene. VHL gene is located on chromosome 3p25-26, and has functions in the hypoxia-inducible pathway. VHL gene product is a multiprotein complex composed of ElonginB, ElonginC, Cul2 and Rbx1, and this complex ubiquitinates transcriptional hypoxia-inducible factor 1α (HIF-1 α). The normal function of HIF, an heterodimer composed of α and β subunits, is to regulate expression of several genes in response to hypoxic stress. In normal conditions, as in presence of wild type VHL and normal oxygen tension, HIF 1α is enzymatically hydroxylated on two proline residues located in the "oxygen dependent degradation domain". Then HIF 1α is hydroxylated and this process allows for hydrogen bond-mediated complex formation between the complex and VHL. HIF 1α is subsequently ubiquinated by the VHL complex and ultimately degraded within proteasomes. Under hypoxic conditions HIF 1α is not hydroxylated and thus cannot bind and be efficiently ubiquinated by the VHL protein complex . Biallelic inactivation of VHL would like wise prevent ubiquination and ultimate degradation of HIF 1α . HIF 1α activity is regulated by growth factor that binding to a tyrosine kinase receptor, increase HIF 1α levels by molecular pathways as PI3K/AKT-mTOR and Ras/Raf/Map kinase. HIF 1α can increase by the integrine-mediated stimulation of PI3K/AKT-mTOR. Once stabilized, HIF 1α traslocates into the nucleus where it complexes with the constitutively present HIF 1β to form the active transcriptional factor HIF 1 heterodimer. HIF 1 binds to different additional transcriptional cofactors, forming a pre-initiation complex of proteins that ultimately activates transcription of Hypoxia-inducible genes, including VEGF, EGFR, PDGF, TGFα, erythropoietin. RCC is a highly vascular tumor, with increased VEGF level, and its growth can be stimulated by factors produced through the HIF 1 pathway[9].

As treatment for RCC, Sunitinib was used for the first time at the dose of 50 mg/die for 28 days every 6 weeks. In a non randomized trial presented at the 2004 ASCO Congress and published on JCO, were enrolled 63 patients with RCC pre-treated with cytokine–based therapy (IFN, IL-2). Primary objective was Response Rate (RR), and Sunitinib obtained PR of 40% and SD of 27%. Median TTP was 8,7 months[20]. Because of these results a second non randomized phase II trial, enrolling 106 patients, confirmed the results of the first trial. On 105 evaluable patients, RR was 43%, with 22% SD and Progression Free Survival (PFS) of 8,1 months[10,11].

The combined analysis of these two phase II trials, on 168 enrolled patients, evidenced objective responses and stable disease for more than 3 months, respectively, in 42% and 24% of patients with PFS of 8.2 months. These results are incredibly important considering that the activity of the drugs in use in metastatic RCC obtain a 5% RR.

Then started an international randomized phase III trial of Sunitinib versus Interpheron alpha (IFN α) in non pre-treated patients with metastatic RCC. On 750 patients, Sunitinib arm RR was 39% versus 8% for IFN α. PFS was 11.2 months for Sunitinib arm versus 5.1 months for IFN α, and the difference is statistically significative ($p < 0.000001$). These data were presented at the Barcelona ECCO Congress of 2007, even if median OS is not still available[12,13,14] (Table 1).

The preliminary Assessment of Safety and Efficacy in an Expanded Access Trial with Sunitinib 50 mg daily, 4 weeks on, 2 weeks off on 3997 patients showed a 55.9% Clinical Benefit (CR + PR + SD) with a median duration of treatment of 6.5 months (0.0 – 23.6). The subgroup analysis showed a Clinical Benefit of 59.2% in cytokine pre-treated RCC patients versus a 47.6% of non pre-treated RCC patients. The Response Rate and the G3/4 Adverse Events rate were not influenced even considering risk factor as brain metastasis, non clear cell hystology, poor PS and elder age. In fact the Clinical Benefit of the subgroups was: 40.3% for Brain metastasis patients, 47.7% for Non-Clear Histology Carcinomas, 33.0% for Poor Performance Status patients and 52.9% for elder patients. This shows that even in presence of non still studied risk factors, Sunitinib is an active drug in metastatic Renal Cell Carcinoma, and that these factors don't influence side effects of the treatment[15].

In another phase II trial of Sunitinib in metastatic RCC resistant to Bevacizumab, was observed reduction in tumor size in 81%. This because Sunitinib could block some molecular pathways involved in resistance to Bevacizumab[17].

Another phase II study, published on 2007, of continuous daily administration of Sunitinib 37.5 mg in patients with cytokine-refractory metastatic Renal Cell Carcinoma showed a 36 weeks PFS comparable to that of Sunitinib 50 mg 4 weeks on, 2 weeks off as second line treatment for mRCC. There were no differences in tolerability or health-related quality of life between patients who took the drug in the continuous daily schedule versus the 4on/2off schedule of treatment[18].

At the 2007 ASCO Congress was presented even a Pharmacokinetic/Pharmacodynamic analisys of different administration schedules of Sunitinib as 50 mg/day 4 weeks on/2weeks off and 37.5 mg/day continuously to evaluate differences in response to Sunitinib as a function of individual exposures to the drug and to evaluate relative efficacy of Sunitinib 50 mg/day on 4/2 schedule vs. 37.5 mg/day on CD schedule. In mRCC, Sunitinib displays greater efficacy in patients with the highest exposures giving higher probability of response with increasing exposure, longer TTP and OS and greater changes in tumor volume; Sunitinib 37.5 mg/day CD appeared equally effective as 50 mg/day 4/2 schedule and similar trends displayed with exposure in GIST. Different works exposure-response are ongoing in breast, lung and colorectal cancer too[19].

**Future Applications**

Several trials are ongoing to test Sunitinib in different types of cancer.

In the treatment of GIST, is in course a trial of Sunitinib versus Imatinib 800 as first line treatment, and will start a trial of Sunitinib versus Imatinib 400.

In the treatment of RCC two trial are ongoing: the first is the ECOG ASSURE trial of Sunitinib versus Sorafenib versus placebo, and the second is Sunitinib versus Placebo, both as adjuvant treatment of renal cell carcinoma.

In the treatment of NSCLC, after preliminary data from phase II trial in heavily pre-treated patients, several trials are going to start: a randomized trial of Sunitinib versus Erlotinib, differentiating for the molecular typization, a trial of Sunitinib as treatment for NSCLC brain metastases and some trials of Sunitinib in association to standard chemotherapy as treatment for metastatic NSCLC.

Different trials are ongoing in metastatic breast cancer too. A trial of Sunitinib as treatment of triple negative patients; a trial of Sunitinib versus Capecitabine as second line treatment, and a trial of Capecitabine with or without Sunitinib. As first line treatment some trials are going to start, using Sunitinib in association to Docetaxel +/- Trastuzumab.

After the encouraging results of a phase I trial presented at the 2007 ASCO G.I about the association of Sunitinib with FOLFOX and FOLFIRI as treatment for metastatic colorectal cancer, different trials will test the association of Sunitinib with standard chemotherapy as first line treatment, will compare the association FOLFOXIRI/Bevacizumab versus FOLFOXIRI/Sunitinib as neoadjuvant treatment for liver metastases from colorectal cancer, will test the efficacy of the combination of Sunitinib and radiotherapy as neoadjuvant treatment of locally advanced rectal cancer.

Other trials are going to test the activity of Sunitinib in the treatment of Gastric Cancer, Sarcomas, NET, Pancreas Cancer, etc.

Best response by RECIST.

| | No. of patients (%) | | | |
|---|---|---|---|---|
| | Investigator assessment | | Independent central review | |
| Response (RECIST) | Sunitinib (n=374) | IFN-α (n=373) | Sunitinib (n=365) | IFN-α (n=346) |
| Objective response* | 175 (47) | 46 (12) | 143 (39) | 29 (8) |
| 95% confidence interval (CI) | (42–52) | (9–16) | (34–44) | (6–12) |
| Complete response | 7 (2) | 4 (1) | 0 | 0 |
| Partial response | 168 (45) | 42 (11) | 143 (39) | 29 (8) |
| Stable disease | 151 (40) | 202 (54) | 146 (40) | 165 (48) |
| Progressive disease | 26 (7) | 69 (18) | 57 (15) | 102 (27) |
| Not evaluable/Missing | 22 (6) | 56 (15) | 20 (5) | 50 (13) |

*P<0.000001 for sunitinib vs. IFN-α.

**Table 1.** Sunitinib versus Interferon Alpha as first line treatment in Metastatic Renal-Cell Carcinoma: Updated Efficacy Results
Motzer RJ et al - 2007

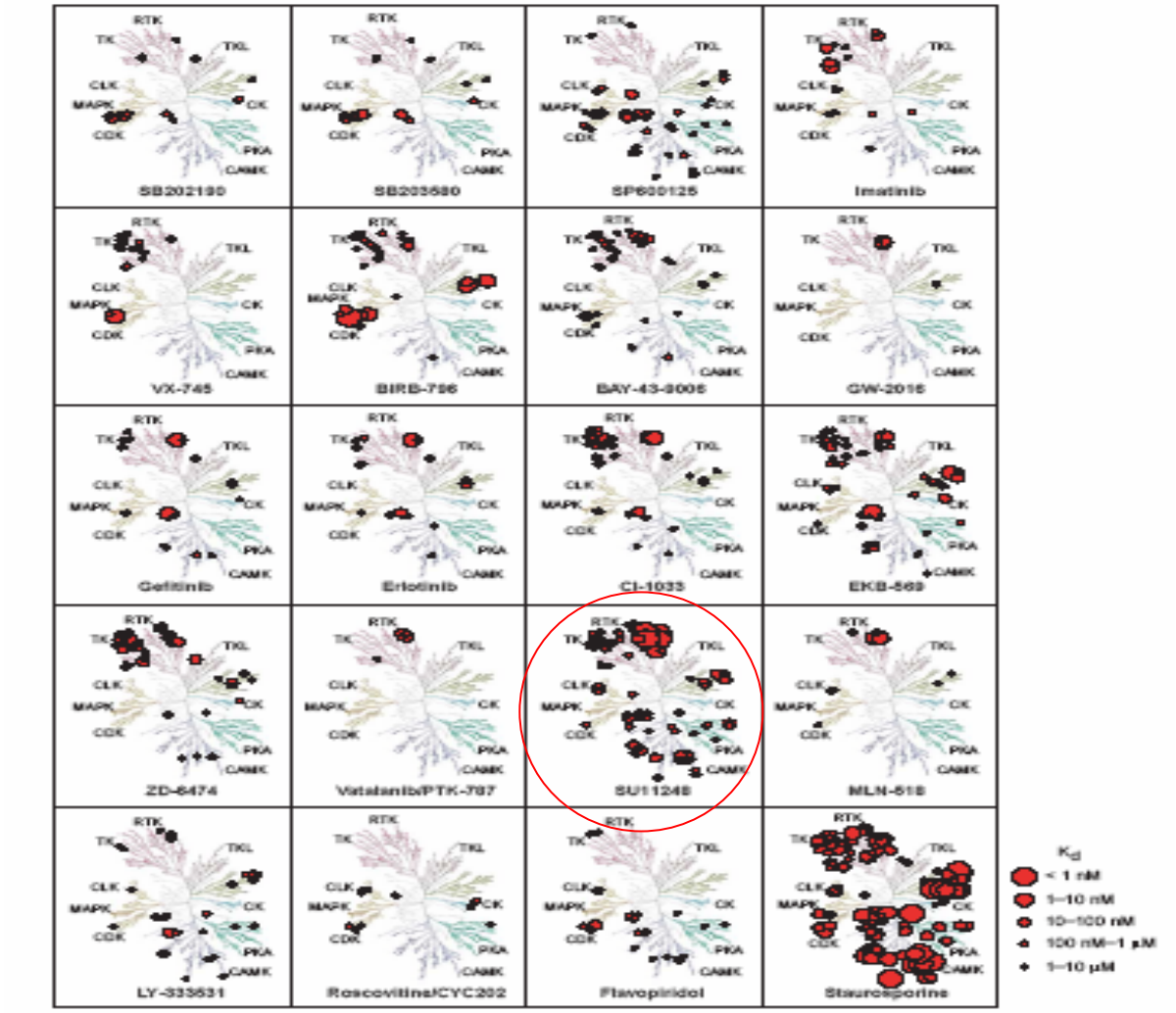


**Figure 3** Specificity profiles of clinical kinase inhibitors. Kinase dendrograms were adapted[3]. TK, nonreceptor tyrosine kinases; RTK, receptor tyrosine kinases; TKL, tyrosine kinase-like kinases; CK, casein kinase family; PKA, protein kinase A family; CAMK, calcium/calmodulin dependent kinases; CDK, cyclin dependent kinases; MAPK, mitogen-activated protein kinases; CLK, CDK-like kinases.

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**New pharmacological and molecular approaches to potentiate bisphosphonates anti-tumour effects.**

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## Introduction

Bisphosphonates (BPs) have emerged in recent years as a highly effective therapeutic option for the prevention of skeletal complications secondary to bone metastases.

BPs bind preferentially to bone at sites of active bone metabolism, are released from the bone matrix during bone resorption and potently inhibit osteoclast activity and survival, thereby reducing osteoclast mediated bone resorption [1]. Newer nitrogen-containing bisphosphonates (NBPs), such as zoledronic acid (ZOL), have a unique mechanism of action and are active at micromolar concentrations compared with the first-generation compounds [2]. ZOL is the only bisphosphonate that has demonstrated significant clinical benefit with prostate, lung and renal cancer [3-5].

Therapy with bisphosphonate is associated to mainly mild to moderate adverse events, while severe adverse events are rare [6-10]. The good safety profile of BPs and the large experience with these agents in clinical practice make them suitable for use in combination with other therapies. There is a growing number of preclinical studies investigating the molecular rationale of combining BPs with several anti-neoplastic agents. In this review we will analyze the data regarding the preclinical anti-tumour effects of combinations of BPs with cytotoxic and biological drugs, as both synergistic and additive effects have been described *in vitro* and *in vivo* and new strategies to increase the distribution of these drugs in the human tissues.

## Mechanism of action of bisphosphonate

NBPs are specific inhibitors of farnesyl pyrophosphate synthase (FPPS), required for the synthesis of farnesyl and geranylgeranyl lipidic residues [11] (Figure1), and thereby suppress prenylation of small GTPases that regulate the proliferation, invasive properties and pro-angiogenic activity of human tumour cells [12,13]. The addition of a lipidic residue to all the small GTP-binding proteins is essential for their correct location on the inner side of the plasma membrane and for their consequent activation by external signals. Exemples of farnesylated proteins are the following: H-, K- and N-Ras GDP/GTP-binding GTPases; the nuclear lamins; and the kinetechores centromere associated protein (CENP)-E and –F. Geranylgeranylated proteins include: GTP/GDP-binding GTPases, RhoA, RhoC, Rac1, cdc-42, Rab and R-Ras [14].

BPs exert their actions either by direct effects on tumour cells, or indirectly by affecting bone resorption, thereby reducing tumor-cell migration to the bone, or possibly both [15].

It has recently been demonstrated in vitro that NBPs, such as Pamidronate (PAM) and ZOL, induce apoptosis and growth inhibition in human epidermoid cancer cells, together with depression of ras signalling and of Erk and Akt survival pathways [16]. These effects occurred together with poly (ADP ribose) polymerase (PARP) fragmentation and the activation of caspase 3 [16]. Moreover, the latter seemed to be essential for apoptosis induced by NBPs in this experimental model. The synthesis of isoprenoids appeared largely responsible for the biological and biochemical effects of NBPs since the addition of farnesol, which restores farnesylation, to tumour cells completely antagonized apoptosis and the inhibition of ras activity in tumour cells exposed to NBPs [16]. These data suggest that the activity of NBPs could be due to the inactivation of the FPPS activity [16].

Moreover, it was reported that ZOL induced growth inhibition on both androgen-dependent LnCaP and androgen-independent PC3 prostate cancer cell lines [17] with G1 accumulation.
One of the primary mechanisms responsible for the direct antitumor activity of bisphosphonates is induction of tumor-cell apoptosis. There are several published reports describing the *in vitro* pro-apoptotic effects of BPs on osteoclasts and tumor cells [15,18]. A recent study showed that the effects of ZOL in human breast cancer cell lines (MDA-MB-231 and MCF-7) were associated with cytochrome c release from the mitochondria, induced by modulating expression of Bcl-2 and subsequent caspase-3 activation. These events might be precipitated by inhibition of Ras activation, which requires protein farnesylation [19].
Several in vitro studies have shown that BPs inhibit adhesion of tumor cells to extracellular matrix (ECM) proteins, thereby impairing the process of tumor-cell invasion and metastasis [20,21]. Recent data indicate that inhibition of tumor-cell adhesion to ECM proteins is dependent on inhibition of protein prenylation. Therefore, inhibition of the mevalonate pathway and induction of caspase activity are important for the inhibitory effects of NBPs. Furthermore, it has been shown that an activating Ras mutation enhanced the adhesion of a normal breast epithelial cell line to ECM proteins, suggesting that increased Ras activation may increase the metastatic potential of breast cancer cells [22]. Thus, by inhibiting protein prenylation and Ras signalling, ZOL should reduce the ability of tumor cells to expand once they colonize bone [21]. In addition to direct antitumor mechanisms, NBPs might modulate the immune system to target and eliminate cancer cells [23].
*In vitro* and *in vivo* studies have shown that ZOL and other NBPs have antiangiogenic effects [22,24,25]. *In vitro* assays showed that ZOL could inhibit the proliferation of human umbilical vein epithelial cells induced by fetal calf serum and basic fibroblast growth factor (bFGF) in a dose-dependent manner. These findings have been confirmed *in vivo*; systemic administration of 3 µg/kg zoledronic acid to mice resulted in potent inhibition of angiogenesis induced by subcutaneous implants impregnated with bFGF [22]. The inhibitory effect of zol on endothelial cell adhesion and migration is mediated, at least in part, by modulation of integrins that are involved in angiogenesis [26].

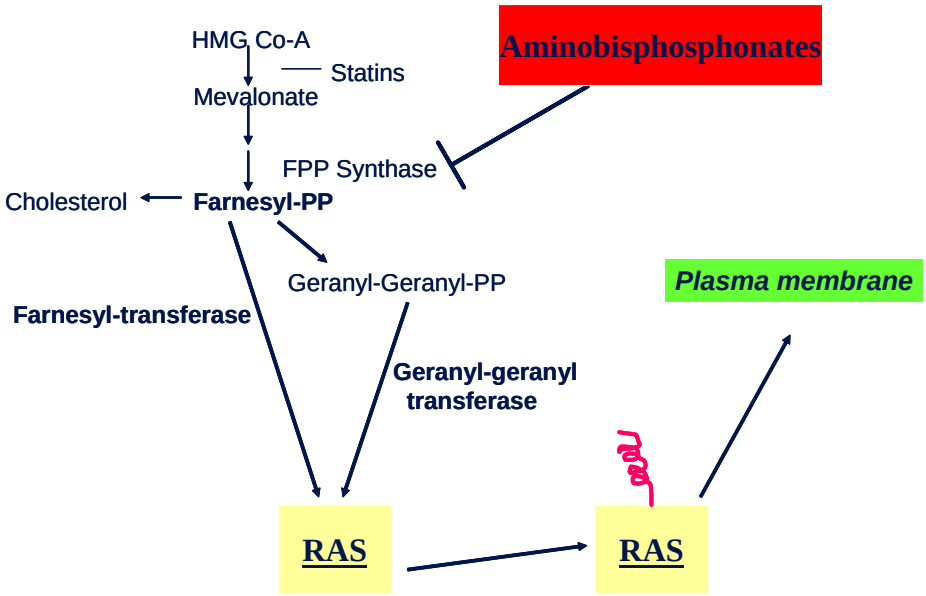


**Figure 1.** Schematic representation of the mevalonate pathway and the effects of nitrogen-containing bisphosphonates.

**New strategies to potentiate bisphosphonates anti-tumour effects.**

Bone metastases are common in patients with many types of malignancies, especially breast and prostate cancer, for both of which the incidence of bone metastasis is approximately 70% among patients with advanced metastatic disease [34,35]. Patients with breast or prostate cancer have a relatively long median survival after diagnosis of bone metastases when compared with patients with other cancers (e.g. lung cancer, renal cancer, and pancreatic cancer), resulting in high prevalence of bone metastases, and these two cancer types account for more than 80% of cases of metastatic bone disease. By contrast, approximately 40% of patients with advanced lung cancer develop bone metastases [27,28]. Bone metastases contribute substantially to the burden of disease, and patients experience considerable skeletal morbidity, including severe bone pain that can require strong narcotics or palliative radiation therapy, pathologic fractures, spinal cord or nerve root compression, and hypercalcemia of malignancy, all of which substantially reduce the patient's quality of life. This skeletal morbidity has led to the development of bisphosphonate therapies, which inhibit bone resorption, thereby reducing the risk of skeletal complications. Consequently, bisphosphonates have become the standard treatment for tumor-induced bone disease [29].

One of the most important limits of NBPs, which makes the direct anti-cancer activity difficult to demonstrate *in vivo*, is their pharmacokinetic profile. In fact, studies on ZOL pharmacokinetics demonstrate that, following infusion of a standard 4 mg dose of zoledronic acid, the drug remains in the plasma for 1–2 h before localization to bone [30,31].

However, due to the intrinsic limitations of current NBPs, further efforts are required in order to allow the clinical translation of experimental results recorded to date and to increase the anti-cancer activity of these drugs. Two possibilities are the improvement of the pharmacokinetic profile and the design of rationale based drug combinations.

**Drugs combination studies.** Evidence from *in vitro* and *in vivo* models indicates a synergistic antitumor activity of BPs when used in combination with cytotoxic drugs, targeted molecular therapies. The demonstration of these synergistic effects could suggest that the low serum concentrations of BPs *in vivo* are still sufficient to exert anti-tumour actions in peripheral tissues.

Based on the relevance of any farnesylation inhibitory effects on anti-tumour activity of the BPs, the FTI R115777 was used together with PAM or ZOL and the effects of the combination treatment on growth inhibition and apoptosis evaluated. BPs and FTI given in combination were strongly synergistic since a $CI_{50}$ (the combination index of the two drugs calculated for 50% cell survival by isobologram analysis using dedicated software) of less than 0.5 was recorded [16,32].

Notably, low concentrations of FTI induced a strong increase of ras expression with only a moderate reduction of ras activity that was, on the other hand, significantly reduced by the combined treatment [16]. These data suggest that escape mechanisms for the inhibition of isoprenylation of ras might be based on the geranylgeranylation or other prenylating processes [33]. The addition of farnesol to cells treated with the combination abolished the effects of the BPs/FTI combination on apoptosis and on the activity of the signalling molecules. These data suggest that the synergistic growth-inhibitory and pro-apoptotic effects produced by the NBP/FTI combination involve the inhibition of both Erk and Akt survival pathways acting in these cells in a ras-dependent fashion [16].

We have recently found a synergistic interaction between R115777 and ZOL on both androgen-independent PC3 and androgen-dependent LNCaP prostate cancer cell lines [34]. These effects were paralleled by disruption of Ras → Erk and Akt survival pathways, consequent decreased phosphorylation of both mitochondrial bcl-2 and bad proteins, and caspase activation. Moreover, ZOL/R115777 combination induced cooperative effects also in vivo on tumor growth inhibition of prostate cancer xenografts in nude mice with a significant survival increase [34]. These effects were paralleled by enhanced apoptosis and inactivation of both Erk and Akt. In conclusions, the combination between ZOL and FTI leads to enhanced anti-tumor activity in human prostate adenocarcinoma cells likely through a more efficacious inhibition of ras dependent survival pathways and consequent bcl-related proteins-dependent apoptosis [34].

Moreover, we have preliminary results about sequence-dependent synergistic effects of ZOL and docetaxel (DTX) combination on growth inhibition and apoptosis of human prostate cancer cells. We have found a synergistic growth inhibition when DTX was administered 24 h before ZOL. On the basis of these results, we have designed a phase I clinical study on the combination between these two drugs metronomically administered in two different sequences in hormone-refractory advanced prostate cancer patients. The aim of this study was to perform a pharmacodynamic evaluation of the effects of the two sequential combinations through the dosage of serum angiogenic, immunologic and bone factors and through the study of both lymphocyte sub-populations and modification of isoprenylation of intracellular proteins. Final endpoint of the study was the evaluation of information for further clinical development of this combination, such as toxicity, as well as information about the mechanism of action of the combination to be translated in the preclinical setting. We have completed the first level without significant toxicities and we have enrolled the first three patients of the second level. In the first level we have completed the 12 cycles in only one patient and 42 cycles were globally administered. The the serum level changes of a biological markers was determined before, during and after the pharmacological treatment. Interestingly, in the patient who completed the 12 cycles of therapy a significant reduction of both interleuckin-8 (a pro-angiogenic factor) and 6 (a prostate cancer growth factor) was recorded. Similar results were obtained in another patient with SD. These findings were also paralleled by a significant reduction of PSA. We are now completing the evaluation of a panel of circulating angiogenic factors (interleukin 8 and 12, VEGF, PDGF), cytokines (TNF alpha, IFN gamma, GM-CSF, RANTES, interleukin-1, 2, 6 and 4) and chromogranin A. We have also collected peripheral blood mononuclear cells on which we will T lymphocyte subpopulation distribution and the isoprenylation of the chaperone protein HDJ2. On the basis of the results of this study we will design a phase III clinical study based on the 2 sequences of administration.

**New molecular targets.** New technological platforms are required to drive molecular research and to identify suitable targets of BPs action and understand cell consequences of the inhibition of the target enzymes. Technological advances are useful for research strategy planning and technologies such as DNA microarrays, and proteomics is particularly useful in this regard. These technologies could represent a relevant opportunity to analyze the post-transcriptional modifications induced by BPs treatment, and to better understand the *in vivo* intra-tumoural molecular pathways involved in the response to these compounds. Moreover, protein microarrays might allow discovery of new BPs targets, identification of new biomarkers predictive of response, and analysis of specific molecular profiling related to the clinical response to bisphosphonate-based therapy. Identification of critical interactions within the proteome network is a potential starting point for drug development, and will aid the design of individual tailored therapies or identification of new molecular profiles, gene profiles, or both that are predictive of response to BPs-based therapy. We have preliminary results about the gene modulation induced by ZOL in androgen-resistant prostate PC3 cell line analysed with cDNA microarray platform. ZOL-treated cells were obtained by Affymetrix HG-U133 chips (including more than 33,000 well-known human genes). The expression of the proteins encoded by the modulated genes was evaluated though western blotting analysis using specific antibodies. We found that the upregulated and downregulated genes were 73/33.000 (four genes were downregulated and 69 genes were upregulated). Among the up-regulated genes, the genes coding for calreticulin gene, n-myc downstream regulated gene 1 (NDGR1) and catenin gene resulted highly up-regulated with a fold-change, respectively of 11.47, 4.81 and 3.25. Among the down-regulated genes, the gene coding for cysteine-rich, angiogenic inducer, 61 (Cyr61) resulted highly down-regulated with a fold-change of 5.58. Cyr61 over-expression in tumor cells promotes tumor growth and vascularization. The evaluation of the involvement of these molecules involved in angiogenesis and in differentiation of human prostate cancer cells treated with ZOL is ongoing. Preliminarily, we have found a reduction of the transcriptional activity of Cyr61 promoter in ZOL-treated PC3 cells in a time-dependent manner (with a peak at 12 hours). Moreover, Cyr61 protein expression was significantly decreased after exposure to ZOL. Interestingly, other inhibitors of ras

(such as R115777) or of the tyr kinase associated to EGF-R (gefitinib) or of C-Raf (BAY 43-9006) used at equitoxic concentrations did not induce or induced less effects on Cyr61 modulation if compared to ZOL (Figure2). Moreover, ZOL down-regulated Cyr61 and blocked the ras-raf-1-dependent pathway. The effects of ZOL were antagonized by the addition of either farnesol or geranylgeraniol. Thereafter, we have investigated on the role of Cyr61 in apoptosis and growth regulation of PC3 cells using a shRNA for Cyr61 in order to down-regulate the expression of the protein. shCYR61 enhanced growth inhibition induced by ZOL with a potentiation factor of 5 while the transfection of PC3 cells with shCYR61 alone is ineffective. The sensitization of PC3 cells to the anti-proliferative effects of ZOL induced by CYR61 knock-down is paralleled by the inactivation of ras, Erk and Akt. Since Cyr61 is a ligand of v3 integrin we have evaluated the effects of Cyr61 knock down on motility and invasion (Figure 3). We have found that shCyr61 transfection induced a 45% and 20% reduction of motility and invasion, respectively, but treatment of shCyr61-transfected cells with ZOL induced a 75% and 30% inhibition of motility and invasion, respectively (Figure 3). Moreover, Cyr61 down-modulation induced by ZOL sensitizes prostate cancer cells to DTX. In fact, the treatment of PC3 with DTX induced an about 2-fold increase of Cyr61. In conclusion, Cyr61 downregulation is involved in the regulation of motility processes and, indirectly, of cell growth inhibition induced by ZOL. We are now evaluating the activity of blocking anti-CYR61 antibodies in inducing the potentiation of ZOL antiproliferative effects. These results could be useful in designing new therapeutical approaches in androgen-independent prostate cancer.

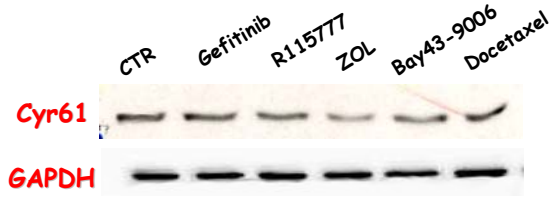


**Figure 2.** ZOL specifically induces Cyr61 decreased expression.

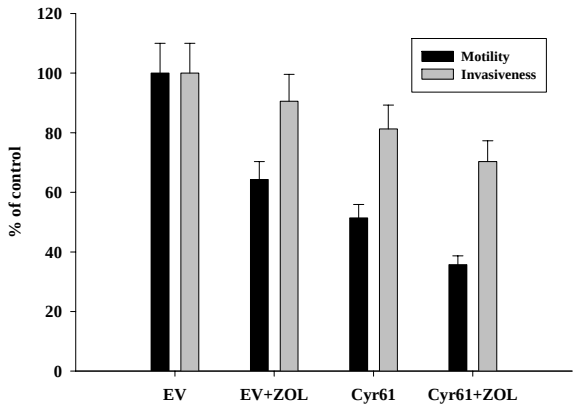


**Figure3.** Effects of Cyr61 on motility and invasiveness of cells treated with ZOL.

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# Polymeric nanoparticles for drug delivery: pharmacokinetics and potential for brain targeting

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## *Nanopharmaceuticals*

The explosive growth of the nanotechnology in the pharmaceutical field is in the process of revolutionizing the way a drug is delivered to the body. Nanopharmaceuticals can be defined as drug delivery systems or biologically active drugs of nanometre size consisting of at least two components, one of which is the active ingredient. In this field the concept of nanoscale was seen to range from 1 to 1000 nm. Nanocarriers include micelles, nanoemulsions, nanoparticles, nanocapsules, nanogels, liposomes, nanotubes, nanofibers, polymer therapeutics and nanodevices. An increased interest is being focused on polymeric nanoparticles which could offer high physical stability and can be designed, using a wide range of materials, to control drug release as well as body distribution of the loaded drug. The fact that nanopharmaceuticals are increasingly complex in their structure and biofate is now forcing the creation of a specific regulatory framework for market approval which can put a further limitation to their development.

In the past 30 years, there has been an explosion of cutting-edge research based on interdisciplinary approaches which have strongly contributed to deep innovation in pharmaceutical technology. A growing number of polymer-based nanosystems for drug and imaging agent delivery has been developed and some products have entered the routine clinical use (table 1).

## *Field of application and classification of drug-loaded nanocarriers*

Nanotechnology can play an important role in developing proper formulations that address the drug delivery issues related to new molecular entities (NMEs) with poor biopharmaceutical properties, such as poor solubility, poor permeability across accessible epithelia (oral or nasal), poor enzymatic or nonenzymatic stability in biological environments. Additionally, nanoparticulate drug delivery systems are being used to alter drug pharmacokinetics improving the treatment ability to target and to kill cells of diseased tissues/organs while affecting as few healthy cells as possible. The opportunity to change radically drug biodistribution in the body is the topic that is attracting much research especially in pathologies such as cancer where the therapy involves the use of highly toxic drugs with severe side effects. In this case, intravenously administered drug-loaded nanocarriers can act as a circulating drug reservoir which is able to protect the drug from degradation and deliver a drug dose to specific tissues or cells. According to their recognition capability nanocarriers can be classified as “non-targeted nanocarriers” and “targeted nanocarriers”. “Non-targeted nanocarriers” are passive carriers loaded with a drug which distribute according to their physico-chemical properties (stability in body environment, surface properties, size) including also active nanocarriers, i.e. stimuli-sensitive carriers. Targeted nanocarriers include a further functionality since they are able to transport their drug cargo in specific body districts. Thus, an active targeting can be accomplished mainly if the carrier is administered directly in the bloodstream. Depending on the properties of the carrier such as size, type of biomimetic coating, surface properties and presence of targeting moieties (figure 1), large variations in drug pharmacokinetics with major clinical implications may occur.

***How affecting biofate of nanosized carriers***

After intravenous injection, drug-loaded nanocarriers can undergo different fates in the body: (i) uptake by cells of reticuloendothelial system (RES) in liver, lung, spleen and, in a minor extent, bone marrow, followed by metabolism and excretion of the drug, (ii) accumulation in tissues other than RES where slow drug release occours, (iii) leakage of the drug from nanocarrier in circulation followed by drug rapid and extensive tissue distribution and elimination. Depending on the relative importance of each of these different pathways, large variations in nanocarrier pharmacokinetics with major clinical implications may occur.

The mechanism of interaction between nanocarriers and blood components is a key factor to understand their fate after intravenous injection. Nanocarriers can be rapidly cleared up in the blood circulation by cells of RES, a process driven by deposition of different proteins present in the blood (opsonins) on particle surface (opsonization). Either polymorphonuclear leukocytes in the blood or fixed macrophages, and particularly Kupffer cells in the liver are involved. RES uptake can be beneficial when targeting phagocytic cells of RES such as in infectious diseases (Trojan horse approach) or in some localized tumor (hepatocarcinoma or hepatic metastasis arising from digestive tract or gynecological cancers, bronchopulmonary tumours, myeloma and leukemia) but unduly disadvantageous when trying to target different organs.

The development of sterically stabilized long-circulating nanocarriers (stealth® nanocarriers) has represented a milestone in the design of carriers which can bypass the RES. At the end of the 80s years, the first liposomal formulations with an increased circulation time have been described. Biomimetic carriers able to escape RES are characterized by a hydrophilic stable coating, mainly generated by low molecular weight polyethyleneoxide (PEO), which limits carrier opsonization. A PEO coating can be generated by either its physical adsorption onto particle surface or chemical grafting of the chains to nanoparticle surface or using amphiphilic polymers to produce nanoparticles. Core-shell nanocarriers are definitely obtained where the hydrophilic surface forms a conformational cloud of flexible chains able to shield opsonin adsorption and a hydrophobic solid core containing the drug to deliver. PEO molecular weight of between 2 and 5 kDa is necessary to suppress plasma protein adsorption. Furthermore, it appears that the higher the PEO density (or the thicker the PEO coat) the slower the clearance and the better protection from liver metabolism. High density mushroom-brush and brush PEO coats are more effective in determining resistance to phagocytosis and avoid complement activation. Pegylated nanocarriers show an increased longevity in the circulation and potential to accumulate predominantly in pathological sites with compromised leaky vasculature which is characteristic for solid tumors as well as infracted tissue, infectious and inflamed sites. Defective vascular architecture coupled with poor lymphatic drainage, for example, is common to many kinds of solid tumours and has been exploited to promote drug accumulation in tumor site (the so-called Enhanced Permeability and Retention (EPR) effect). Of course, carrier distribution will be unduly affected by cutoff size of tumor blood vessel wall, being this parameter variable for different tumors. To date, almost all long-circulating nanoparticles are cleared from the systemic circulation of test animals within 8-10 h after IV administration. Direct correlations between the longevity of a nanocarrier in the circulation and its ability to reach the target have been observed on multiple occasions.

Recently, a lot of studies have been performed on surface modification of superparamagnetic nanopartilces, which are now considered as promising agents for drug delivery into regional lymphnodes and for diagnostic imaging purposes.

It is worth of note that nanocarriers with a similar structure (size, coat type, zeta potential) but a different core composition (liposome versus nanoparticles) can show a completely different biological behaviour.

The further development of the concept of pharmaceutical biomimetic nanocarriers implies their surface engineering with specific ligands able to interact with cell surface-characteristic structures (active targeting) thus imparting recognition capability. This approach has been extensively applied

to improve specificity of anticancer treatments by increasing drug dose able to accumulate specifically in the diseased site by exploiting differences between cancer and normal cells. Since cancer cells overexpress particular antigens on their surface, antibody-bearing nanocarriers can be constructed once a specific target has been identified with confidence and is not expressed in significant quantities elsewhere in the body. Along this line, prostate specific antigen and erbB2 have been selected as possible targets. Immunoliposomes bearing anti-erbB2 antibodies have demonstrated their potential in human breast cancer models in immunotherapy. Several targets have been used for this purpose comprising peptide receptor, folate, transferrin and integrin surface receptors as well as the asyaloglicoprotein receptor of liver cells. In the case of folate-bearing nanoparticles, it has been demonstrated that the way the ligand is exposed on carrier surface strongly affects target cell recognition and carrier internalization. It has been suggested too that drug accumulation and slow release inside tumour cells can be useful to circumvent Multi Drug Resistance.
It is worth of note that whether the concept of active targeting has led to many liposomal-based constructs, there are much less data in the literature concerning the active targeting performed with polymer nanoparticles for cancer treatment probably because of the needing of tailor-made materials with well-defined properties.

***Nanoparticles and brain delivery***

Recently, a great deal of interest is focusing on the possibility to deliver drugs to brain through non-invasive techniques. This approach is intended for local diseases which can be cured through drugs which normally cannot pass blood brain barrier (BB). In this context, specifically designed polymeric NP have been designed and tested. The most well studied system is polysorbate-coated poly(butylcyanoacrylate) (PBCA) nanoparticles which are able to deliver peptide and peptide-like drugs, lipohilic small drugs into the brain in mouse and rat models. The advantage of nanoparticulate systems is related mainly to the fact that drug action in the brain is prolonged due to its slow release from the carrier. It is believed that Polysorbate 80 is able to anchor Apo B/E on nanoparticle surface in the blood and encompass receptor-mediated endocythosis of LDL in BBB. In other studies lipid-coated nanoparticles, solid lipid nanoparticles, thiamine-coated nanoparticles have been tested. Efforts are also devoted to assess nanoparticle toxicity, and mainly the effect on BBB integrity, and understand the mechanism underlying their transport in the brain.

***Conclusions***

The advances in pharmaceutical nanotechnology have been undeniably sustained by a strict interaction with biomaterial science which have contributed to develop structures more and more tailor-made on the need of specific applications. Despite the fact that significant progresses have been made in the field of nanotechnology, one century has gone by since the concept of “magic bullet” was proposed and we are still very far from being able to direct a drug to the desired site of action. As a natural consequence, a great effort of research is now focusing on how to make this concept a reality. A careful design of tailor-made polymeric materials with different architectures and bearing functional groups which can be exposed on nanoparticle shell will strongly support the development of more efficient targeted nanocarriers and help the understanding of crucial problems relating their structure and physico-chemical properties to pharmacological performance.

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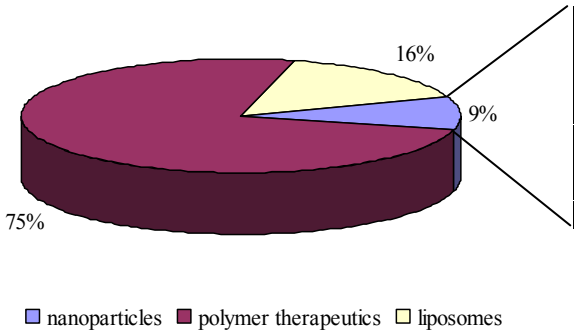
**Table 1.** Nanomedicines in routine clinical use or development.




| Product | Compound | Status | Use |
|---|---|---|---|
| *Imaging Agents* | | | |
| Endorem® | Superparamagnetic iron oxide nanoparticle | Market | MRI agent |
| Gadomer® | Dendrimer-based MRI agent | Phase III | MRI agent-cardiovascular |
| *Drug delivery* | | | |
| Abraxane® | Albumin nanoparticle | Market | Breast Cancer |

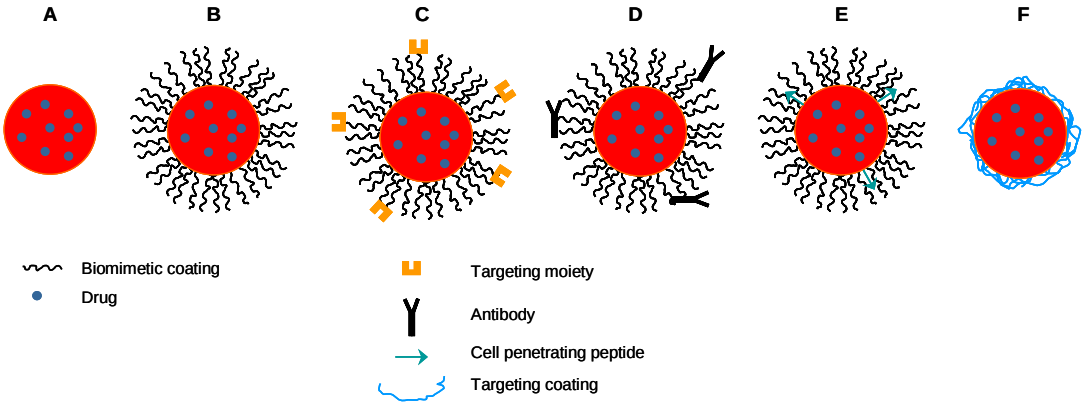



**Figure 1.** Schematic structure of different drug-loaded nanoparticles. A. Conventional nanoparticles; B. Long-circulating nanoparticles; C Targeted nanoparticles; D. Immunonanoparticles; E. Cell-penetrating nanoparticles; F. Polymer adsorbed nanoparticles. Core-forming polymer is represented in red. Adapted from reference 6.

**Delivery of oligodeoxyribonucleotides and siRNAs: opportunities from micro and nanotechnologies.**

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Oligodeoxyribonucleotides (ODNs) are synthetic fragments of DNA, from 13 to about 25 nucleotides, able to selectively modulate gene expression in mammalian cells. Gene silencing can be achieved with ODNs by inhibition of transcription factor binding to promoter region of target gene (decoy ODNs), inhibition of gene transcription (antigene ODNs), inhibition of the translation process (antisense ODNs). Recently, great attention have been paid to short double stranded RNAs (small interfering RNA or siRNAs) able to induce cleavage of specific mRNA, mediated by RNA Induced Silencing Complex (RISC). Compared to ODNs, siRNAs can provide silencing effects at doses significantly lower. Due to the high selectivity, a growing number of ODNs and siRNA sequences are proposed for the treatment of several diseases, such as cancer, inflammation, viral infection. However, despite the encouraging results obtained on cell cultures, the key hurdle is to move from the lab to the bedside of patients. Indeed, due to the rapid degradation in biological fluids and to the macromolecular and polyanionic character, nucleic acids (NAs) are characterised by very short half-life and low bioavailability. Thus, a therapy with these molecules require high doses and frequent administrations by invasive routes. Chemical modifications have shown to increase ODN and siRNA stability against enzymatic degradation and improve their cell uptake. However, modification of the chemical backbone could affect NA interaction with the target, with consequent reduced activity or appearance of side effects (Agrawal 1999). Moreover, issues such as aspecific biodistribution in the body remains unresolved with this approach.

The use of delivery systems represents a promising strategy to improve ODN and siRNA pharmacokinetics. An ideal delivery system for ODNs and siRNAs should be safe, improve stability toward nucleases, accumulate into targeted tissues/cells, improve cellular penetration and release the drug in the right intracellular compartment. Moreover, a sustained NA release is desirable with the aim to avoid frequent administrations. Micro and nanotechnologies have been extensively investigated for the NA delivery in order to meet all the requirements mentioned above (table 1).

Cationic liposomes are certainly the most diffused nanocarriers for ODNs and siRNAs delivery (figure 1A). Different liposome-based formulations are today available for *in vitro* transfection experiments. Liposomes are lipidic bilayer vesicles delimiting one or more aqueous cavities. Bilayer generally comprises cationic lipids (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride or DOTMA, 1,2-dioleoyl-3-trimethylammonium-propane or DOTAP, 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride or DC-Chol, etc.) able to impart a positive charge to the colloid. Cationic liposomes electrostatically interact with NAs and the resulting complexes, also called lipoplexes, enter into cells by endocytosis. NA escape from endosomes/lysosomes and dissociation from cationic lipids have been explained by a "flip-flop" mechanism in which anionic lipids diffuse from endosomal membrane into the complex, neutralizing the positive charge of cationic lipid and leading to NA release into the cytoplasm (Zelphati et al. 1996). Transfection efficiency of liposomes can be strongly improved by associating cationic lipids to neutral lipids such as cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). However, cytotoxicity of cationic liposomes as well as physical instability of lipoplexes in serum remain unresolved issues. Several studies have been focused on the development of liposome-based delivery systems able to overcome these limits. "Stabilised liposomes" that is characterised by the presence of a poly(ethylenglycol) (PEG) coating realized by adding PEG-conjugated lipids in the formulation prevent vesicles aggregation and reduce binding to serum proteins, thus reducing opsonization process (Meyer et al. 1998). Recently, liposomes

containing an UV-inactivated Haemagglutinating virus of Japan or HJV (HJV-liposomes) have showed an efficient delivery of ODNs *in vivo*, although the risk of immunogenicity should be seriously taken into account. Attachment of ligands (e.g., folate or transferrin) or an antibody fragment directed against a cell surface receptor, can facilitate lipoplex entry into cells via receptor-mediated endocytosis. With this approach ODNs and siRNAs have been systemically delivered to primary and metastatic tumor cells (Rait et al. 2002, Pirollo et al. 2006).

An alternative to cationic liposomes is represented by cationic polymers able to interact with NAs forming nanosized complexes, also called polyplexes. Among cationic polymers, polyethylenimine (PEI) (figure 1B) has been largely investigated for ODN (Boussif et al. 1995) and recently also for siRNAs (Subramaniam V et al. 2007) delivery. PEI is a polymer, available in different molecular weights and in linear or branched form, characterised by a high density of positive charges. PEI capacity to effectively transfer NA to mammalian cells, has been attributed to the "proton sponge effect" of the polymer, which leads to endosome rupture and consequent release of NA into the cytoplasm (Boussif et al. 1996). Cytotoxicity and transfection efficiency of PEI are linearly related to its molecular weight (Godbey et al. 2001). Many studies have been aimed to reduce its toxicity by chemically grafting linear PEG to PEI (Petersen et al. 2002), using low molecular weight PEI, PEI glycosylation (Leclercq et al. 2000). Efficient delivery of ODNs and siRNAs by using PEI have been demonstrated in vivo (Pirollo et al. 2005, Grzelinski et al. 2006). Another class of cationic polymer is represented by dendrimers, among which starburst polyamidoamine (PAMAM) dendrimers are the most successful in NA transfection. PAMAM are synthetic branched polymer characterized by a spherical shape and a high density surface charge. Depending on the layers of branched chains added with the synthesis, different dendrimer "generations" can be distinguished. The different generations correspond to carriers with a different transfection efficiency. Dendrimer/ODN complexes are stable in the presence of serum, although transfection efficiency as well as cytotoxicity, depend on the generation used (Melik et al. 2000).

Different types of nanoparticles have been used as delivery systems for ODNs and siRNAs. Cationic nanospheres can be made of either cationic polymers or not charged polymers on which cationic surfactants are adsorbed (figure 1C). In this last case, nanospheres can be composed of synthetic biodegradable polymers such as poly(lactic acid) (PLA) or poly(alkylcyanoacrylate) (PACA) (Fattal et al. 1998) on which an ion pairing agent, such as the cationic surfactant cetyltrimethylammonium bromide (CTAB), can be adsorbed onto the surface (Fattal et al. 1998). ODNs and siRNAs complexed with cationic nanoparticles are efficiently uptaken into cells and this effect was also demonstrated *in vivo* (Schwab et al. 1994). However, for optimal activity, NAs should not be adsorbed but rather entrapped within particles. Polyisobutylcyanoacrylate (PIBCA) nanocapsules containing an aqueous core (figure 1D) have been successfully used to encapsulate both ODNs and siRNAs. These systems showed an increased NA stability in the presence of serum as compared with NA adsorbed on nanoparticles (Lambert et al. 2000). Encapsulation into PIBCA nanocapsules importantly increased cell uptake of both ODNs and siRNAs (Toub et al. 2005, 2006), although the mechanism by which encapsulated ODNs can escape from endosomes is not yet fully understood. It has been shown that the use of nanocapsules allows a strong reduction of NAs administered dose in an *in vivo* model (Toub et al. 2006).

Beyond protection against degradation and improved uptake into cells, NA encapsulation into nano or microparticle (figure 1E) based on biodegradable polymers allows to release NAs for long time frames, thus reducing the need of frequent administrations. Among the biodegradable polymers, PLA and its copolymers with glycolic acid (PLGA) have been extensively used in the last three decades as pharmaceutical excipients. This family of polymers is available on the market at different molecular weights, lactide to glycolide ratios and hydrophilicity (i.e. capped and uncapped end-groups) offering the possibility to select among several biodegradation rates. Once entrapped into PLGA particles, ODN is also efficiently protected by degradation in serum (Lewis et al. 1998, De Rosa et al. 2003). It has been shown that ODNs can be slowly released from microspheres for more than one month (De Rosa et al. 2005). When incubated with cells, the *in vitro* fate of the NAs

entrapped into PLA/PLGA particles can vary according to particle size. In particular, PLGA nanoparticles (mean diameter lower than 1 μm) and small microparticles (mean diameter between 1 and 5 μm) are taken up by cells, while large microparticles (diameter higher than 5 μm) are unable to enter into cells and are rather developed for sustained NA release. Although these systems have been developed as depot devices, it has been demonstrated that the slow release from microspheres strongly increases cell uptake of ODN as compared with naked ODN (De Rosa et al. 2003, De Rosa et al. 2005). *In vivo* studies confirmed the long lasting pharmacological effect of ODN when delivered as PLGA microspheres (Khan et al. 2000).
In spite of many efforts for the development of nano and microsized delivery systems for NAs, at the moment no medicinal product is available, although the number of clinical trials has increased in the last years (Li et al. 2007). However, the relatively high transfection efficiency together with the absence of risks of nano and microcarriers should incite researchers to further optimize their performance, finally transferring ODNs and siRNAs "from the lab bench to the bedside".

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| Delivery system | Support material | Type of interaction with ODNs/siRNAs | Effect |
|---|---|---|---|
| Cationic liposomes | Cationic lipids (e.g. DOTMA, DOTAP, DC-Chol, etc.) frequently associated to helper lipids (DOPE or cholesterol) | Electrostatic interaction | Improved NA uptake<br>Increased NA stability against nucleases |
| Cationic polymers | Dendrimers, Polyethylenimine | Electrostatic interaction | |
| Cationic nanoparticles | PIBCA or PLA associated to cationic surfactant (e.g., CTAB) | Electrostatic interaction | |
| Nanocapsules with an aqueous core | PIBCA | Encapsulation | |
| Nano/microparticles | PLA/PLGA | Encapsulation | Long term delivery<br>NA protection against nucleases<br>Improved NA cellular uptake |

**Table 1.** Characteristics of delivery systems more commonly used for ODN and siRNA delivery.

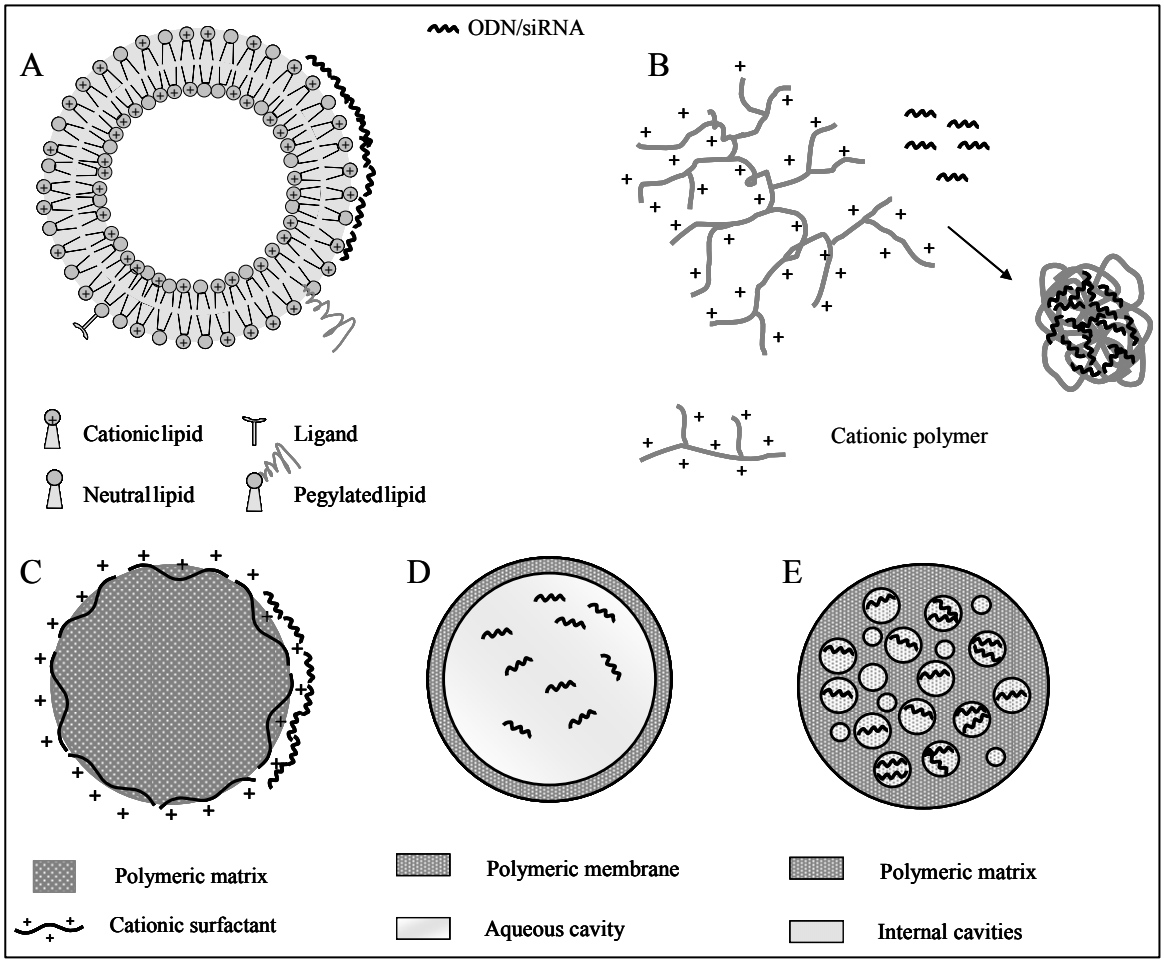


**Figure 1.** Schematic representation of the delivery systems more commonly used for ODN and siRNA delivery. A) Cationic liposomes; B) cationic polymers; C) cationic nanoparticles; D) nanocapsules; E) nano/microspheres.

## Interferon and liposome pegylation

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Many methods have been studied in the last three decades in an effort to modify the molecular and biochemical characteristics of proteins and macromolecules in order to prolong the permanence of substances in circulation. Poly(ethylene glycol) (PEG) modification had been used for many years to prolong the half lives of biological proteins and to reduce their immunogenicity [1] PEG is a linear polyether diol with many useful properties such as biocompatibility, solubility in aqueous and organic media, lack of toxicity, very low immunogenicity and antigenicity, and good excretion kinetics. These properties allow its use in a variety of applications, including the biomedical field, after FDA approval for internal administration. PEG moieties are inert, long-chain amphiphilic molecules produced by linking repeating units of ethylene oxide. A large number of potential PEG molecules are available, and they can be produced in different configurations, including linear or branched structures, and in different molecular weights. *In vivo* PEG does not exhibit specific affinity for any organ and its accumulation in the tumor tissue is mainly governed by the level of hyperpermeable tumor vasculature (enhanced permeability retention, EPR effect), irrespective of the molecular mass of the polymer and the tumor loading site. Conversely, the higher the molecular mass of PEG, the longer the retention time in the blood. The polymer backbone is chemically inert, and the terminal primary hydroxyl groups are available for derivatization.
Goals for chemically coupling PEG to protein drugs [2] include decreased clearance, retention of biological activity, and enhanced water solubility without significantly altering bioavailability (for example by subcutaneous injection). These changes can produce a number of clinical advantages, such as sustained plasma concentrations, decreased adverse effects, improved patient convenience and enhanced quality of life. Large proteins generally have more attachment sites and, therefore, are commonly multipegylated. Attachment at multiple sites, however, increases the likelihood of steric interference at the active site of the native protein, resulting in a possible inhibition or reduction of activity. Small proteins generally have fewer attachment sites and can be effectively pegylated with a single large (possibly branch-chained) PEG moiety. A stable linkage between the PEG moiety and the drug is important to ensure that PEG-induced pharmacological changes are maintained. Protein pegylation is generally achieved by formation of linkages between an amino group on the protein and an active carbonate, active ester, aldehyde or tresylate derivative of PEG. The use of a functional amino group is justified by the high number of residues bearing amino groups present on the protein surface exposed to the solvent. According to the procedure used for PEG activation, the final conjugate may have amino groups no longer in the nucleophilic form (as in the case of all reactions based on amino group acylation) .This must be taken into consideration when amino group acylation is associated with a decrease or even loss of protein activity. Pegylation produces alterations in the physicochemical properties of the parent molecule [3], these include changes in conformation, steric hindrance, changes in electrostatic binding properties, hydrophobicity, local lysine basicity and pI (the pH at which a protein's charge is neutral). These physical and chemical changes reduce systemic clearance by a number of mechanisms, including decreases in renal clearance, proteolysis and opsonisation (macrophage uptake), and can influence the binding affinity of the therapeutic protein to cellular receptors, resulting in changes in the bioactivity of the agent. In addition, pegylation may increase the absorption half-life of subcutaneously administered agents, and is sometimes associated with a decreased Vd. Pegylation may decrease cellular protein clearance by reducing elimination through the reticulo-endothelial system or by specific cell-protein interactions. In addition, pegylation forms a protective 'shell' around the protein. This shell and its associated waters of hydration shield the protein from immunogenic recognition and increase resistance to degradation by proteolytic enzymes.

**Interferons** (IFNs) represent, so far, the most effective drugs in inducing remission of chronic hepatitis C, but they have very short half-lives and persist in serum for only a few hours. They are administered three times a week, which leads to wide fluctuations in the serum IFN concentration [4]. Unfortunately, this treatment has significant limitations and is effective in a minority of patients. When IFN is used alone, approximately 40% of patients initially respond to treatment with normalisation of the ALT level and loss of detectable HCV-RNA. Treatment with IFN monotherapy has produced sustained virological response rates only in 15 20% of patients [5].

Effectiveness of IFN alfa has been limited by protein characteristics that include poor stability, a short half-life and immunogenicity. The resulting fluctuations in exogenous IFN alfa concentration during the course of treatment are believed to negatively impact the anti-viral efficiency of the drug. Maintaining drug concentrations at or near a target concentration for an extended period of time is often clinically advantageous, and is particularly useful in antiviral therapy, since constant antiviral pressure should prevent replication and may thereby suppress the emergence of resistent variants Additionally, pegylation modification may decrease adverse effects caused by large variations in peak-to-trough plasma drug concentrations associated with frequent administration and by the immunogenicity of unmodified proteins

Manufacturers of the two PEG-IFN products (Fig. 1) have selected two different PEG-chain sizes: PEG-IFN alfa 2b has a straight PEG chain of approximately 12,000 daltons (12kDa); PEG-IFN alfa 2a has a branched PEG chain of approximately 40,000 daltons (40kDa). PEG-IFN alfa 2a is produced by attaching PEG at multiple lysine sites of IFN alfa 2a (94% Lys 31, Lys 121, Lys 131, Lys 134; 6% Lys 70, Lys 83), and PEG-IFN molecule retains only 7% of IFN alfa 2a's native bioactivity [6]. In contrast, the production of PEG-IFN alfa 2b utilises a unique process to attach PEG at multiple positions including 45% at histidine residues, with the remainder primarily at lysine residues and a small amount elsewhere. This attachment pattern retains 37% of IFN alfa-2b's native bioactivity. Increasing the PEG size or the number of PEG attachments is not always advantageous. For example, as the PEG size increases, the antiviral activity of the molecule decreases. Therefore, a PEG that is too large will severely restrict the antiviral activity of the molecule, thus requiring higher and higher doses to achieve the same physiologic effect. A larger PEG molecule directly obscures the binding portion of the IFN molecule by steric hindrance so that IFN cannot reach the cell receptor. As would be expected, the larger PEG molecule (40kDa) results in significantly more hindrance than the smaller one (12 kDa): while PEG IFN alfa 2b retains 37% of the activity of standard IFN alfa 2b, PEG-IFN alfa 2a retains less than 7% of the activity of nonpegylated IFN. This helps to explain why PEG-IFN alfa 2a doses must be much higher than PEG-IFN alfa 2b doses to obtain an effect. However, although a larger PEG molecule may be associated with a lower *in vitro* specific activity, it may not be associated with less activity *in vivo.* In general, the reduction of potency at the molecular receptor should be compensated by the prolonged residence in blood, or by the higher total drug exposure. Another factor must be considered when selecting the size of the PEG chain: the ability to dose-reduce or discontinue therapy quickly: if the molecule is too large and half-life significantly prolonged, it becomes difficult to ameliorate adverse events quickly after dose reduction or treatment discontinuation [7]. The volume of distribution is considerably greater for PEG-IFN alfa 2b (69 liters) than PEG-IFN alfa 2a (6-14 liters) [8]. Drugs with a high volume of distribution have the best potential to infiltrate peripheral tissue, drugs with a low volume of distribution offer much less chance to infiltrate extravascular tissue. Multiple studies demonstrate that HCV can be widely distributed to tissues and organs, in fact, in hepatitis C, viral suppression in blood alone may not be enough: thus, reservoirs outside the blood may play a role in both HCV persistence and reactivation of infection. HCV is able to infect different extrahepatic sites such as peripheral blood mononuclear cells (PBMCs), renal cells, thyroid cells, and gastric cells, and there is mounting evidence that these could represent replicative compartments for the virus [9]. Due to its restricted volume of distribution the PEG-IFN alfa 2a molecule accumulates preferentially in the blood, which would suggest that the molecule would then fail to penetrate other specialised compartments of the body where HCV might be

lurking. The lesser volume of distribution of PEG-IFN alfa 2a has been invoked also as an argument to support fixed, rather than weight based dosing. With fixed-dose treatment, there is a direct correlation between increasing body weight and decreasing rate of SVR [10]. Individualising therapy by using weight-based dosing provides a consistent rate of SVR across the broad spectrum of body weight in a given patient population. Therefore, individualised weight adjusted dosing of PEG-IFN might represents the best treatment strategy to assure that all patients have the same opportunity to achieve a SVR [11]. Moreover, lighter weight was associated with more grade-3/4 neutropenia than heavier weight.

In conclusion the two available PEG-IFN products confer enhanced therapeutic efficacy when compared with their IFN counterparts. Both have the added convenience of once-weekly dosing with no novel toxicities. PEG optimises the action of the IFNs by decreasing clearance rates, thereby allowing serum concentrations to remain constant over the dosing period. The length and shape of each PEG moiety are crucial in determining the effect on pharmacokinetic and pharmacodynamic properties. The design of PEG-IFN must balance the loss of activity due to the increase of PEG size against the benefits of prolonged circulation times. PEG-IFN alfa 2a has a large chain, which allows for a prolonged half-life, but it becomes difficult to ameliorate adverse events quickly after dose reduction or treatment discontinuation. In hepatitis C, viral suppression in blood alone may not be enough: HCV reservoirs outside the blood may play a role in HCV persistence, therefore drugs with a high distribution volume like PEG-IFN alfa 2b may infiltrate extravascular tissues, which may explain the lower relapse rate as compared to derivatives with small distribution volume.

**Classical liposomes** (phospholipid bilayer vehicles) have been shown to alter biodistribution by reducing drug clearance, decreasing the volume of distribution (Vd) and shifting the distribution in favour of diseased tissues that have increased capillary permeability [12].

The use of the first liposomes as drug carriers is supported by the following properties: intravenously administered liposomes are captured by monophagocytes in the reticulo-endothelial system (RES) and can be used for efficient delivery of antiparasitic and antimicrobial drugs in treating infections localized in this system. However, the efficient uptake of liposomes by the RES, probably due to their opsonization by plasmaproteins, results in their rapid removal from circulation and is the main disadvantage for the possible use of liposomes as delivery systems when the target sites beyond the RES. In addition, liposomes can induce complement activation, resulting in enhanced clearance as well as a risk of cardiovascular and haematological adverse events. Although they were first described in the 1960s, only at the beginning of 1990s, did the first therapeutic liposomes appear on the market. The first-generation liposomes (conventional liposomes) comprised a liposome-containing amphotericin B, Ambisome (Nexstar Co., Boulder, CO, USA), used as antifungal drug [13] and Myocet [14] (Elan Pharma Int. Princeton, NJ, USA), a doxorubicin-containing liposome, used in clinical trials to treat metastatic breast cancer. The second-generation liposomes ("pure lipid approach") were long-circulating liposomes, such as Daunoxome, a daunorubicin-containing liposome, approved in US and Europe to treat AIDS-related Kaposi's sarcoma (ARKS) [15]. The third-generation liposomes were surface-modified liposomes with gangliosides or sialic acid that can evade the immune system responsible for removing liposomes from circulation (RES system) [16]. The fourth-generation liposomes, pegylated liposomes, were called "stealth liposomes" because of their ability to evade interception by the immune system, in the same way as the stealth bomber was able to evade radar [17] (Fig.2). To obtain stealth liposomes is necessary to decrease the interaction with serum proteins, the so-called opsonins such as complement (C3a and C3b), fibronectin and immunoglobulin (mainly IgG). Actually the only stealth liposome on the market is Caelyx/Doxil (pegylated liposomal doxorubicin, PLD), (Schering-Plough Corp., Madinson, NJ, USA), used to cure ARKS, resistant ovarian cancer and metastatic breast cancer [18-20]. PLD is characterized by very long-circulation half-life, favourable pharmacokinetic behaviour and specific accumulation in tumour tissues.These features account for the much lower toxicity shown by Caelyx in comparison to free doxorubicin, in terms

of cardiotoxicity, vesicant effects, nausea, vomiting and alopecia. PLD also appeared to be less myelotoxic than doxorubicin. Typical forms of toxicity associated to PLD are acute infusion reaction, mucositis and palmar plantar erythrodysesthesia (PPE) occurring especially at high dose or short dosing interval.

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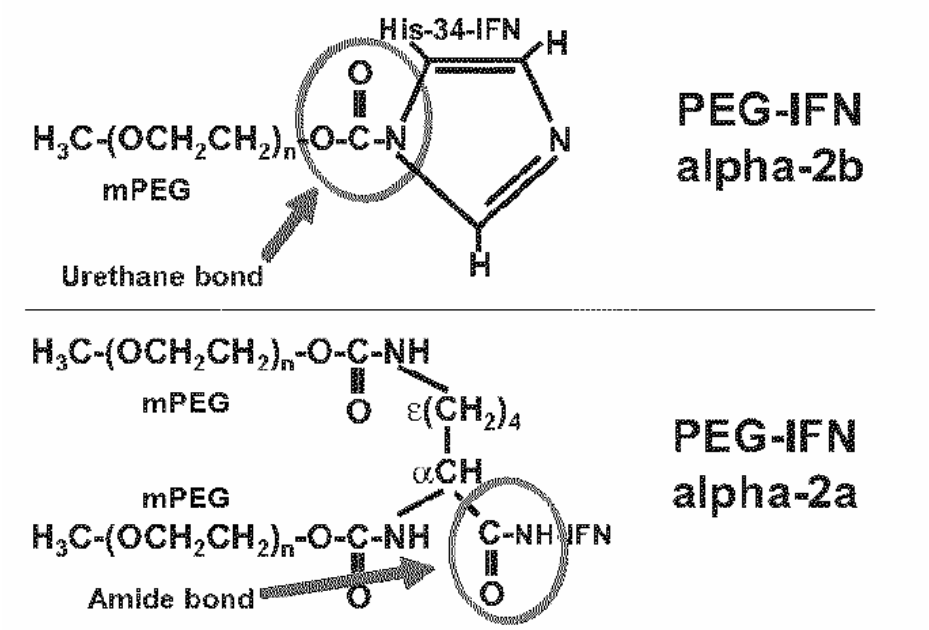


**Figure 1** - Structure of the main products obtained by interferon pegylation with PEG 12kDa and 40kDa.

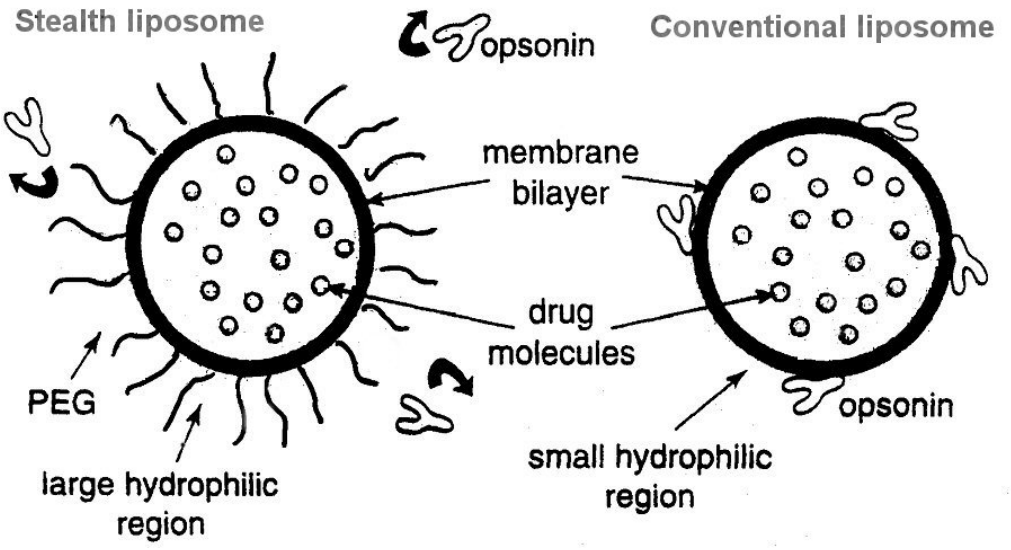


**Figure 2 –** Stealth and conventional liposomes

**Single nucleotide polymorphisms (SNPs) and prediction of anti-cancer drug activity and toxicity**

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Cancer chemotherapy has progressed since its introduction into clinical practice and represents the most promising treatment modality. Despite the availability of a wide range of different anti-cancer drug classes, several cancer patients do not respond to therapy and/or develop serious adverse effects, although diagnosis and drug dosage are correct. Therefore, the wide variability in response to anti-cancer therapy may compromise the pharmacological and clinical management of patients by limiting the ability to predict drug activity and toxicity.

The molecular mechanisms responsible for the individual variability of the response to anti-cancer drugs are not fully elucidated, but increasing evidence suggests that genetic background plays a determining role (Danesi et al., 2001). In this respect, it should be pointed out that clinical outcome is thought to result from the combinatorial effect of a number of key genes, involved in part in drug metabolism and disposition (pharmacokinetics), in part in drugs' mechanism of action (pharmacodynamics). Genetically induced alterations of pharmacokinetic pathways can either lead to the accumulation of the drug in the organism, thus increasing the risk of adverse drug reactions, or they can determine a lack of response by inducing an abnormally high rate of drug clearance. Genetic alterations of pharmacodynamic pathways, on the other hand, can alter target molecules' ability to interact with the drug, thus affecting its activity (Evans and McLeod, 2003).

Dihydropyrimidine dehydrogenase (DPD), i.e., the rate-limiting enzyme of pyrimidine catabolism, represents a therapeutically promising example of genetic variations in genes that code for proteins involved in key pharmacokinetic pathways of anti-cancer drugs. This enzyme is responsible for the elimination of approximately 80% of administered dose of 5-fluorouracil (5-FU); however, enzyme activity is highly variable and DPD deficiency predispose patients to severe adverse events, including neurological and gastrointestinal toxicities. Such a pharmacogenetic syndrome has been detected in 3% to 5% of the Caucasian population. Genotypic studies have identified several sequence variants within the DPYD gene, some of which include G62A, T85C, ΔTCAT295-298, A496G, C703T, G1003T, G1156T, G1601A, A1627G, T1679G, ΔC1897, G2194A, G2657A, A2846T, G2983T (Danesi et al., 2001). The mutation IVS14+1 G>A (DPYD*2A), the most common mutation associated with DPD deficiency, was detected in 24-28% of all patients suffering from severe 5-FU toxicity (van Kuilenburg, 2004). Specifically, a G>A base change at the splice recognition sequence of intron 14, leads to exon skipping and results in a 165-bp deletion in the DPD mRNA. As a consequence of this, the homozygote DPYD*2A genotype results in complete deficiency while the heterozygous DPYD*2A genotype results in partial deficiency of DPD (Saif et al., 2007). More recently, methylation of the DPYD promoter region has been also recognized as a potentially important regulatory mechanism of DPD activity and basis for 5-FU toxicity in cancer patients (Ezzeldin et al., 2005).

The toxicity of thiopurine drugs has been correlated to the activity of thiopurine S-methyltransferase (TPMT), whose interindividual variation is a consequence of genetic polymorphisms. TPMT has been demonstrated to inactivate 6-mercaptopurine and 6-thioguanine by S-methylation at position C-6 of the purine ring. The genetics of TPMT deficiency have been defined with the identification of the wild-type allele TPMT*1 and three mutant alleles, TPMT*2 (G238C transversion), TPMT*3A (G460A transition) and TPMT*3C (A719G transition). Reduced activity of TPMT caused by polymorphisms in the TPMT gene has been associated to severe or

potentially lethal neutropenia in leukaemia patients administered with either 6-mercaptopurine or 6-thioguanine, or in those where azathioprine was given for immunosuppression following organ transplant (Danesi et al., 2001). Similarly, polymorphism of methylenetetrahydrofolate reductase (MTHFR), an enzyme which catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, predisposes to severe bone-marrow toxicity during treatment with an inhibitor of folate synthesis, as observed in breast cancer patients given methotrexate in combination with cyclophosphamide and 5-FU (Toffoli et al., 2000).

Another pharmacokinetic pathway of major relevance to cancer pharmacotherapy involves glucuronidation. The DNA topoisomerase I inhibitor, irinotecan, is mainly detoxified by UDP-glucuronosyltransferase 1A1 (UGT1A1) and decreased enzyme activity caused by UGT1A1*27 and UGT1A1*28 variant genotypes are related to severe toxic effects of this drug (Fujita and Sasaki, 2007).

Glutathione S-transferases (GSTs), a family of enzymes composed of alpha (GSTA), mu (GSTM), pi (GSTP) and theta (GSTT) classes, have been recognized to play an important role in drug detoxification and are associated with tumour-cell resistance to anti-cancer agents. For instance, GST polymorphisms can alter the risk of cisplatin ototoxicity because individuals with the GSTP3*B allele are less susceptible than others (Peters et al., 2000).

CYP enzymes, particularly the CYP3A subfamily, are responsible for activation or inactivation of several anti-cancer agents. CYP3A4 catalyses the activation of the prodrug ifosphamide, raising the possibility that tumour cells could generate cytotoxic metabolites, depending on CYP enzyme activity (Murray et al. 1999), while interpatient variation in susceptibility to the leukaemogenic effect of epipodophyllotoxins might be related to the polymorphism in the 5′ promoter region of CYP3A4 (leading to the variant allele CYP3A4*V), which in turn, is associated with decreased enzyme activity and reduced production of DNA-damaging catechol and quinone metabolites of this drug class. This finding might explain the increased risk of leukaemia in individuals previously given teniposide or etoposide for other cancers and carrying the wild-type CYP3A4 genotype (CYP3A4*W) with respect to CYP3A4*V (Felix et al., 1998).

Concerning the relationship between genotype and drug efficacy, it has been widely recognised that the cellular thymidylate synthase (TS) level is one of the determining factors for the antitumor activity of fluoropyrimidines. Polymorphism in the 3'-untranslated region of the TS gene, classified according to the presence or absence of a 6 bp nucleotide fragment, has been related to sensitivity of stomach cancer to fluoropyrimidine-based chemotherapy. In particular, the response rate of the -6/-6 bp and -6/+6 bp groups was found to be significantly higher than the +6/+6 bp group, thus suggesting that the TS 3'-UTR polymorphism profile can be used to guide the choice of 5-FU-based chemotherapy, in this type of neoplasia (Lu et al., 2006). The variable number of tandem repeat (VNTR) of TS gene, mainly 2 repeat (2R) and 3 repeat (3R), is one of the genetic variations that can potentially predict the effectiveness of 5-FU-based chemotherapy. Interestingly, the selection of patients who are likely to respond to 5-FU therapy may be substantially improved by considering an additional SNP (G/C polymorphism) within the 28-bp repeat component of TS VNTR (Kawakami and Watanabe, 2003; Marcuello et al., 2004).

A recent study performed in 210 esophageal cancer patients demonstrated that MTHFR Glu429Ala variant genotypes were associated with significantly improved survival (hazard ratio = 0.56; 95% CI, 0.35 to 0.89) in patients treated with 5-FU (Wu et al., 2006). According to this, pretherapeutic determination of the MTR A2756G polymorphism may predict survival of multimodally treated oesophageal squamous cell carcinomas (Sarbia et al., 2006). Furthermore, the response rate was significantly higher in patients with metastatic colorectal cancer who carry MTHFR 677 TT mutation compared with the other groups (Jakobsen et al., 2005).

A multivariate analysis of genomic polymorphisms aimed to investigate whether distinct patterns of functional genomic polymorphisms in genes involved in drug metabolic pathways and DNA repair could predict clinical outcome to 5-FU/oxaliplatin chemotherapy in patients with advanced colorectal cancer, suggests that the genomic polymorphisms xeroderma pigmentosum group D

(XPD)-751, excision repair cross complementing group 1 (ERCC1)-118, GSTP1-105, and TS-3'UTR may be useful as predictive markers for overall survival and time to progression (Stoehlmacher et al., 2004).

Although such remarkable advances in the understanding of genotype-drug-response phenotype associations may represent a rational basis towards a personalized medicine, the realization of these expectations seems to be more challenging than some initially expected. Indeed, several studies investigating the association between genotype and therapeutic results in cancer patients show contrasting data, thus determining increasing uncertainties in the identification of reliable predictive genetic markers of drug response which cannot yet be easily translated into treatment recommendations. This is probably related to difficulties in replication of findings from association studies in prospective trials from diverse populations and the small numbers of patients examined in some studies. Furthermore, multiple SNPs within a haplotype may have a higher predictive pharmacogenetic power than individual SNPs (Seck *et al*., 2005; Herrlinger et al., 2005; Lankisch et al., 2006; Jones *et al*., 2007) by highlighting that coinheritance of SNPs on these haplotypes, if not considered, may yield to misleading information on the causative role of individual SNPs in drug response phenotype. Finally, the complex mechanism of action of drugs currently used in the treatment of cancer and the intricate interplay between different drug targets including enzymes, signal transduction intermediates, transcription factors as well as molecular and cellular immunosystem entities, underline the key problem of multigenic influences in anti-cancer drugs' therapeutic outcomes.

Therefore, the future challenge of cancer pharmacogenetics relies on the identification of polygenic determinants of drug effects by an innovative multidisciplinary approach for the development of a genotype platform aimed at predicting drug response in the individual patient. Towards this ambitious goal, the synergistic interaction between clinicians, pharmacologists, molecular biologists and computer scientists as well as the availability of emerging powerful technologies for high-throughput SNP genotyping (Twyman, 2004; Koch, 2004), web-based programs to automate the process of selecting SNPs for genetic association studies (Xu *et al*., 2005), and information contained in the recently developed haplotype map (Altshuler *et al.*, 2005) may offer the opportunity to create, in a not-too-distant future, a prototype genetic test containing several polymorphisms in a relative wide number of candidate genes to be validated in well-designed randomized clinical trials (Figure). The NIH Pharmacogenetics Research Network (PGRN), a group of investigators with various approaches to ultimately identify genetic variants that predispose an individual to nonresponse to or toxicity from drugs, represents an illustrative example of the integrative-collaborative model towards genotype-based therapeutic drug optimization. Indeed, the collective expertise of the network may allow for synergy in developing methods and in populating a knowledgebase in pharmacogenomics (Giacomini et al., 2007).

This perspective implies that the classic approach of dosage regimen optimization by a trail-and-error method for the treatment of cancer may be abandoned in favour of individually-tailored anti-cancer drugs based on an individualized genotype-phenotype map.

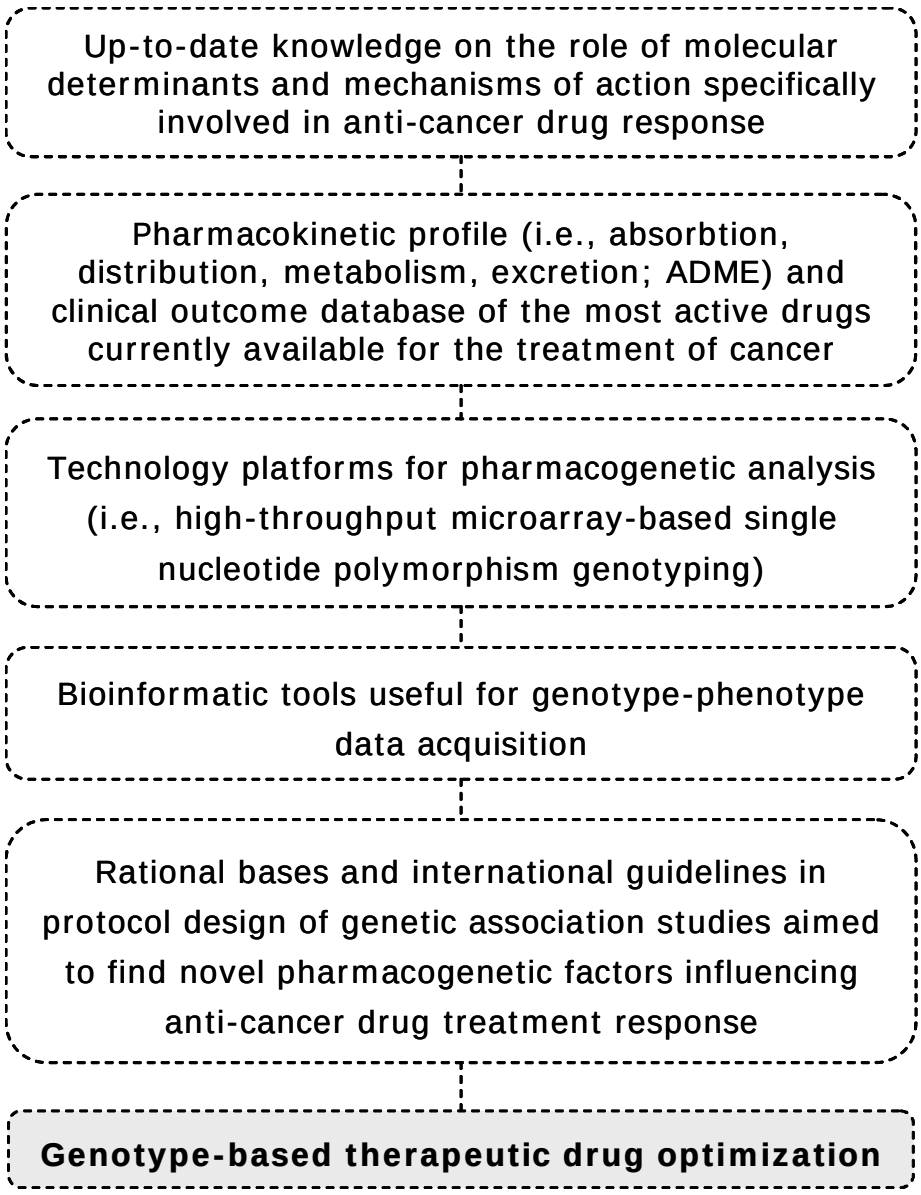


**Figure 1**
A representative schema of the multidisciplinary steps toward genotype-based therapeutic drug optimization.

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**Molecular predicting factors of responsiveness to monoclonal antibodies in colorectal carcinomas**

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Monoclonal antibodies anti-epidermal growth factor receptor (EGFR) have been recently approved for the treatment of metastatic colorectal cancer (CRC) patients with EGFR positive tumors at immunohistochemistry (IHC). Data demonstrated a lack of correlation between the tumor's EGFR expression at IHC and outcome. The identification of predictive factors is mandatory being these agents not chip, not lacking in side-effects and effective only in a minority of patients. Several potential clinical and biological predictive markers of activity and/or efficacy have been analysed in retrospective series with promising results. These potential predictive factors can be divided in EGFR's ligands expression, *EGFR* gene copy number and mutations, proteins of the Ras/Raf/MAPK and the PI3K/Akt signalling pathways, germ line genes polymorphisms and angiogenesis related cytokines. A recent study (1) pointed out that epiregulin (EREG) and amphiregulin (AREG) tumoral overexpression plays a central role in determining responsiveness to and benefit from cetuximab treatment. Fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH) techniques are used to study *EGFR* gene copy number and mutations. The role of *EGFR* copy number as measured by FISH or CISH analysis in the prediction of response to anti-EGFR MoAbs has been retrospectively investigated. In these studies increased *EGFR* copy number has been demonstrated to be correlated with anti-EGFR activity and efficacy (2,3). On the other hand, the *EGFR* activating mutations in tyrosine kinase domain don't seem to have any impact on sensitivity/resistance to cetuximab in clinical retrospective analyses (4). The Ras/Raf/MAPK cascade regulates cell proliferation and survival. Several retrospective studies addressed the question of whether *KRAS* mutations can predict the outcome of metastatic CRC patients treated with anti-EGFR monoclonal antibodies. Considering the entire population analysed in these studies the incidence of *KRAS* alteration is significantly higher among non responder when compared to responder patients (2, 5,6). Nuclear factor kB (NF-kB) is a downstream effector of the EGFR signalling pathways aberrantly activated in many human malignancies. Scartozzi et al. (7) demonstrated a significant correlation between NF-kB activation status and poor survival in cetuximab treated patients. In the last years, some polymorphisms/allelic variants involved in EGFR signalling have been described and their influence in EGFR inhibitors' prediction of benefit investigated (8,9). Graziano et al. (in press on J clin Oncol) investigated several polymorphisms in 110 assessable patients who underwent cetuximab-irinotecan salvage therapy for disease progression after two previous lines of chemotherapy; a significant association with favourable survival was observed with two copies of EGFR allele containing less than 17 CA each, and with EGF 61 GG genotype. The inhibition of tumor angiogenesis could play a role in the anticancer proprieties of cetuximab. Several studies report that cetuximab is able to decrease tumor-cell production of angiogenic growth factors such as basic vascular endothelial growth factor (bVEGF), fibroblast growth factor (FGF), and interleukin-8 (IL-8) (10,11). With this rational, Vincenzi et al.(12) investigated in humans the modulation of VEGF seric levels in advanced colorectal cancer patients at different time points before and after the beginning of cetuximab plus irinotecan treatment. The median VEGF basal values showed a long lasting significant decrease persisting 92 days after the start of therapy. In order to verify in humans the role of these VEGF seric modifications related to cetuximab based therapy as a prognostic and predictive factor in advanced

colorectal patients, the same research group designed a specific prospective trial (13). The study demonstrated that, in patients treated with cetuximab based therapies, a VEGF reduction of at least 50% was correlated with time to progression and overall survival, both in univariate and in multivariate analyses. These first evidences of a predictive role of circulating VEGF and its intratumoral expression levels in patients treated with cetuximab based therapy are interesting and promising, but these results require larger confirmatory studies. Considering the above described data from various retrospective analysis, every effort should be performed in the future with the aim to conduce prospective, well designed and adequately powered clinical/translational trials to confirm these promising tools. The identification of the molecular and genetic predicting factors of responsiveness to such monoclonal antibodies is mandatory for selecting responsive patients in order to avoid useless toxicities and to increase the cost-effectiveness of these agents.

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**Valutazione delle cellule endoteliali circolanti in pazienti trattati con terapie anti-tumorali: studio della neo-angiogenesi *in vivo*.**

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The formation of new blood vessels is a fundamental process that occurs during embryonic and post-natal development but also in a number of pathologies ranging from cancer to chronic inflammatory disease. Cancer is still the second most prevalent cause of death after cardiovascular disease in the industrialized word, whereby the primary cause of cancer is not attributed to primary tumor formation but rather to the growth of metastases at distant organ site. New blood vessel formation, is a crucial event in the process of tumor growth and metastatic dissemination. In fact, sustained expansion of a tumor mass requires new blood vessel formation to provide rapidly proliferating tumor cells with an adequate supply of oxygen and metabolites and also to influences the dissemination of tumor cells to distal sites.

This process is orchestrated by a variety of activators and inhibitors. Activators are the ligands for receptor tyrosine kinases (Carmeliet P., 2000) such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), or molecules of different origin such as lysophosphatic acid (LPA), and affect mainly proliferation and migration of endothelial cells, the basic components of blood vessels. The first described angiogenic inhibitor is thrombospondin-1 which modulates endothelial-cell proliferation and motility (Volpert O.V., 1995). Interestingly, many inhibitor molecules derived from cleavage of longer protein not involved in angionenesis. For example the statins such as angiostatin, derived from cleavage of plasminogen, or endostatin, tumstatin and canstatin that are fragments of collagens. The levels of activators and inhibitors determines if an endothelial cell will be in a quiescent or an angiogenic state and changes in balance between these molecules determine the induction of a tumor vasculature.

The "switch" (Folkman J., 1995) to an angiogenic phenotype is considered an hallmark of malignant process. In general, increased tumor vascularization and tumor expression of proangiogenic factors has been associated with advanced tumor stage and poor prognosis in a variety of human cancers. Tumor blood vessels are architecturally different from their normal counterpart: they are disorganized, irregular in caliber, tortuous, and do not have specialized features of normal arterioles such as capillaries or venules. Neo-angiogenic tumor vessels have large gaps between or through cells, loose pericytes, and discontinuities or redundant layers within the basement membrane, rendering these vessels hyper-permeable. Moreover, tumor vessels have also been reported to have cancer cells integrated into the vessel wall (Folberg R., 2000; McDonald DM, 2000).

Depriving a tumor of its vascular supply by means of anti-angiogenic agents has been of great interest since 1971, when J. Folkman hypothesized that tumor growth could be dependent on blood supply and proposed for the first time the treatment of cancer with drugs that prevent the formation of blood vessels (Folkman J., 1971). In the next decades a greater number studies focused on angiogenesis and the possibility to design drugs for the inhibition of this process. The discovery of key molecular modulators of angiogenesis, notably vascular endothelial growth factor (VEGF), has catalyzed the development of numerous neutralizing therapeutic agents. The validity of VEGF inhibition as a therapeutic strategy has been well supported in randomized clinical trials, and U.S. Food and Drug Administration approved recently several anti-angiogenic drugs such as anti-VEGF antibody bevacizumab, or small molecules interfering with its receptors such as sunitinib malate and sorafenib.

Since anti-angiogenic agents have entered the clinical oncology arena the classic tumor size measurements defined to monitor the efficacy of chemotherapy might not be appropriate for these

newer therapeutics. Indeed new surrogate pharmacodynamic markers of anti-angiogenic drugs activity are needed. The classic maximum tolerable dose (MTD) approach, as defined in the past for cytotoxic drugs, might not be adequate or relevant for this class of drugs which in general have considerably less toxicity than conventional cytotoxic used at MTD, and therefore may be used for prolonged periods to obtain inhibition of new vessel growth, and, in turn, tumor stabilization or shrinkage. Also the concepts of induction of dose limiting toxicity (DLT) and "objective tumor response", are not sufficient to evaluate the early clinical development of the new generation of antiangiogenic or other types of molecularly targeted drugs, since such drugs frequently have optimal therapeutic activity below a maximum tolerated dose. Another clinical need is to identify early reliable biomarkers for the angiogenic state of a tumor so that salvage therapies could be initiated in a more timely fashion for patients with tumors with evidence of angiogenic escape.
It has been postulated that antiangiogenic therapy resistance could develop with an activation of compensatory pathways driving angiogenesis in the setting of VEGFR block. Resistance could develop with an incomplete inactivation of VEGF pathway or activating other hypoxia-inducible factor-driven genes such as CXCR4 and TGF-α or through the selection of a sub-population of tumor cells able to survive in the presence of a VEGFR blockade probably by activation of pathways that are independent by VEGF such as FGF and IL-8 (Casanovas O., 2005; Mizukami Y., 2005). These observations also underline the importance of surrogate biomarkers for angiogenesis modulation.

Different preclinical strategies has been generated to measure angiogenesis but often are either difficult to be reproduced in clinical practice or are not adaptable to patients. Currently, angiogenesis and anti-agiogenic drug activity are empirically measured by microvascular density, or by measuring the circulating levels of angiogenic growth factors or by functional approaches such as magnetic resonance imaging of blood flow and vascular permeability. All these approaches have difficulties for an accurate evaluation of angiogenesis and anti-agiogenic drug activity.
The measurement of microvessels density in biopsy samples visualized by immunoistochemistry using endothelial-associated antigens such us CD31 (PECAM-I), CD34 (an immature hematopoietic/stem cell marker), or CD105 (Endoglin), is an invasive approach but is also difficult to standardize; moreover the heterogeneity of tumor tissue can be the cause of a lack of correlation between the microvessels density in biopsy samples and that in the entire lesion. Moreover, since also platelets contain and could release angiogenic and antiangiogenic factors, the measurement in patient plasma of cytokines/growth factors such as VEGF and its family member basic fibroblast growth factor, or chemokines, could not be a correct way to monitor anti-angiogenic anti-tumor therapy. Currently vessels anatomy and flow are also being assessed by novel imaging modalities such as diffusion contrast-enhanced magnetic resonance imaging, arterial spin-lebeling magnetic resonance imaging and computed tomographic perfusion; but these methods requires expensive instrumentations and the effectiveness of this type of approach remains to be validated.

Since 1973 Hladovec and Rossman (Hladovec and Rossman,1973) reported that cells with endothelial characteristics circulate in the blood. Over the past decade several reports demonstrated that circulating endothelial cells (CECs) are increased in many pathological conditions, including cancer (Blann AD, 2005). The circulating endothelial cells are thought to originate from sloughing off the vessel wall following some forms of pathological insult. The majority of CECs shows characteristics of mature, terminally differentiated and frequently apoptotic cells, however a sub-population express antigens that suggested a stem-like or progenitor-like phenotype. These latter cells were designated as circulating endothelial progenitor cells (CEPs) and are believed to arise not from the vessel wall but from the bone marrow, and are supposed to be important in repair following vascular damage. The presence of CEPs in peripheral blood indicates that vasculogenesis is not restricted to embryonic development and that circulating endothelial progenitor cells (CEP) recruitment participates in the generation of new vessels in adults. It has also been shown that tumor angiogenesis is associated with recruitment of hematopoietic and circulating endothelial precursor

cells (CEPs) from bone marrow as well as sprouting of preexisting endothelial cells (Lyden D, 2001).

It was also reported that CEC levels are increased in the peripheral blood of cancer patients at diagnosis, and chemotherapy can reduce the amounts of mature viable CECs determining the return to normal values in patients undergoing complete remission. In particular, Bertolini and colleagues have recently demonstrated that CEC count and viability represent a very promising tool to select cancer patients who might benefit from the new generation of anti-angiogenic therapies (Bertolini F., 2006). On the other hand, chemotherapy can mobilised CEPs that may contribute to neovascularisation indicating that an early anti-angiogenic therapy in combination with chemotherapy could be beneficial for the success of cancer therapy (Fürstenberger G, 2006).

Three methods for monitoring CECs and their viability as well as CEPs, in blood samples of patients, have been tested. In details CEC enumeration can be performed by immunohistochemistry, a technique that allow morphological confirmation of the endothelial nature of the cells, but it is subjected to reader-dependent variability (Dignat-George F., 2000; Woywodt A., 2006). It is also possible to perform quantitative enumeration of endothelial-specific or cancer endothelial-specific transcripts, a method with high specificity but with the limitations of transcripts instability in the blood and the restrictions to be subjected to the activation or viability status of endothelial cells. The third possibility of enumeration of CECs and CEPs is represented by a four color-flow cytometric analysis. Recently the availability of six-color flow cytometry might be a major step forward in this area. However also cytometry analysis had some limitation since it requires standardization (Bertolini F., 2007).

For flow cytometric analysis peripheral blood is collected in EDTA tubes through 21G needles and cell suspensions are evaluated after red cell lysis by FACSalibur (BD, San Josè, CA). After acquisition of at least 1.000.000 cells per blood sample, analyses are considered as informative when adequate numbers of events (i.e. > 100, typically 300-400) are collected in the CECs enumeration gates. Gates are used in order to exclude platelets, dead cells and debris.
CECs are defined as negative for hematopoietic marker CD45, positive for endothelial markers P1H12 and CD31. Moreover, vitality of CECs are evaluated by DNA staining with syto16. Nuclear staining for DNA is crucial to exclude aggregated platelets and/or endothelial microparticles from CECs counts. The CEPs are define as negative for hematopoietic marker CD45, positive for endothelial markers CD31, positive for VEGFR-2 and for immature haemopoietic/stem cell marker CD34 or positive for progenitor marker CD133.

We are currently measuring the number of CECs and CEPs in the blood of patients enrolled in the BRANCH study, a phase II radio-chemotherapy plus bevacizumab neo-adjuvant trial for rectal cancer recently started at National Cancer Institute Pascale of Naples, Italy. The blood samples are collected the day of the first scheduled bevacizumab delivery (repeated every 14 days), and at the $3^{th}$, $15^{th}$, $18^{th}$, $29^{th}$, and $33^{th}$ day after first scheduled bevacizumab delivery, and the day before surgery.

## Technologies in proteomics: DIGE and MALDI-TOF

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The proteomic approach to the analysis of complex biological samples aims to the large-scale visualization and characterization of the global gene expression pattern. In this way the temporal, spatial and functional properties of a high number of different proteins can be annotated, including information on primary gene products as well as on post-translational modified products that a genome can express in a specific moment, cellular compartment, or in the context of a specific biological process.

Proteomics can be considered as the study of the proteome using a combination of different technologies for high-throughput protein separation (2D electrophoresis, multidimensional chromatography, protein arrays, etc), protein characterization and identification (mass spectrometry) and bioinformatics.

Two-dimensional gel electrophoresis is still considered one of the "core" technique for protein separation in global proteomic studies, thanks to its very high resolving power that permits to visualize simultaneously primary and post-translational modified gene products, in a single gel. Currently, with 2D electrophoresis, where the position of each spot is determined by its pI and Mr, quantitative and qualitative variations and post-translational modifications (phosphorylation, glycosylation, oxidation, etc..), involving single or groups of spots, can be monitored in different experimental conditions.

Although this procedures have been standardized, high reliability of the data can be achieved only by a very time-consuming user-dependent analysis. Moreover, in order to minimize the experimental error in the quantitative measurement, innovative procedures for fluorescent chemical labelling of proteins before the electrophoretic separation have been recently introduced: 2D-DIGE (Differential In-Gel Electrophoresis) [1].

The 2D-DIGE procedure currently represents the most reliable method for the detection of differential quantitative variations by 2-D PAGE. The DIGE procedure is based on a sample-specific fluorescent labelling before the electrophoretic separation (Fig 1). In this way two different samples can be loaded and separated on the same electrophoretic gel. The gel is then scanned at two different wave length in order to produce, from a single gel, two different digital images. In this procedure a sample A comes marked with the Cy3 fluorescent molecule and a sample B comes marked with the Cy5 fluorescent molecule. A third fluorescent molecule (Cy2) comes used for the labelling of the mixture of the two samples, to be used like an internal standard for the differential quantitative analysis between the images obtained from the sample A and from the sample B. This kind of in-gel differential quantitative analysis demands a commercially available specific software, the DeCyder Differential Analysis software, that contains specific algorithms for the co-detection of the fluorescent images and for differential quantitative analysis of images produced by the DIGE system. Such algorithms use the mixture of the two samples, labelled with the third fluorescent molecule, as an internal standard in which all the proteins of each sample are represented. With inclusion of an internal standard DIGE technology also allows for repetitive measurements and multivariable analyses to be quantitatively analyzed in one coordinated experiment, yielding statistically-significant changes in protein expression related to many disease states [2].

The DIGE/DeCyder system currently represents the most reliable system for differential quantitative analysis of complex protein samples, since allows an in-gel differential quantitative analysis, eliminating therefore the experimental variability noticeable in standard gel-to-gel comparison.

All separation systems relies on mass spectrometry to unambiguously identify proteins. Matrix Assisted Laser Desorption Ionisation (MALDI) [3], probably the widest spread MS technology in proteomics, deals well with thermolabile, non-volatile organic compounds especially those of high molecular mass and is used successfully in biochemical areas for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides**.**

This technology relies on the 'peptide-mass fingerprinting" approach. In this approach the mass spectrum of the eluted peptide mixture is acquired, which results in a 'peptide-mass fingerprint' of the protein being studied. This mass spectrum is obtained by the matrix-assisted laser desorption/ionization (MALDI) — which results in a time-of-flight (TOF) distribution of the peptides comprising the mixture usually sufficient for a database identification.

Trypsin cleaves the protein backbone at the amino acids arginine and lysine, the masses of tryptic peptides can be predicted theoretically for any entry in a protein sequence database. Specific on-line available software allow the screening of the theoretical "peptide mass fingerprinting" of all the proteins in protein database, obtained by "in silico tryptic digestion" . The identification is achieved with the protein showing the theoretical peptide mass fingerprintg corresponding to the experimental one. As more full-length human genes are represented in the database, the success rate of identification by MALDI will increase further.

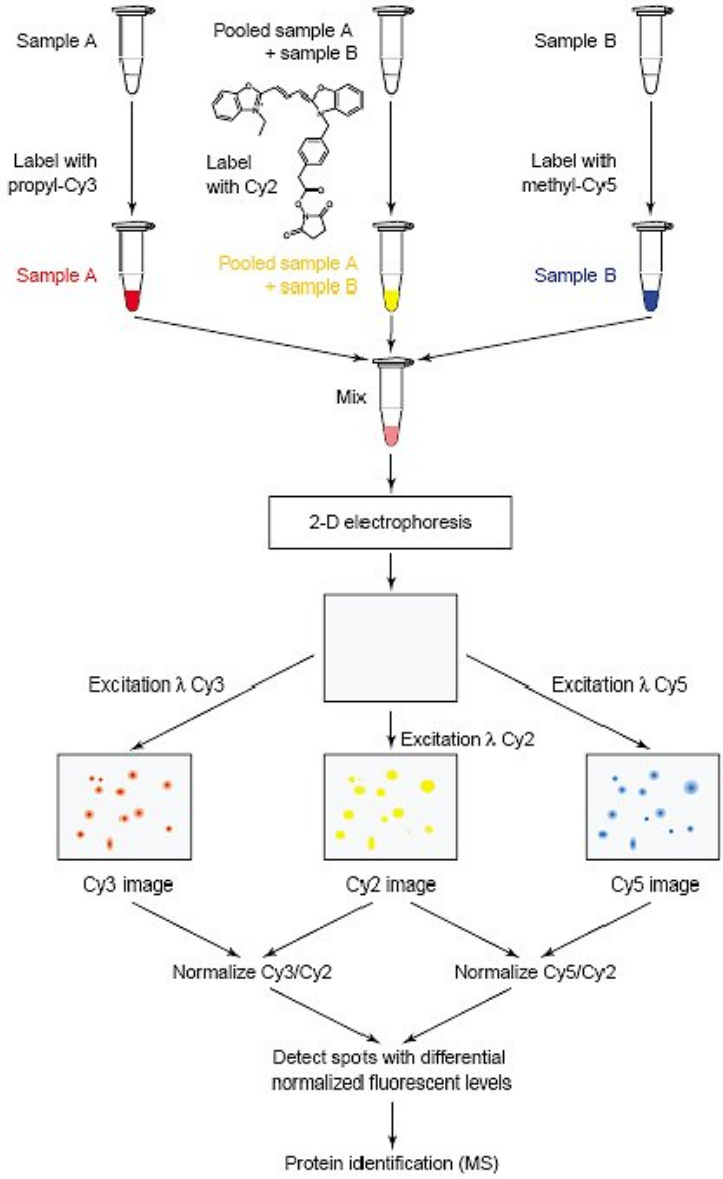


**Fig 1.** Representation of a typical 2D-DIGE experiment procedure

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**Discovery of Cancer and Autoimmune Biomarkers Utilizing Serum Antibody Profiling on Protein Microarrays**

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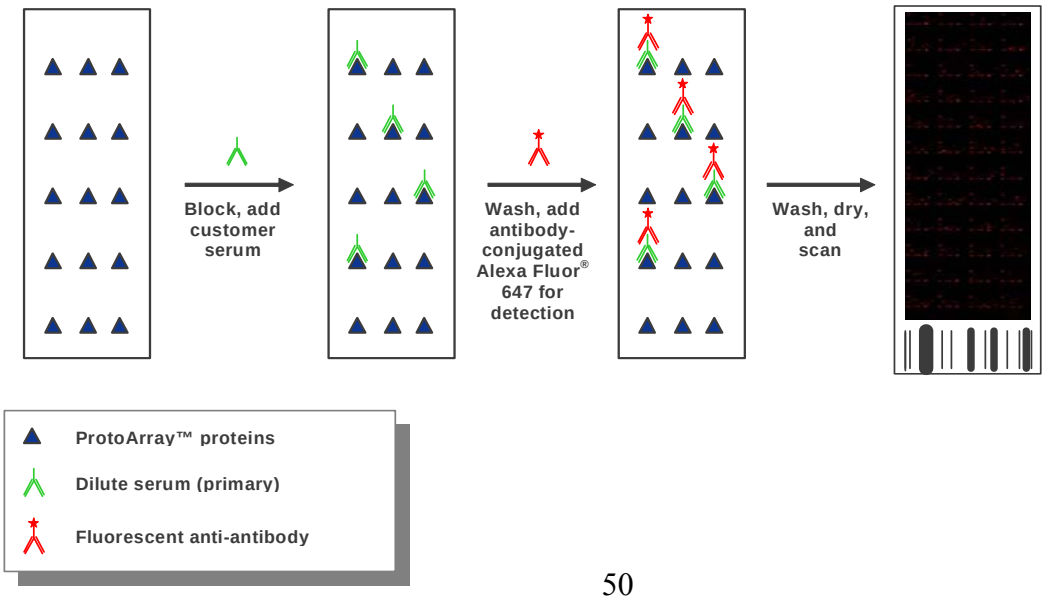
## Abstract

It has been well established that serum autoantibodies have important diagnostic value for many diseases, including cancer and autoimmune diseases. Identifying the antigens which elicit an autoimmune response can yield sets of biomarkers that provide classifiers for particular diseases and disease stages as well as predictors of patient outcomes and patient responses to therapeutics. Protein microarrays are valuable tools that have been successfully applied to investigate the circulating antibody profile in several disease states.

The ProtoArray® Protein Microarray from Invitrogen allows analysis of large numbers of proteins in an easy-to-use format. The ProtoArray® Human Protein Microarray v4.0 consists of approximately 8,000 highly purified full-length human proteins expressed in insect cells as N-terminal glutathione S-transferase (GST) fusion proteins, purified under native condition, and spotted in duplicate on
glass slides (1 inch x 3 inch). Since all proteins on the array are purified under native conditions, the immobilized proteins are expected to maintain their native conformations and therefore should be recognized by antibodies requiring structured epitopes.
To discover potential autoantibody biomarkers specific to ovarian cancer, sera from 30 ovarian cancer patients and 30 healthy patients were profiled on protein microarrays. Patient and control sera were diluted and applied to protein microarrays according to a simple profiling protocol depicted in Figure 1.

**Figure 1—Protocol for serum profiling application.** Briefly, the protein microarray is blocked with a nonspecific protein blocker, treated with diluted serum, and incubated for 90 min. The array is washed to remove any unbound proteins/antibodies. The bound serum antibodies are then detected with anti–isotype-specific secondary antibodies conjugated to Alexa Fluor® 647.



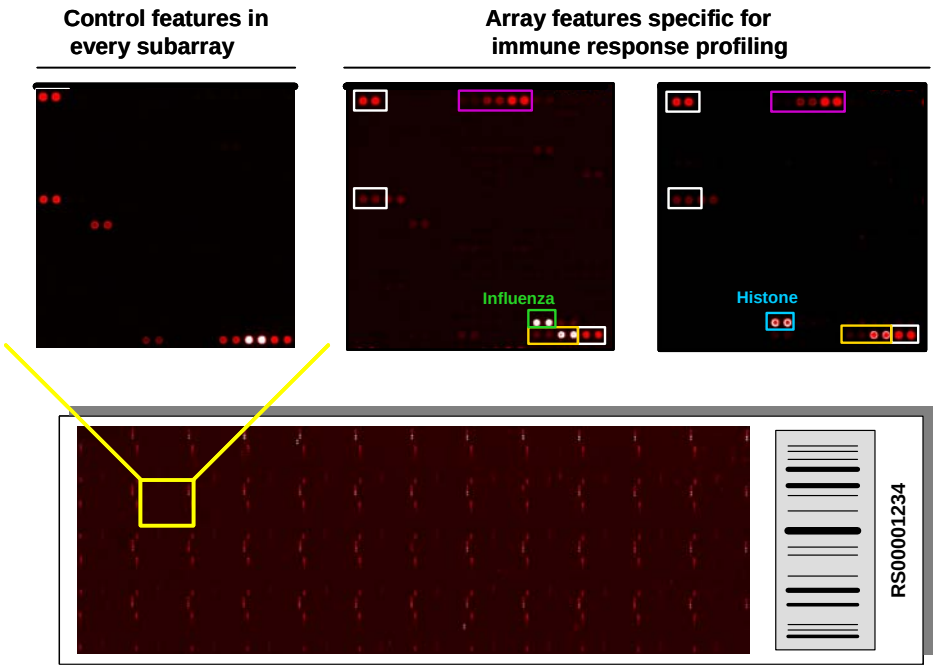
Inter-and intra-assay reproducibility is highly important to the success of biomarker discovery projects in which numerous samples are profiled over various days and by multiple operators. Specific protocol steps, normalization algorithms, and protein QC standards have been included in the development and production of the ProtoArrays that ensure reproducible profiling capabilities important to the success of biomarker discovery projects. Table 1 shows assay reproducibility within and across ProtoArray microarray lots.

**Table 1**

| | |
|---|---|
| Same lot, same operator | Median CV = 12 % |
| Same lot, different operator | Median CV = 15 % |
| Across lots, same operator | Median CV = 16% |

A number of control elements have been built into the ProtoArray that are specifically tailored to the immune response profiling application. As shown in Figure 2, control features that are present in every subarray include AlexaFluor647 labeled protein which serves as "landing lights" for proper alignment during data acquisition. Additionally, every subarray contains a gradient of human IgG, which is bound by the anti-human IgG detection reagent and serves both as a control for proper performance of the detection reagent, as well as a measure of the appropriate setting for scanning, facilitating use of the maximal dynamic range of the scanner. In addition, several control elements appear in each subarray. These include an anti-human IgG antibody which serves as a positive assay control, and influenza antigen, which serves as a secondary positive assay control. Studies carried out at Invitrogen have demonstrated that approximately 90-95% of samples are seropositive for influenza.

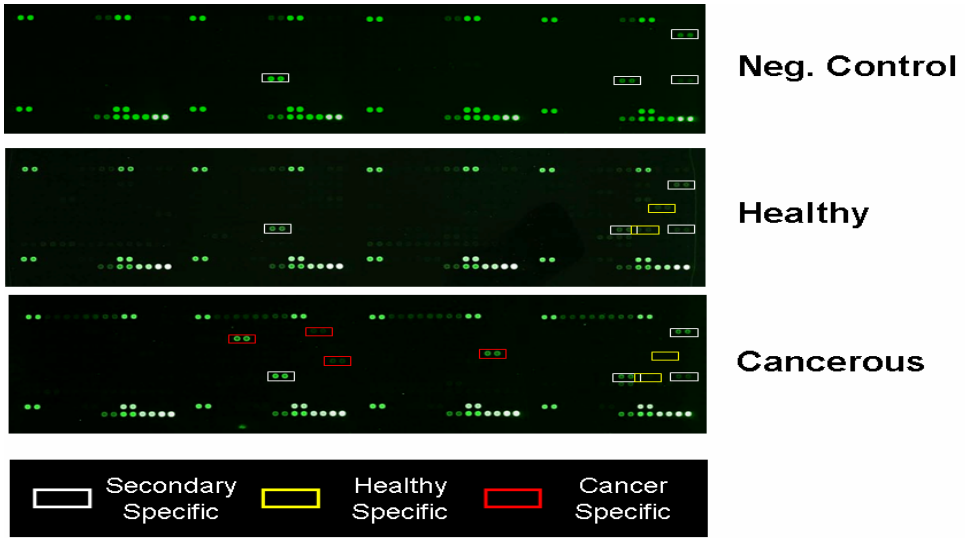
**Figure 2**




Over 95 candidate biomarkers were identified that exhibited enhanced reactivity in sera from cancer patients relative to that of the control individuals (Figure 3). Antibodies to four antigens were

tested for differential reactivity in tissue samples of cancer patients relative to healthy patients using immunoblot analysis and tissue microarrays. Three biomarkers exhibited elevated expression in the cancer tissue relative to controls. Signal observed for the biomarker antigens appeared significantly stronger than signal corresponding to the existing ovarian cancer antigen, CA-125.

**Figure 3**




Additional studies focused on identifying biomarkers associated with autoimmune diseases, such as Rheumatoid Arthritis and Systemic Lupus Erythematosus, have recently been completed and will be discussed in this presentation. The application of protein microarray technology for serum antibody profiling will be discussed along with data from on-going validation studies.

# USE OF DNA MICROARRAYS IN THE STUDY OF TUMOR ANGIOGENESIS

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## Abstract
Angiogenesis is inhibited by many promising cancer chemopreventive compounds, a concept we termed "angioprevention". Endothelial cells are altered in numerous pathological states, among these are chronic inflammatory conditions, atherosclerosis, cardiovascular diseases, rheumatoid arthritis and cancer. We have used DNA microarray technologies and transcriptome analyses to understand the molecular mechanisms regulating endothelial cell gene expression after treatment with chemopreventive drugs or during senescence. These approaches provided key insights toward elucidating the molecular targets of "angiopreventive drugs" in order to develop novel clinical approaches.

## Introduction

Cancer is a multi-factorial disease that requires modulation of multiple pathways and biological processes, in particular angiogenesis. Solid tumors require blood vessels for their growth, as they are essential for the supply of tumors with oxygen and nutrients, and for the spread of metastatic cells. Novel new anti-cancer therapies are being directed against the tumor vasculature in order to deprive the tumor of oxygen and nutrients. Angiogenesis is promoted by numerous factors including cytokines and growth factors, such as VEGF and bFGF; it is also negatively regulated by endogenous inhibitors such as thrombospondin, interferons, some cytokines, angiostatin, endostatin and TIMPs. The factors released by tumor cells as well as by surrounding stromal cells act in concert to promote either pro-angiogenic or anti-angiogenic processes.
The endothelium is located in a strategic anatomical position, within the blood vessel wall, acting as a barrier between the blood flow and vascular smooth muscle cells. The functional integrity of the endothelial monolayer is essential to prevent vascular damage and the formation of atherosclerotic lesions. The role of endothelial cells is pivotal in many fundamental processes of the body, controlling metabolic exchanges, leukocyte translocation, inflammation and immune responses. Furthermore, aging of endothelial cells is associated with changes related to an increased risk of chronic diseases, in cardiovascular morbidity, diabetes, rheumatic diseases and cancer.
In oncology, endothelial cells are involved in tumor angiogenesis, one of the main "rate limiting steps" in tumor progression and spreading. For this reason over the last several years a number of agents have been patented for cancer therapy based on their ability to inhibit tumor angiogenesis.
Apoptosis of endothelial cells may critically compromise the integrity of the endothelial monolayer and thereby contribute to vascular injury. Aging is also one of the major risk factors for the development of cardiovascular disease, in fact aged endothelial cells show enhanced apoptosis sensitivity with dramatically increased caspase-cascade activity in response to inflammatory stimuli when compared with relatively "young" cells. Aged endothelial cells are known to reveal significantly increased levels of apoptotic cell death, with down-regulation of eNOS expression, and accumulation of age-related damage to mitochondria, leading to enhanced reactive-oxygen species (ROS) formation.
Senescence of the endothelium is linked to regenerative capacity reduction and a higher rate of endothelial cell apoptosis. Increased oxidative stress is closely related to the endothelial aging,

apparently due to a progressive dysfunction of the endogenous free radical scavenging system and impairment the regulation of cell-cell adhesion. The most common alteration associated with endothelium functional impairment is that of changes in cell-adhesion molecule expression. Cell adhesion molecules are cell surface proteins involved in the cell-to-cell communication, contributing to leukocyte trafficking and movement between body compartments.
Chronic inflammation is also related to endothelial dysfunction and atherosclerosis: the pathogenesis of cardiovascular and oncological diseases is frequently associated with the expression of pro-inflammatory cytokines, such as type I interferons, associated with endothelial cell aging. Endothelium aging is also associated with a reduction in the regenerative capacity of the endothelium and endothelial senescence, which is characterized by an increased rate of endothelial cell apoptosis. Increase of oxidative stress is tightly related to endothelium aging due to the malfunction of endogenous free radical (ROS) scavenger system. Chronic exposure of endothelium to ROS drives the cells to morphological changes and the impairment of cell-cell adhesion. Specifically oxidative stress increases vascular endothelial permeability, which is coupled with alterations in endothelial cell signal transduction. Compromised cellular redox balance lead to elevated activation of nuclear transcription factors, such as NF-kB, that regulate numerous extracellular signaling molecules involved in processes as inflammation, tissue remodeling, hyper-proliferation and apoptosis. The cell stress associated with compromised redox balance together orchestrate a degenerative response leading to cellular aging.
Substantial epidemiological data support the potential chemopreventive effect of dietary components and non-toxic drugs; many of these compounds are anti-oxidants, acting as a scavenger system for free radicals, involved in the carcinogenesis process. It is intriguing that many chemopreventive drugs, such as the anti-oxidant thiol *N*-acetyl-L-cysteine (NAC) or epigallocatechin-3-gallate (EGCG), the predominant active polyphenol derived from green tea, exert anti-angiogenic effects. These molecules form part of the group of "angioprevention" agents, based on the concept that effective chemoprevention agents target tumor angiogenesis, thus indirectly tumor growth and metastatic dissemination. These and other anti-oxidant drugs inhibited tumor growth, in the highly angiogenic Kaposi's sarcoma xenograft model, and tumor vascularization by reducing endothelial cells migration and invasion, but do not affect the growth of endothelial cells. Given their efficacy and the relation to anti-angiogenic therapy, NAC and EGCG appear to be suitable at least for adjuvant therapy in combination with classical chemotherapy.
The mechanisms of the angiopreventive drugs NAC and EGCG on endothelial cells was examined by microarray gene expression profiling. RNA was extracted from the cells treated and from untreated controls. cRNA was synthesized from the two populations. and used for screenings of micro-array chips containing probes for essentially all annotated protein encoding genes and other UniGene clusters containing a total of non-redundant genes (Affymetrix, HG-U95Av2 GeneChips, Analysis with MAS 5.0 and GeneSpring 4.2). To validate the microarray data quantitative Real-time PCR was performed using SyBr-Green method, based on amplification primers already in use for the key proteins involved in angiogenesis; we observed a significant correlation between microarray and real-time PCR data. Semi-quantitative analyses were performed in triplicate and quantifications was performed considering the number of cycles at which a given threshold level is reached. GAPDH and β-actin were amplified in parallel as standards for normalization. We observed that the treatment of endothelial cells with NAC or EGCG induced quite similar changes in their transcriptome gene expression profile (Figure 1). In contrast, the retinoid 4HPR, which is an oxidant able to inhibit angiogenesis, showed a completely different pattern (Figure 1). This microarray approach was useful for identifying molecular mechanisms by which these chemopreventive agents can influence endothelial cell behavior. We identified a number of angiogenesis-related genes that were similarly regulated by these two compounds; in particular anti-oxidant treated endothelial HUVE cells showed a gene expression pattern that was compatible with a less activated and apoptotic phenotype, which correlated with reduced NF-κB activation in these treated cells. This was quite evident observing the effects on TNFα responsive pro-metastatic and

pro-inflammatory genes such as E-selectin and urokinase plasminogen activator (UPA) (Table 1). These common targets identified could significantly contribute to the anti-angiogenic and chemopreventive effects observed with a broad range of phytochemicals, and lead to combination prevention approaches.

Focusing on the role of E-selectin in endothelial cells and its modulation during endothelial aging, we observed that E-selectin is one of the main genes whose expression was also modulated during aging with culture passages in vitro. E-selectin is an endothelial cell-specific membrane glycoprotein involved in leukocyte adhesion processes during inflammation; it provides the ligand for the initial rolling steps in movement of innate immune cells from the blood flow into sites exposed to inflammatory stimuli. At the same time, this protein also appears to be often used by tumor cells during the tumor metastatic process. While E-selectin gene expression decreased with aging in terms of number of population doubling in culture, we also noted that NAC had a further inhibitory effect on E-selectin expression, and the effect of NAC was more marked on aged endothelial cells in respect to the cells at low passages in culture.

These data define molecular targets for future therapeutic implications, toward the safe therapeutic use of angioprevention drugs in cancer chemoprevention. Furthermore, the maintenance of an intact endothelial monolayer and endothelial function is necessary to protect against cardiovascular damage and several diseases associated with the endothelium, such as atherosclerosis, providing a key clinical tool for the prevention of diseases.

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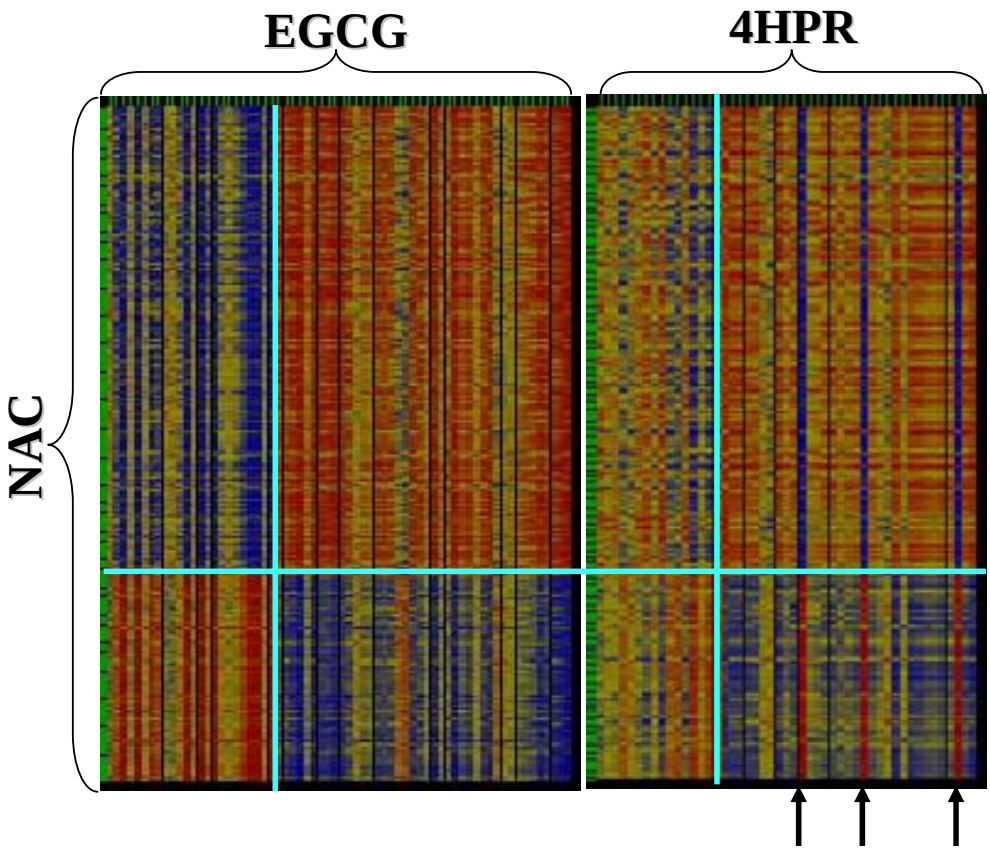
**Table 1**

| **EGCG and NAC Down-Regulated Angiogenesis Related Genes** | | |
|---|---|---|
| Fold Expression | | Description |
| **EGCG** | **NAC** | |
| 0.29 | 0.29 | E-selectin (endothelial adhesion molecule 1) |
| 0.41 | 0.53 | gap junction protein, alpha 4 |
| 0.57 | 0.31 | tumor necrosis factor (ligand) superfamily member 10 |
| 0.64 | 0.53 | CED-6 protein |
| 0.69 | 0.56 | cyclo-oxygenase 2 |
| 0.71 | 0.54 | cas-like docking |
| 0.74 | 0.65 | Carboxypeptidase E |
| 0.76 | 0.67 | ligand for the Notch receptor |
| 0.76 | 0.52 | ATP-binding cassette, sub-family G |
| 0.77 | 0.66 | collagen, type IV, alpha 1 |
| 0.78 | 0.57 | modulator of apoptosis 1 |
| 0.79 | 0.59 | TRAF interacting factor |
| 0.8 | 0.58 | cadherin 13 |
| 0.81 | 0.71 | thioredoxin-like |
| 0.82 | 0.66 | integrin, alpha V |
| 0.83 | 0.71 | HIV-1 Tat interactive protein 2 |
| 0.85 | 0.63 | basic fibroblast growth factor |
| 0.86 | 0.69 | death receptor adaptor protein |
| 0.9 | 0.67 | hyaluronoglucosaminidase |
| 0.9 | 0.65 | transmembrane 4 superfamily member 6 |
| 0.91 | 0.65 | plasminogen activator, urokinase |
| 0.96 | 0.69 | class I cytokine receptor |
| 0.96 | 0.58 | interleukin 10 receptor, beta |
| 0.98 | 0.68 | endothelial monocyte-activating |

This table shows genes whose expression is down-modulated by more than 1.4 fold by the anti-oxidants EGCG and NAC treated HUVE cells.

**Figure 1**




Genes regulated by one factor as compared to those regulated by another. Red = high correlation; Yellow = medium Correlation, Blue = No correlation (i.e. induced vs reduced). Confrontation of NAC vs EGCG shows a strong correlation pattern (red and blue). Instead, quite different molecules, NAC vs 4HPR, show limited correlations (yellow) and some with quite opposite behavior (arrows).

**Espressione differenziale di geni in patologie del tratto gastroenterico: la strana storia del carcinoma del colon e del carcinoma del retto**

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INT Pascale, Napoli

Colorectal cancer is the third most common cancer worldwide, and in many countries in the western world is the first or the second cause of cancer death. More than one third of the colorectal cancer arise in the rectum, the fifteen most distal centimetres of the large bowel. The issue of whether colon and rectal cancer should be considered as a single entity or two distinct entities is still debated. The good correlation between cancer incidence rates for both the sites observed in different ethnic populations and the shared similar etiology, type of precancerous lesion as well as mode of spread, all give evidence in favour of the first assumption. However, differences exist between colon and rectal carcinomas with respect to age and gender of the patients as well as the clinical behaviour and treatment. Prominent among these is the tendency for rectal cancer, but not colon cancer, to recur locally. At a molecular level, much progress has been made in the last two decades in the identification and characterization of the genetic changes involved in the malignant colorectal transformation process. Kinzler and Volgenstein have proposed a genetic model, describing the transition from healthy colonic epithelia through increasingly dysplastic adenoma to malignant cancer, that identifies a number of key oncogenes and tumour suppressor genes and the progressive acquisition of activating or loss of function mutations in which drives the adenoma to carcinoma transition Inactivation of the tumour suppressor genes APC and p53, and activation of the oncogene K-ras are thought to be particular important determinants of tumour initiation and progression. A second pathway of colorectal tumorigenesis has been depicted in cases with a normal karyotype but carrying genetic instability at microsatellite loci attributable to alterations in the DNA mismatch repair (MMR) genes. (Fig. 1).

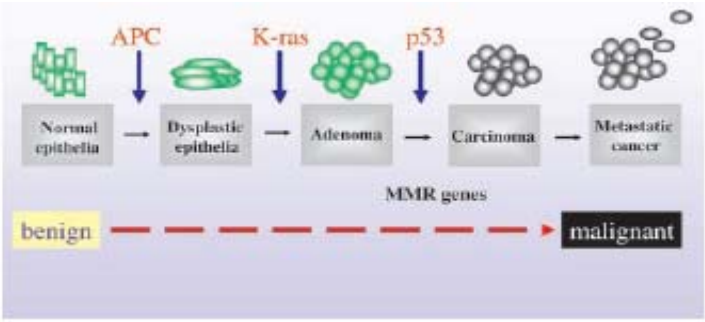


Fig 1. A model of the genetic changes required for progression from adenoma to carcinoma in the development of colorectal cancer , with the proposed order of mutations in APC, K-ras, p53, and the DNA MMR genes

The model was considered also valid for familial colorectal syndromes. However, this stepwise model of colorectal tumorigenesis has been mainly validated conceptually, and there is mounting evidence that alternative genetic events may occur during colorectal carcinogenesis, sometimes preferentially, sometimes randomly, and sometimes with an overlap. In a recent study based on an analysis of *APC*, K-*ras*, and *TP53* genes in the same samples, multiple alternative genetic pathways were shown to lead to tumour progression, and more importantly, the originally postulated pathway including simultaneously all of the three genetic alterations was found in only 6.6% of the analyzed cases. These data suggesting that the model originally proposed is not representative of the majority of colorectal tumours. Therefore there is a growing interest to study the heterogeneity of the pathogenetic pathway leading to colorectal cancers as well as to identify possible biological and/or molecular differences between colon and rectal cancers. On this regard, it has reported, in a

recent contribution, a significantly different β-catenin and TP53 expression in a series of colon and rectal cancers and concluded that these two diseases could follow different mechanisms of oncogenesis. Expanding the spectrum of molecular alterations, an Italian group have confirmed the presence of significant differences between colon and rectal tumours. The most striking difference was seen in the K-ras-dependent pathway, which is preferentially followed by colon cancer, in agreement with the model of Kinzler and Vogelstein. In contrast, the K-ras independent pathway was predominant in rectal cancer, because in this tumour the alterations in the four molecular markers were rarely detected. Overall, the different pathways observed can be summarized as follow:

a) K-ras mutations were more commonly detected in colon than in rectal samples.
b) The number of mutations detected was significantly higher in colon than in rectal tumors, irrespectively of the genes involved.
c) A mutational pattern restricted to the *APC* gene was more common in rectal than in colon tumours.

Microsatellite instability (MSI)-positive colon tumours display a low incidence of mutations in the APC and p53 tumor suppressor genes and K-ras oncogene. Instead, they carry a plethora of different mutated genes, TGFβRII and Bax, which are rarely found in MSI-negative tumours. This is because in the MMR deficiency background, mutations occur preferentially in genes with simple repeats in their coding or regulatory sequences. The differences in genotype can explain the differences in phenotype displayed by MSI-positive tumours, such as poorly differentiated histological features, a less advanced stage of tumour progression, and a better survival. Of note, MSI-positive tumours showed a preferential location in the proximal region of the large bowel.

Moreover, epigenetic alterations have been also associated with tumorigenesis. Hypermethylation has been linked to tumour suppressor gene silencing, such as hMLH1 DNA mismatch repair (MMR) gene. The anatomical distribution of hypermethylation overlaps the preferential localization of MSI in the proximal colon. The reason for the exacerbated asymmetry in anatomical location for both mutator and methylator phenotypes is intriguing and it could be due to an intrinsic asymmetry in some critical cellular processes between proximal and distal colon, such as stem cell renewal and mitotic activity. A higher mitotic activity may be sufficient to increase the probability of occur-rence of both mutator mutations (somatic structural alterations inactivating MMR genes) and epigenetic silencing of hMLH1 and more importantly, the necessary additional cell replications before neoplastic transformation can eventually occur

On the whole, it is emerging biological and molecular differences between colon and rectal cancers. However, further study will be required to clarify the importance of these difference and to translate the molecular knowledge into a clinical setting to improve the diagnosis and to direct a rationale pharmacological treatment.

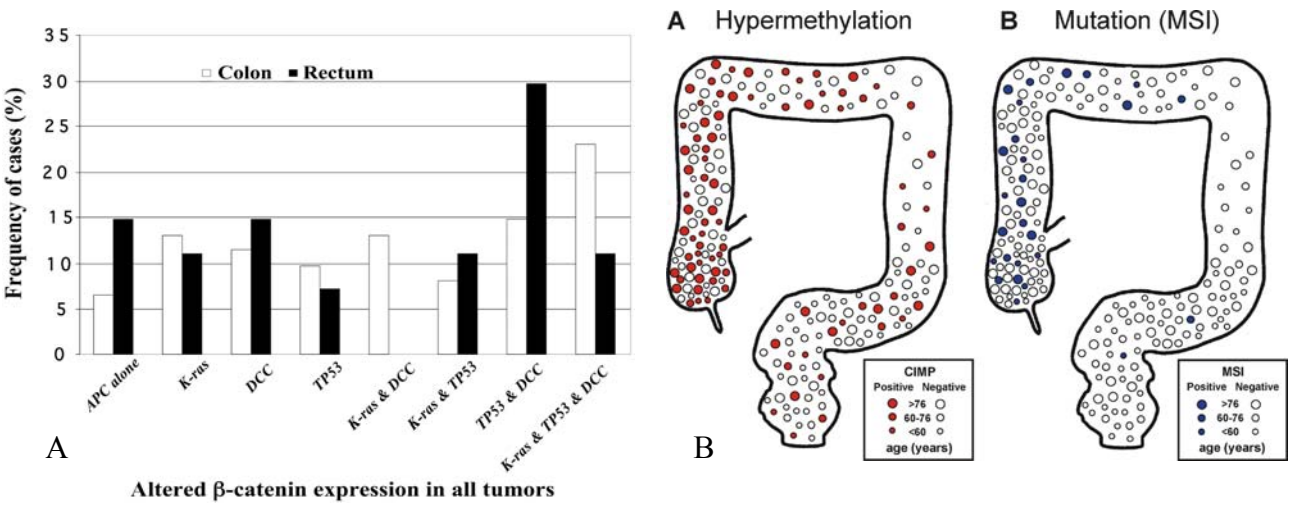


Fig 2. A)Comparison of genetic pathway frequencies on the basis of tumor location (colon and rectum). B) Gradient of DNA methylation and MSI in colorectal cancer.

## The role of genomic pattern in prognosis and prediction of response in breast cancer

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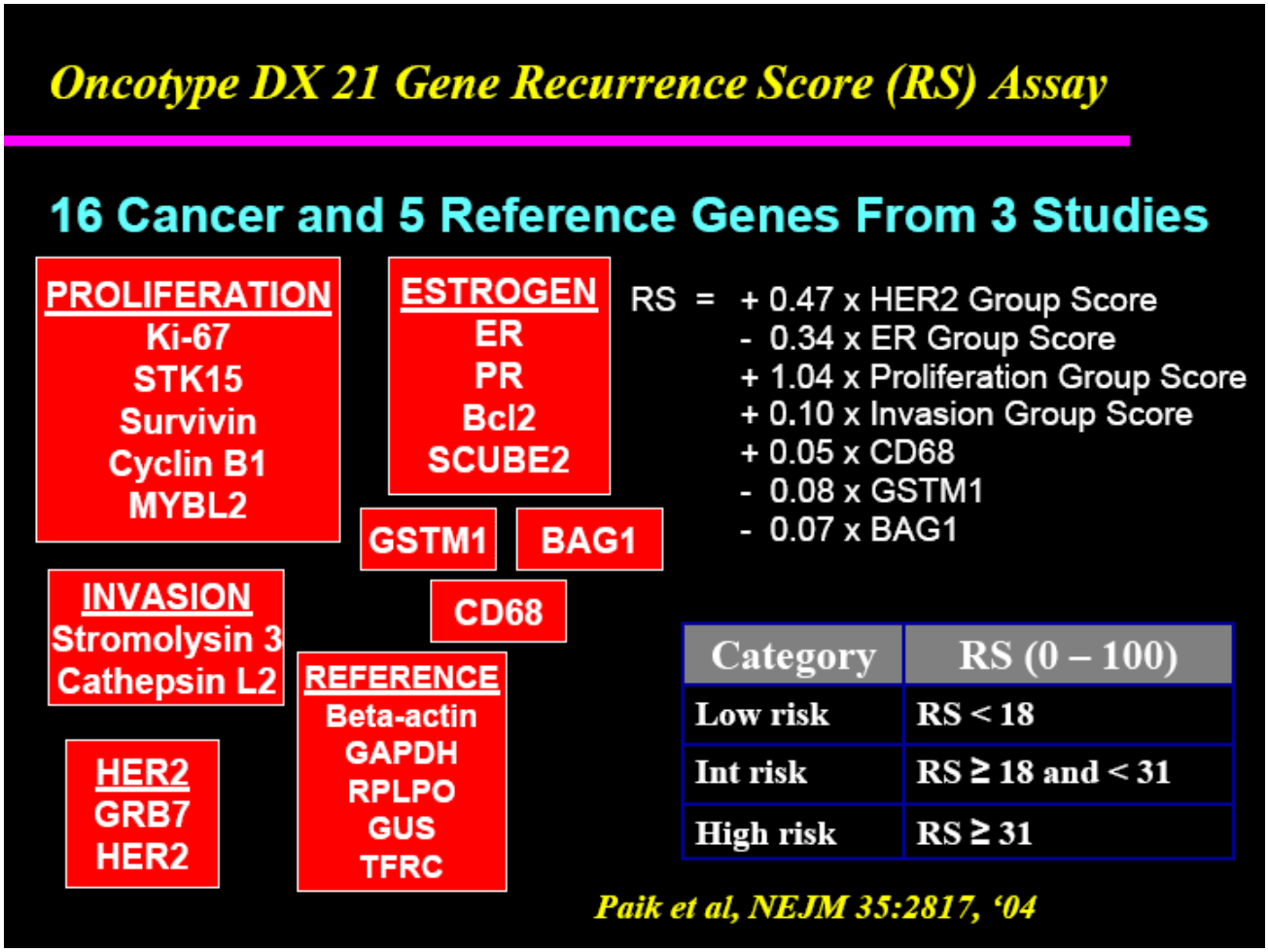
Breast cancer is a clinically heterogeneous disease and existing histological classifications are not fully associated with the varied clinical course of this disease. Histological type, grade, tumor size, lymph node involvement, and estrogen receptor α (ER) and HER-2 receptor status all influence prognosis and the probability of response to systemic therapies. These clinical variables can be combined into multivariate outcome prediction models, as The Nottingham Prognostic Index and Adjuvant! Model for early breast cancer [1,2]. However, regardless of what outcome prediction model is used, it remains substantial variability in disease outcome within each risk category. The different clinical course of patients with histologically identical tumors is a result of molecular differences among cancers. So detailed molecular analysis of the cancer could give information on prognosis and prediction. Recently, proteomic and gene-expression profiling methods are being explored as diagnostic tools. These emerging tests simultaneously quantify the expression of multiple genes and combine the gene expression measurements into prediction scores that may foretell clinical outcome more accurately than any of the genes alone [3]. Determination of ER status is an essential part of the diagnostic workup of all breast cancer patients. The information is most useful to determine whether a patient is a candidate for endocrine therapy. This diagnostic test is routinely used in the clinic, but its reliability is far from perfect. The existing immunohistochemical (IHC) assays have only modest positive predictive value (30%–60%) for response to hormonal therapies [4,5]. Furthermore, there are intra- and interlaboratory variation in ER results because fixation, antigen retrieval and staining methods may differ among laboratories [6]. The modest positive predictive value of current methods and the variable reproducibility of results encourages to develop more accurate and more reliable predictors of benefit from hormonal therapy. Several studies examining the expression of thousands of genes simultaneously via gene expression arrays suggest that breast cancer is not a single disease, but it contains multiple biologically. There are now several methods that can measure ER mRNA expression, such as quantitative reverse transcription polymerase chain reaction (RT-PCR) and DNA microarrays. Oncotype DX™ (Genomic Health Inc., Redwood City, CA) represents an important advance in the diagnosis of ER-positive breast cancers. This RT-PCR-based assay not only measures ER mRNA expression in a highly quantitative and reproducible manner, but it also measures the expression of several downstream ER-regulated genes (PR, BCL-2, SCUBE-2) that may contain information on ER functionality. The same assay also quantifies HER-2 expression and proliferation-related genes. Combining information from each of these measurements into a single prediction score can provide a superior method of outcome prediction than ER IHC alone (Figure 1). A study examined the correlation between the Oncotype DX™ recurrence score and the likelihood of distant relapse in 668 ER-positive, node-negative, tamoxifen-treated patients who were enrolled in the National Surgical Adjuvant Breast and Bowel Project (NSABP) clinical trial B14. 51%, 22%, and 27% of these ER-positive patients were categorized as low, intermediate, and high risk for recurrence after tamoxifen therapy, respectively. The observed 10-year distant recurrence rates were 6.8%, 14.3%, and 30.5% in the three risk categories, respectively ($p < 0.001$). In a multivariate analysis, the recurrence score predicted relapse and overall survival independently of age and tumor size [7]. A recent report examined the value of the recurrence score for predicting benefit from adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy in 651 patients with ER-positive, node-negative breast cancer included in the NSABP B20 randomized study. Higher recurrence scores were associated with greater benefit from adjuvant CMF chemotherapy ($p = .038$). The hazard ratio for distant recurrence after CMF chemotherapy was 1.31 for patients with a recurrence score <18 and 0.26 for patients with a recurrence score >31. The absolute improvement

in 10-year distant recurrence-free survival was 28% (60% vs. 88%) in patients with a recurrence score >31, while there was no benefit in patients with a recurrence score <18 [8]. Several other multigene prediction scores have been developed in order to refine the predictive value of ER and better estimate who will benefit of endocrine therapy. In one study, 81 genes were found to be differentially expressed between tamoxifen-sensitive and tamoxifen-resistant, ER-positive breast cancers (n = 46) and a 44-gene predictive signature was developed from the data. This signature was tested on 66 independent ER-positive cases and could select patients who had prolonged progression-free survival (odds ratio, 3.16) [9]. Another prediction score is the "Amsterdam Signature" (Mammaprint ®), a DNA microarray based test that measures the activity of 70 genes to define a prognostic profile. This gene profiling was developed from frozen tumors from women under the age of 55 years old with Stage I or II primary breast carcinoma and it was to identify genes that most distinguished a short interval to distant metastasis from a good prognosis [10]. The Microarray for Node-Negative Disease may Avoid Chemotherapy (MINDACT) trial has been designed to compare the ability of the 70-gene prognostic profile versus clinical and pathological criteria to identify women with node-negative breast cancer who are unlikely to benefit from adjuvant chemotherapy. Enrollment of 6000 patients is planned, the test will be performed to assign patients to endocrine therapy or chemotherapy. If the hypotheses are confirmed, the "Amsterdam Signature" could be considered as a prognostic and predictive test with relevant clinical utility [11]. Gene profiling has also been used to predict metastasis to distant organs. In fact genes associated with bone metastases were identified in experimental models [12]. In humans, Smid et al. identified a 69-gene panel associated with bone metastasis in patients with node negative breast cancer. TFF1 was the most differentially expressed gene in an independent patient cohort (p = 0.0015). A classifier of 31 genes was identified, which in an independent validation set predicted all tumors relapsing to bone with a specificity of 50% [13]. Moreover, Minn et al. identified a set of genes that mediates breast cancer metastasis to the lungs. In fact they found significant differences in lung-metastases-free survival between patients with or without expression of lung metastasis gene signature, especially in patients with poor prognosis (p= 0,008) or ER-negative (p=0,004) [14]. The advent of gene-expression profiling technologies allowed investigators to identify different molecular subtypes of breast cancer associated with different natural histories. Moreover, the molecular signatures that define particular groups may lead to the discovery of new therapeutic targets and treatments that are effective in particular molecular subsets. The first study to examine comprehensive gene-expression patterns of breast cancer suggested that at least four major molecular classes of breast cancer exist: luminal-like, basal-like, normal-like, and HER-2 positive [15]. Subsequent studies confirmed that there are large-scale gene-expression differences between ER-positive (mostly luminal-like) and ER-negative (mostly basal-like) cancers and suggested that further molecular subsets also exist [16, 17]. Many studies have shown that the prognosis and chemotherapy sensitivity of the different molecular subgroups are different. Luminal-like cancers tend to have the most favourable long-term survival compared with the others, whereas basal-like and HER-2-positive tumors are more sensitive to chemotherapy [18, 19]. In a recent study by Carey et al, the primary tumor chemosensitivity of breast cancer subtypes has been evaluated. They showed that basal-like and HER2+/ER– subtypes are more sensitive to anthracycline-based neoadjuvant chemotherapy than luminal breast cancers. Patients that had pathologic complete response to chemotherapy had a good prognosis regardless of subtype. So the poorer prognosis of basal-like and HER2+/ER– breast cancers could be explained by a higher likelihood of relapse in those patients in whom pathologic complete response was not achieved [20]. Other molecular markers have been recently identified as prognostic and predictive factors in patients with breast cancer. Geisler et al. showed that p53 mutations were associated with lack of response to chemotherapy (p=0,063 for all mutations and p=0,008 for mutations affecting the loop domains L2 or L3). These results were confirmed by Di Leo et al, that evaluated 108 patients with metastatic breast cancer treated with doxorubicin or docetaxel. P53 gene mutations were observed in 20% of patients. In patients with a mutated p53, a lower percentage of responders was observed in the

doxorubicin arm (17% VS 27%), compared with the docetaxel arm (50% VS 36%). So p53 gene mutations compromised the efficacy of doxorubicin, not interfering with the antitumor activity of docetaxel [21]. Another study evaluated the prognostic relevance of a novel semiquantitative classification of Bcl2 immunohistochemical expression in breast cancer. Bcl-2 expression was evaluated in 442 patients, resulting an indipendent predictor of clinical outcome in both node-negative and node-positive patients. In fact patients with Bcl-2 negative immunostaining had higher probability of relapse (5 times) or death (7 times) [22]. A recent meta-analysis showed the prognostic value of Ki-67 in early breast cancer. 68 studies were identified and 46 studies including 12 155 patients were considered for evaluation of correlation between KI-67/ MIB-1 + and DFS and OS. Ki-67/MIB-1 + resulted associated with higher probability of relapse (P<0.001) and worse survival (P<0.001) in all patients, indipendently from nodal status [23]. Thanks to these new technologies, new perspectives are opening. Trials based on genetic profiling will also serve as an important resource for evaluating new molecular signatures and other technologies such as proteomics, epigenomics and pharmacogenomics as these technologies evolve. A co-primary objective of these studies will be to create a biologic specimen bank that would facilitate the evaluation of other tumor markers as they are developed. So promising markers may be evaluated in the laboratory and, if appropriate, may be brought back to the clinic as novel diagnostic markers or new drugs. Molecular analyses, including the subtyping of breast cancer, furthers end points "personalizing" each woman's therapy based on specific profiling of the patients and the tumor.

**Figure 1:** Oncotype DX Gene Recurrenca Score Assay: This test evaluates the expression of 16 cancer genes and 5 Reference Genes providing A Recurrence Score from 0 to 100. Thanks to this score, population is stratified in 3 risk categories, useful to understand patient outcomes and the potential benefit of chemotherapy



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**Recent metanalysis showing the prognostic value of Ki-67 in patients with early breast cancer**

**Predictive role of genomic microarrays in urological malignancies.**

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One of the major task of the modern oncology is to study genomic alterations underling cancer development. The possibility to have high-density oligonucleotide microarrays have provided readily available methods and genomic resource for identifying and monitoring DNA expression. Using these assays, we are able to characterize gene expression with high resolution, and compare alterations in DNA copy number and gene expression associated with cancer. That is important for both the basic understanding of cancer and its therapy.
Another intriguing possibility is to improve the prediction of clinical outcome of tumors i.e. progression, survival, response or resistance to therapy. In the present summary we will focus on the role of wide-genomic analyses by microarrays in the prediction of response to therapies and/or stage of disease and/or clinical outcome of urological malignancies. This report will describe results obtained in the clinical setting on the most common urological malignancies which are in order of reference: bladder, renal, prostate, and testis cancer.
Bladder cancer is a common malignant disease with 357000 new cases and 145000 deaths worldwide annually. The overall cause specific 5-year survival rate is about 70%. Clinical risk factors for distant recurrence are high grade, tumor size and presence of regional lymph nodes metastases. A recent study on 404 patients affected by bladder cancer validated previously reported gene signatures using a 52-gene tumor extension classifier and an 88-gene progression classifier. The first was found to be highly significantly correlated with pathologic stage ($p<0.001$) and with disease progression of Ta or T1 tumors ($p<0.001$). The molecular 88-gene progression classifier was highly significantly correlated with progression-free survival ($p<0.001$) and cancer-specific survival ($p=0.001$). Multivariate Cox regression analysis showed the progression classifier to be an independently significant factor associated with disease progression after adjustment for age, sex, stage, grade, and treatment (hazard ratio, 2.3; $p=0.007$). Another study examined the gene expression patterns in the development of bladder cancer from preneoplasia along papillary and nonpapillary lesions. The authors reported expression profiles of 19 pairs of RNA samples from adjacent urothelium and tumors using cDNA microarrays. They selected 6 genes and verified the expression of their products on a cohort of 251 bladder cancer patients using tissue microarray and immunohistochemistry. The expression of CDH1 (e-cadherin) and TOP2A (DNA-alfa-topoisomerase II) were found to correlate with metastasis-free and overall survival. In particular, tumors characterized by the low expression of e-cadherin and the high expression of DNA alpha-topoisomerase II had a high propensity for distant metastasis and were associated with poor survival. Dyrskjot et al reported the identification of clinically relevant subclasses of bladder carcinoma using expression microarray analysis of 40 well characterized bladder tumors. Hierarchical cluster analysis identified three major stages, Ta, T1 and T2-4, with the Ta tumors further classified into subgroups. A 32-gene molecular classifier was able to classify benign and muscle-invasive tumors with close correlation to pathological staging in an independent set of 68 tumors. This classifier provided new predictive information on disease progression in Ta tumors compared with conventional staging ($p<0.005$). A cDNA microarray study in 80 bladder tumors, 9 bladder cancer cell lines and 3 normal bladder samples identified two clusters for pTa and pT1 versus pT2-pT4 muscle-invasive tumors. CTSE was the most highly expressed gene in the superficial tumors, and RGS1 the most highly expressed in muscle-invasive tumors. Seven genes were able to discriminate between good- (alive after 18 months of follow-up) and bad-prognosis (death occurring <18 months) tumors. Validation of these results on an independent set of tumors was also conducted. Another interesting report identified genes differentially expressed between early-stage (n=8) and invasive bladder cancer (n=9) using cDNA microarrays. Then, the selected

genes were validated on a large cohort of 173 patients to delineate any associations with clinical outcome. Ninety-two genes provided no misclassification among early versus invasive tumors and 4 genes were studied by immunohistochemistry on tissue microarrays (cytokeratin, neuropilin-2, ninjurin and p33ING1). Significant correlations were found between the expression of these proteins with tumor stage and grade. Only expression of p33ING1 was associated with overall survival ($p=0.02$). A large study including 105 patients was conducted using a similar selection and validation two-stage strategy. At the selection phase top-ranked genes differentially expressed between superficial versus invasive bladder cancers were: peptidylprolyl isomerase A, tetratricopeptide repeat domain G, nuclear RNA export factor 1, hematopoietic-cell specific Lyn substrate 1. Top-ranked differentially expressed genes regarding survival in patients with invasive tumors were: HCLS1, ankyrin, BIRC3, ICAM-1, and TP53-AP1. The products of these genes were studied by immunoistochemistry in 294 primary tumors by tissue microarrays and all resulted significantly predictive of clinical outcome (survival). Other genes differentially expressed were synuclein and its receptor (CNR1). Synuclein was associated with pRB inactivation ($p<0.0005$) and KI67 ($p=0.007$).

Renal cell carcinoma (RCC) is the most common malignancy of the adult kidney, representing 2% of all malignancies and 2% of cancer-related deaths. RCC is a clinicopathologically heterogeneous disease subdivided into clear cell, papillary, chromophobe, collecting duct and unclassified (WHO, 1998). In a recent study, Takahashi examined the molecular signatures of 70 kidney tumors from seven different subgroups: clear cell, papillary, granular, chromophobe, sarcomatoid RCC, oncocytoma, transitional cell carcinoma (TCC) of the renal pelvis and pediatric Wilms' tumors by using cDNA microarray analysis using the expression ratios of a selected 3560 cDNA set. They found distinct molecular signatures in clear cell, papillary, chromophobe RCC/oncocytoma, TCC and Wilms' subtypes. In the clear cell RCC clustering, two subgroups emerged that correlated with clinical outcomes, confirming the potential use of gene expression signatures as a predictor of survival. In the so-called granular cell RCC, none of the six cases clusters together, supporting the current view that they do not represent a single entity. Four genes were selected in order to identify novel subtype-specific markers. The results were consistent with the gene expression microarray data: glutathione S-transferase was highly expressed in clear cell RCC, methylacyl racemase in papillary RCC, carbonic anhydrase II in chromophobe RCC and K19 in TCC. Another study compared by cDNA microarray analyses 32 clear cell RCC, 11 papillary RCC, 6 cromophobe RCC, 8 TCC, 12 oncocytoma and 23 normal tissues. Gene signatures based on a 30-gene predictor for each histotype was found (oncocytomas and cromophobe RCC grouped together). Subsets of these genes code for secreted proteins involved in angiogenesis and membrane receptors involved in migration, adhesion and proliferation. This metastatic signature was able to predict tumors with and without metastases at the time of surgery.

Prostate cancer represents the second leading cause of cancer-related deaths. Prostate specific antigen screening has resulted in earlier disease detection but 30% of men die of metastatic disease. Slow disease progression, an aging population and associated morbidity and mortality underscore the need for improved disease classification and therapies. A recent study analyzed a cohort of 64 patients using array comparative genomic hybridization (CGH). Half of patients recurred after radical prostatectomy. Analysis of the CGH profiles revealed numerous recurrent genomic copy number aberrations. Specific loss at 8p23.2 was associated with advanced stage disease, and gain at 11q13.1 was found to be predictive of postoperative recurrence independent of stage and grade. Moreover, comparison with an independent set of metastases revealed approximately 40 candidate markers associated with metastatic potential. Thus, copy number aberrations at these loci may predicts metastatic genotypes.

Testicular cancer is a relatively rare malignancy; however, it is the most common cancer of men between the ages of 15 and 34. Based on morphological features, germ cell tumors are divided into seminomatous and non-seminomatous tumors. The latter can be divided into: mixed, yolk sac tumor, embryonal carcinoma, choriocarcinoma, and teratoma according to the WHO International

Histological Classification of Testicular Tumors (3). The unique study reporting data on the prognostic role of genome-wide expression profiling in testicular cancer has been recently conducted. Gene expression profiles of 17 retroperitoneal NSGCTs (Non-Seminomatous Germ Cell Tumor) (10 yolk sac tumors, 3 embryonal carcinomas, 4 teratomas) and 2 PNETs (Primitve Neuroectodermal Tumor of the testis) were obtained from patients with two clinical outcomes. One group of NSGCTT and PNET patients developed metastases within 2 years (early-relapse) of initial successful treatment, and the other group developed metastases after 2 years (late-relapse). They identified 13 genes that can distinguish between early-relapse and late-relapse yolk sac tumors. The most over-expressed gene in the late-relapse group was small nuclear ribonucleoprotein 70kD polypeptide. Glutaredoxin (GRX) and aldehyde dehydrogenase (ALDH)-1 family member A2 were the most differentially underexpressed genes in the late-relapse group.
Some criticisms can be raised on the methodologies of published studies. The first consists on the number of analyzed cases which often is less than 100 cases (a table of small studies is here reported, see table 1). Furthermore, the studies are often retrospective and lacking of a prospective and independent validation of genomic signatures.

**Table 1.**

| Author | Methods | No. | Tumor | Predictor | Predictive role |
|---|---|---|---|---|---|
| *Gottardo et al* | Micro-RNA profiling | 27<br>27 | Kidney<br>Bladder | miR-26b | Stage (bladder cancer) |
| *Takahashi et al* | cDNA microarray | 29 | Kidney | 40 genes | Prognosis |
| *Gashaw et al* | cDNA microarray | 40 | Seminoma | ETV4 | pT1-pT3<br>pT2-pT3 |
| *Tamura et al* | cDNA microarray | 25<br>10 | HRPC<br>HSPC | 106 genes | Hormone-refractory phenotype |
| *Xin et al* | cDNA microarray | 20<br>16<br>21 | HRPC<br>NPC<br>NP | Annexins 1,2,4,7,11 | Hormone-refractory phenotype |
| *Takata et al* | cDNA microarray | 18 | BC | 14 genes | Response to neoadjuvant M-VAC |
| *Modlich et al* | cDNA microarray | 20<br>22 | IBC<br>SBC | 41 genes | Superficial vs Invasive |

HRPC: Hormone-Refractory Prostate Cancer; HSPC: Hormone-Sensible Prostate Cancer; NPC: Naïve Prostate Cancer; NP: Normal Prostate; BC: Bladder Cancer; IBC: Invasive BC; SBC: Superficial BC

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**TMA Role in diagnostic and prognostic validation of translational clinical studies.**

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## 1. Introduction.

The large use of genomics and proteomics addressed to biomarkers identification for new diagnostic and therapeutic applications has been greatly increased, leading to the development of high-throughput technologies. But determination of which genes impact really in terms of diagnosis, prognosis and therapy in different cancers represents a bottleneck (1-3). First, the clinical relevance in the expression of single gene is often which cell expresses it both in normal and diseased human tissues (2). In addition, the significance of biomarker expression need large patient group with long-term clinical follow-up for definitive validation (3). In this view the concept of DNA microarrays was extended to embedded tissue samples of pathology archives. Tissue microarray (TMA) technology, described by Kononen *et al* in 1998, is an array-based, high-throughput technology used to examine molecular alterations in a large number of tissues on parallel-obtained slides (4). Many advantages are recognized in TMA experiments with respect to experiments driven of whole sections from different samples, mainly because of possibility to analyze different markers in the same area previously selected and reducing variability linked to pre-analitic conditions (1-5).

As the number of cancer studies using high-throughput technologies increases, TMA technology has been proven to be a high-throughput validation tool of the marker genes identified in DNA microarray experiments. In fact candidate genes identified in other high-throughput technologies, such as serial analysis gene expression and array comparative genomics hybridization, were frequently validated in TMA studies (6).

## 2. TMA design and experiment

TMA design requires careful selection of block donors, through identification of representative areas in relative haematoxylin and eosin stained slide, and precise recording of their localisation details. Core needles keeping in donor tissue vary in diameters from 0.6 to 2.0 mm. Tissue cores are transferred to a recipient paraffin block, into a made hole, guided by a defined x–y position. Core thick is between 3 and 5 mm. Considering a standard section of 4 μm, TMAs could produce more 1250 sections. In this manner a TMA block of 300 cores would produce 375,000 different results (1,6).

Currently, most TMAs (80%) are being used for immunohistochemistry with the remaining 20% being investigated by *in situ* hybridization techniques. Immunohistochemistry and in situ techniques are used in the standard way, testing simultaneously all cores in an standardisation of condition in all methodic steps (6).

1-2 cores are generally enough to represent a specific tumour. Nevertheless high heterogeneity of some tumours, like breast carcinoma and renal cell carcinoma, lead to select 2-4 cores for recipient block. Analysis of Estrogen Receptor, Progesteron Receptor (PgR) and p53 in a large series of invasive breast cancer in a TMA reveals overlapping results in term of prognosis, even considering one spot, although the frequency of positive staining becomes similar to analysis of large standard sections, when using more spots, above all for p53 expression (7).

In the case of prostate cancer, the use of three-four cores provide the optimal sampling for TMA, in term of analysis directed to correlation to PSA recurrence following radical prostatectomy for clinically localized prostate cancer. Fewer TMA samples significantly increased Ki-67 variability and a larger number did not significantly improve accuracy (5,6, 8).

In hemathological disease immunohistochemical expression evaluated in two cores do not show deviation from immunohistochemical data from whole classical section. Franco et al in a ocular adnexa B cell lymphoma TMA, analysed for main markers of cell cycle and apoptosis control (Table 1), observed that the reproducibility of the results obtained was confirmed by comparing them from whole sections for markers related to lymphoid cell differentiation (CD20, CD3, CD138, CD79a, bcl6 and CD5) and, in ten case comprehending Burkitt lymphoma, for EBER-ISH (9). In this same series also MALT related translocations, t(11;18) and t(14;18), could be easily evaluated in one core as representative of whole section (Figure 1).
From a theoretical point of view, the adequate numbers of cores in a TMA depends upon the variability of the parameters object of analysis. In fact in the case of homogeneous markers, a single TMA spot per case will be adequate (5).

Control normal tissue cores were generally recommended.
Array construction could be performed in association to a computer file, containing tissue block coordinates with basic patient data. Division in quadrants and space without cores represent valid strategies to orientate recipient block (1,6).

**3. TMA types**

*Normal tissue arrays* are used in order to identify distribution of specific molecules in all human tissue. It represents a valid tool in definition of topographic localization of antigens through new developed antibody (10).
*Multitumor TMA* favours qualitative and quantitative screening of target distribution within different tumors. In addition because multitumor TMA are designed including more tumors of the same hystotype associated to relative normal tissue, they provide the possibility to analyse the deviation of target distribution between specific tumors and normal tissue from which it derives. The use of Multitumor TMA has also shown that antigens considered neoplasm-specific were widely distributed. In fact analysis of CD117 expression in 3556 primary tumors from 134 different tumor types included in a multitumor TMA, demonstrated its unequivocal positivity in gastro-intestinal stromal tumors (100%), in seminomas (84%), and decreasing proportions from 65 to 17%, in adenoid-cystic carcinomas, malignant melanomas and large-cell carcinomas of the lung (11).
*Progression TMA*s are particularly useful to asses morphological and molecular changes through the different stages of tumour progression. They contain samples of different stages, from normal tissue, preneoplastic lesions until tumoral lesions in the spectrum of different grading score. This approach recognizes a remarkable role in definition of potential diagnostic and therapeutic targets associated with progression. In this view many paper were published in order to propose critical biomarkers linked to progression. Varambally at al. in a study of prostate cancer, in which TMA include benign parenchyma, hyperplasia, PIN, cancer in different Gleason grade and metastasis, the protein EZH2 seems to be much more expressed in advanced Gleason grade adenocarcinoma and in metastasis with respect to localized disease (12). Similarly IGFBP 2 and HSP 27 were demonstrated to be correlated with progression by Bubendorf et al. (13).
*Prognosis (patient outcome) TMA* is surely the most common constructed and reported TMA, associated to clinical follow-up data. In addition to its use as a marker of prostate cancer progression, the degree of expression of EZH2 was related to outcome after radical prostatectomy, being strong expression related to disease progression (12). In their study of 532 RCC samples and six normal kidneys, Hoch *et al.* found an association between vimentin expression and poor prognosis of patients with kidney cancer (14). Combining in situ detection of chromosomal alteration with studies of immunohistochemical expression of product of gene involved, it is possible to evaluate in a structured manner in the same series the impact on tumoral progression. In this way, Richter et al demonstrated that Cyclin E gene amplification and relative protein overexpression directly correlate with bladder cancer outcome (15).

**4. Multiple Biomarker approach. Molecular predictive models.**

The possibility of analyse different critical marker in tissues of the same typology of tumours could offer the realize a predicting model. An accurate system for predicting survival is useful for patient counselling, planning follow-up, and selecting patients for additional treatment.

This approach has been variously applied in different neoplastic pathologies. Rhodes et al analyzed expression of 14 candidate prognostic biomarkers for prostatic cancer progression in a TMA containing more than 2,000 cores from 259 patients and defined a moderate or strong expression of EZH2 coupled with at most moderate expression of E-cadherin as the biomarkers combination mostly related to prostatic cancer progression after prostatectomy (16). Kim et al constructed a multimarker nanogram predicting progression in renal cell carcinoma after nephectomy. This model comprehends expression of makers considered significantly related to prognosis in multivariate analysis, i.e. Carbonic Anydrase IX, p53, Vimentin and Gelsolin. The risk to progression was calculated attributing specific points to single markers positivity. Authors also proposed another nanogram in which clinical features flank markers expression, improving the possibility of predicting risk to progression (17).

Predictive models in hematologic malignancies appear particularly interesting, because actual classification of this group of neoplasias includes yet homogeneous clinic-biologic categories. Saez et al performed a TMA study of 52 selected molecules (mainly cell cycle and apoptosis regulator and B cell differentiation markers), proposing a statistical predicting model with definitive 8 markers (cyclin E, CDK1, SKP2, EBER, MUM1, CDK2, bcl-6, and Rb-P). Combining this biological score and the International Prognostic Index (IPI) improves the capacity for predicting failure and survival. This predictor was then validated in the independent group (18).

A statistical predictor model was also proposed for melanoma in a TMA based study. Alonso el al identified among 39 markers (cell cycle, apoptosis, melanoma antigens, transcription factors, DNA mismatch repair, and other proteins), definitive markers of a statistical predictor model (Ki67, p16, p21 and Bcl-6), also validated in an independent series of 72 vertical growth phase melanoma patients (19).

## 5. Special array types. Frozen and cell lines TMAs.

The quality of some epitopes for immunohistochemical analysis and of DNA/RNA for in situ detection are generally much higher. Thus technologies for obtaining TMA from frozen tissue were developed and proposed (20).

Analysis on cell lines could provide excellent immunohistochemical positive controls. Moreover identification of induced proteins could be an interesting tool in translational studies. In both cases isolated cells grown in culture were fixed in formalin, embedding them in paraffin or agarose (21).

**6. Conclusion**

High throughput molecular screenings have provided information of many markers involved in specific neoplastic disease pathogenesis and progression. But clinical relevance of these biological markers cannot easily determined. TMA technology offer a powerful method to validate biological markers in order to define their impact in development and progression of neoplasia. Moreover the integrated analysis of multiple markers could generate multimarkers model of progression with interesting implications in management of patients. At the end recent studies showed that TMAs could accelerate the process of drug discovery, by validation of drug targets, determination of molecular epidemiology and development of diagnostic assays (22).

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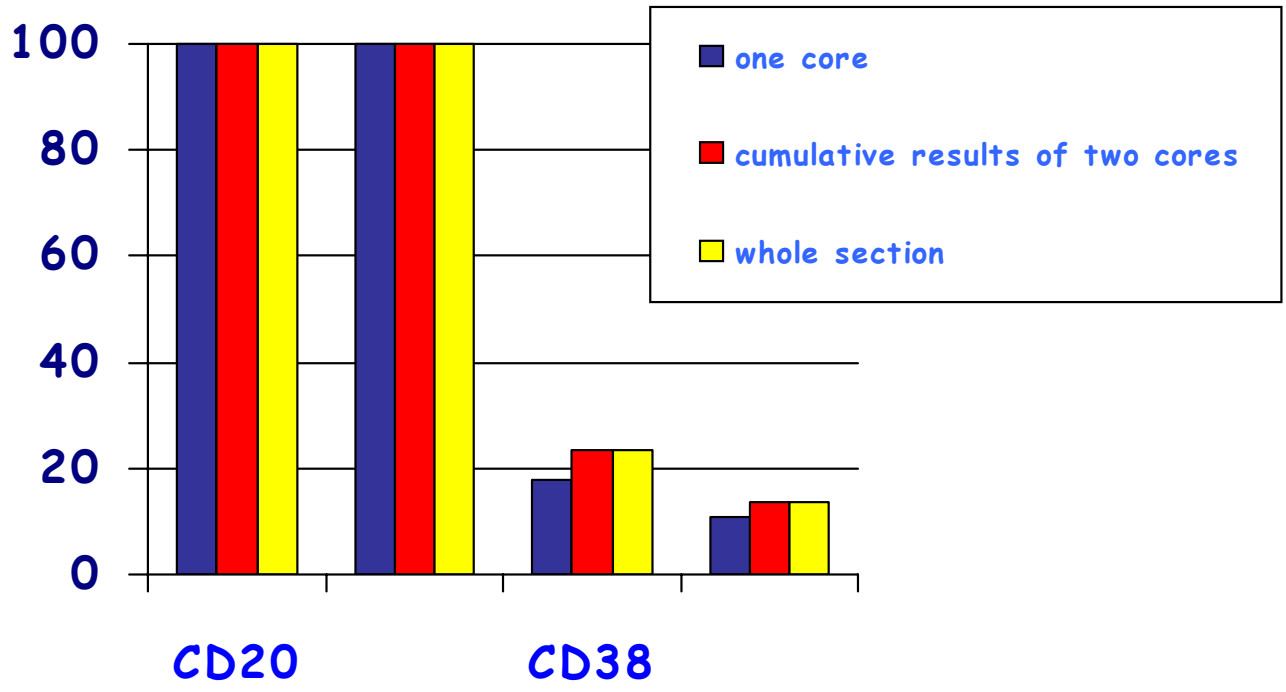
**Table 1.** Analysed Antigens in Ocular Adnexa B cell Lymphoma TMA

| Antigen | Reactivity | Thresold | Internal control |
|---|---|---|---|
| **Bcl2** | High/low | >50% neoplastic cell | Small lymphocyte |
| **BAX** | Positive/negative | >10% neoplastic cell | Benign small lymphocyte |
| **Bcl-XL** | High/low | >10% neoplastic cells | TMA Controls |
| **Survivin** | High/low | >10%neoplastic cells | TMA controls |
| **NFkB** | Positive/negative | Nuclear expression | TMA control |
| **Caspase-3** | Positive/negative | >10% positive neoplastic cells | TMA controls |
| **BCL10** | Positive/negative | >10%neoplastic cells | Reactive lymphocyte |
| **MUM1** | High/low | >80% positive cells | Plasma cells |
| **Bcl6** | Positive/negative | >10% neoplastic cells | GC (germinal center) B cells |
| **CD38** | High/low | >80% positive cells | Plasma cells |
| **CD5** | Positive/negative | >10% positive cells | Reactive lymphocyte |
| **CD10** | Positive/negative | Any tumoral cell positive | Gc B cells |
| **CD20** | Positive/negative | Any positive neoplastic cells | Reactive lymphocyte |
| **Cyclin A** | Positive/negative | >10% neoplastic cells | Proliferatine cells(G2/M) |
| **Cyclin B1** | Positive/negative | >50% positive cells | Proliferatine cells(G2/M) |
| **Cyclin D1** | Positive/negative | Any positive neoplastic cell | Macrophagee and endothelian cells |
| **Cyclin D3** | Positive/negative | >50% positive cells | Proliferatine cells |
| **Cyclin E** | Positive/negative | >80% positive cells | TMA controls, proliferatine cells |
| **CDK1** | Positive/negative | >80% positive cells | TMA controls, proliferatine cells |
| **CDK2** | Positive/negative | >50% positive cells | TMA controls, proliferatine cells |
| **CDK6** | Positive/negative | >80% positive cells | TMA controls |
| **P21** | Positive/negative | >10% neoplastic cells | Scatterei GC |
| **P16** | High/low | >10% positive cells | Normal cells |
| **P27** | High/low | >10% positive cells | Restino lymphoid cells |
| **Ki67** | High/low | >505 positive cells | Proliferatine cells |
| **P53** | High/low | >80%positive cells | Scatterei GC cells |
| **Hdm-2** | High/low | >10%positive cells | Macrophages |
| **Rb** | High/low | >80% positive cells | Proliferating cells |
| **CD3** | Positive/negative | Any tumoral cell | Reactive lymphocyte |
| **p-IkB** | Positive/negative | Antiapoptotic marker | TMA control |

**Figure 1**. Proportion of positive cases to immunoistochemical detection of CD20, CD79a, CD38 and FISH detection of t(14;18) in analysis of one core, two cores and whole section from Ocular Adnexa MALT B cell Lymphoma. No difference in positive cases between analysis of two cores and whole section. Relative lower sensibility of analysis of CD38 (77%) and t(14;18) (80%) with respect to analysis of two cores/whole section.



**Bovine serum amine oxidase, spermine and lysosomotropic compounds in therapeutic implications**

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In tumours polyamines and amino oxidases increase if compared with normal tissue. The *in situ* formation of cytotoxic metabolites by an enzyme-catalyzed reaction is a recent approach in cancer chemotherapy. Cytotoxicity induced on human cancer cells by bovine serum amine oxidase (BSAO, E.C. 1.4.3.6) and spermine is attributed to $H_2O_2$ and aldehydes produced by the reaction. The results demonstrate that cytotoxicity caused by spermine metabolites was greater in multidrug resistant cell (MDR) than the corresponding wild-type ones (WT), due to an increased mitochondrial activity (1,2). This represents an aspect of particular importance with respect to the potential therapeutic applications of the method (1,3), since conventional cancer therapy suffers from the development of drug resistance. In fact, the purpose of this research is to suggest a new strategy to overcome MDR in human cancer cells by using drug combinations with the aim to increase the induction of cell death by toxic polyamine metabolites. Association with MDL 72527 ($N^1$,$N^4$-bis(2,3-butadienyl)-1,4-butanediamine dihydrochloride), a lysosomotropic compound, improves the cytocidal effect of polyamines oxidation products, more effectively in MDR cells than their wild-type counterparts, as was evident from the decrease of cell survival. In particular, cancer cells were pre-treated with (MDL 72527) and cytotoxicity resulted greater by the combined treatment than by BSAO/spermine alone (2,4). In fact, pre-treatment with MDL 72527 300 μM, for 6, 24 or 48 h sensitized WT and MDR human cancer cells (2,4) to the subsequent exposure to BSAO/spermine enzymatic systems. TEM observations showed that the pre-treatment of the cells with MDL 72527 caused the temporary formation of cytoplasmic vacuoles, of lysosomal origin (2,4). Confocal microscopy analysis confirmed that the pre-treatment with MDL 72527 increased the number of lysosomal structures, indicating a contribution of the lysosomotropic properties of MDL 72527 to the sensitization of the tumour cells to spermine enzymatic oxidation products (5). In fact, the release of lysosomal enzymes into the cytosol by MDL 72527 is the major reason for its sensitizing effect. Experiments with acridine orange-stained cells show that MDL 72527 releases acridine orange from the lysosomes into the cytoplasm. The release of lysosomal enzymes produces oxidative stress and apoptosis (5). Instead, mitochondrial damage, as observed by TEM, seemed to correlate better with the cytotoxic effects induced by the ROS (6), generated during the treatment, than with the formation of vacuoles (2,4). In fact, severe changes of the mitochondrial structure, such as dilatation of the *cristae* and disruption of membranes, were mainly observed in MDR cells. Our studies carried out on colon adenocarcinoma (LoVo) and melanoma (M14) cells suggest that the anti-malarial drug chloroquine (CQ), in association with BSAO/spermine, could also potentiate the effects of the enzymatic oxidation products of spermine. In our opinion, the systematic exploration of CQ in combination with conventional anticancer drugs promises new and efficient anti-cancer therapies. The combination of BSAO/spermine is not only able to prevent tumour cell growth, but also prevents mass tumour growth, particularly well if the enzyme has been conjugated with a biocompatible hydrogel-polymer. In fact, the growth of a mouse melanoma (B16-F0) was reduced by 70% after injection of the immobilized enzyme, in comparison with 32% inhibition after injection of native enzyme. Since the tumour cells release endogenous-substrates of BSAO, the administration of spermine is not required (7). The findings, lysosomotropic effects caused by MDL 72527 or CQ and mitochondrial alterations induced by spermine oxidation products, make this new approach attractive in combating cancer and in treating MDR cancer patients.

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**RITA, a small molecule which induces accumulation of p53 in tumor cells, showed anti-tumor effect and modulation of tumor susceptibility to natural cytotoxicity**

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Inactivation of p53 by mutations or deregulation of HDM2 (human double minute-2) protein, which by binding the amino terminus inhibits p53 transactivation function and targets its proteosomal degradation, have been described in several human tumors. RITA is a small molecule which can bind p53 and induces its accumulation in tumor cells through a disruption of p53-HDM2 interaction. We demonstrated that RITA induced perturbation of cell cycle and growth inhibition in cell lines derived from several tumors with different p53 status. Since a functional p53 pathway is important for a 5-Fluorouracil (5-FU) -induced cytotoxic effect, we evaluated RITA and 5FU combination treatment, showing synergistic anti-proliferative and pro-apoptotic effects in colon and head an neck human cancer cells. In addition, RITA positively modulate, on cancer cell surface, the expression of ligands for NKG2D (MICs, ULBPs), a key NK cell's receptor required for the recognition of tumor cells. Recently, it was shown that DNA damaging agents, including 5-FU, induce in mouse tumor cell system NKG2D ligands expression. Here we have investigated whether RITA/5FU combination treatment, could have a synergistic effect also in the induction of human NKG2D ligands homologues. Our data demonstrate that indeed the combinated 5-FU and RITA treatment increased the MICs and ULBP1-4 expression on several tumor cell lines. Finally, we demonstrated that RITA pre-treatment of HT29 colon target cells increases their susceptibility to resting and IL-2-activated natural lymphocyte- cell cytotoxicity. Therefore these data add new insight in the RITA antitumor effect mechanism and showed the potentiality of this small molecule in combining direct anti-tumor effect and triggering the immune surveillance against cancer.

**Anti-tumor activity of the new dual PI3K/mtor inhibitor, NVP-BEZ235, in sarcomas**

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Osteosarcoma and Ewing'sarcoma are the most common primary malignant bone tumors in children and adolescents whereas rhabdomiosarcomas are the most common soft tissue sarcoma of childhood. Combination chemotherapy associated with local control with surgery or radiation therapy has become a standard practise in the treatment of patients with sarcomas. However, treatments for high-risk sarcoma are still completely inadequated since one third of patients with non metastatic disease and the great majority of patients with metastases at diagnosis do not survive. Although recent clinical studies indicated that the cure rate of patients with local disease can be improved with dose intensification of conventional therapeutics; neverthless this raise with severe toxicity and secondary tumors. For these reasons, identification of new selective drugs are urgently needed for these tumors. We have previously reported that all these tumors are characterized by the presence of insulin-like growth factor-1 receptor (IGF-IR) and the autocrine production of its ligands IGF-I and/or IGF-II. The interaction between IGF-IR and its ligands anduces autophoshorilation , stimulation of intrinsic tyrosine kinase activity and activation of downstream effector molecules, such as phosphatidylinositol 3-Kinase (PI3K). PI3K contributes to the recruitment and activation of a wide range of downstream targets, including the serine-threonine protein kinase AKT/PKB (protein kinase B). Activated AKT/PKB (pAKT) regulates many biological processes, such as proliferation, apoptosis, migration, invasion as well as angiogenesis. Thus, the IGF-IR- induced PI3K/AKT signal transduction has a critical role in the prevention of apoptosis and regulation of cell cycle progression. Disruption of normal PKB/Akt signaling has been documented as a frequent occurrence in several human cancers and the enzyme appears to play an important role in their progression.
In this study, we analyzed the preclinical therapeutic potential of a novel inhibitor of PI3K/mTOR, NVP-BEZ235, in Ewing's sarcoma, osteosarcoma and rhabdomyosarcomas. The compound induced a $G_1$ cell cycle block in all cell tested, whereas apoptosis rate remained unchanged. Growth in soft-agar, as well as migration and adhesion to ECM components were significantly reduced in cells treated with NVP-BEZ235. Combined *in vitro* treatments identified in the interaction of NVP-BEZ235 with vincristine the best drug-drug combination and this was confirmed against Ewing's sarcoma xenografts in nude mice. No toxic effects were observed with respect to metabolism. These results encourage the potential inclusion of this drug in the treatment of patients with musculoskeletal tumors.

**PPAR-γ reactivation by troglitazone sensitizes human pancreatic cancer cells to stat-1-mediated antitumor activity of Interferon-β**

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Pancreatic adenocarcinoma is a highly aggressive malignancy poorly sensitive to chemo- and radio-therapy. We recently showed that interferon-β (IFN-β) induced a potent inhibitory effect on the proliferation of pancreatic cancer cell lines (Vitale G et al. Ann Surg 2007). In the present study, we investigated the potential interaction between IFN-β and troglitazone (TGZ), a PPAR-γ agonist, in BxPC-3, a human pancreatic cancer cell line.

Simultaneous exposure of BxPC-3 cells to IFN-β and TGZ produced synergistic antiproliferative activity compared with single drug treatment, as evaluated by the dedicated software Calcusyn.

It is well known that the antiproliferative activity, mediated by the activation of type I IFN receptors, is classically modulated by the phosphorylation of STAT-1 and STAT-2. However, IFNs can also recruit and phosphorylate STAT-3 protein, counteracting the antitumor activity promoted by the STAT-1 and STAT-2 activation. In fact, STAT-3 phosphorylation seems to be an inducible survival response, protecting cancer cells from the antiproliferative activity of type I IFNs.

In BxPC-3 IFN-β induced growth inhibition paralleled by STAT-1 and STAT-3 activation, while the combination of IFN-β and TGZ resulted in increased STAT-1 activity and antagonism on STAT-3 activation, as evaluated by western blotting with phospho specific antibody. In addition, we have found that IFN-β alone increased both ERK1/2 and AKT phosphorylation, while the combined treatment decreased these effects. The transfection of pancreas cancer cells with a dominant negative (DN) STAT-3 antagonized the activation of ERK by IFN-β and potentiated its inhibition by the combination. These effects occurred together with an increase of the cell growth inhibition induced by IFN-β and the combination. On the other hand, the transfection with DN STAT-1 antagonized the synergism of the combination on the cell growth inhibition. The synergistic effects induced by the combination were likely due to perturbation of cell cycle. In fact, FACS analysis after staining with propidium iodide showed a delay of G1/S phase progression, that occurred together with increased expression of p21 and p27 proteins. The increase of p21 and p27 is mainly due to a decreased proteasome-dependent degradation of both proteins.

In order to study an eventual influence of IFN-β on the modulation of PPAR–γ activity TGZ inducible, we evaluated the effects of treatment with IFN-β and/or TGZ on the binding of PPAR-γ to ARE6, a specific natural PPAR-γ response element, by EMSA. The IFN-β + TGZ mediated ARE6-binding activity of the nuclear extracts resulted to be higher than the activity of both single drugs.

In conclusion, this preclinical work represents the first demonstration of a synergistic antitumor interaction between IFN-β and PPAR-γ agonists in pancreatic cancer, and could represent a rationale for further in vivo investigation.

**Cationic Liposomes for the Delivery of the Photosensitizer *m*-THPC in Photodynamic Therapy of Tumors**

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Malignant gliomas represent the most common primary brain tumor: more than 50% of them are glioblastoma multiforme (GBM). GBM are generally considered "high grade" tumors and the poor prognosis is due to the tumor cells invading normal brain tissues, beyond the reach of resection, leading to the recurrence of tumor. Among adjuvant therapies, photodynamic therapy may offer a very good chance of targeted destruction of infiltrating GBM cells, thus increasing the survival time and recurrence-free interval of GBM patients. Meta-tetrahydroxyphenylchlorin (*m*-THPC), due to its photophysical properties and good affinity to tumor tissues, is a promising photosensitizer for the treatment of brain tumors.
In this study we investigated the performance of liposomes composed by different ratios of a natural phospholipid, dimyristoyl-*sn*-*glycero*-phosphatidylcholine (DMPC), and a cationic gemini surfactant, as vehicles for *m*-THPC on human and murine glioblastoma cell lines. The uptake (flow cytometry) and the intracellular distribution (confocal microscopy) of *m*-THPC in cationic liposomes, were compared to those of the same chlorin in the pharmaceutical form (Foscan®). Moreover, by cloning efficiency assay the potential therapeutic efficiency of chlorin delivered by liposome formulations was compared to that of the pharmaceutical compound, before and after irradiation with laser light at 652 nm.
The obtained results indicated that cationic liposomes (i) transferred *m*-THPC in glioblastoma cells more efficiently than pharmaceutical formulation; (ii) significantly ($p << 0.001$) increased the *m*-THPC cytotoxic effect after laser irradiation; (iii) seemed to exert their cytotoxic action in the early phase of interaction with the cells, during adhesion to the plasma membrane.

**HIV-1 Virus-like Particles as biological vehicle for antigen delivery to elicit systemic and mucosal Immunity.**

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**Background:** Virus-like particles (VLPs) are based on the expression of virus capsid proteins, which spontaneously assemble in particles structurally similar to native virus particles, representing a non-replicating, non-infectious particulate antigen delivery system able to present conformationally structured proteins to the immune system. VLPs enter into professional APCs, such as macrophages and dendritic cells, and deliver antigens both to the MHC class I and class II antigen presentation pathway inducing CD8 and CD4 T cell responses. Furthermore, VLPs are particularly efficient as mucosal vaccines due to their size as well as their capacity to activate the innate immune response. Indeed, they can be phagocytosed by M cells in the nasal lumen and then directly deposited to the NALT via M cell transcytosis, which preferentially drains into cervical lymph nodes. This process induces strong local (NALT) and distant immune responses in both peripheral and mucosal immune compartments.

**Results**: Pr55gag Virus-Like Particles (HIV-VLPs) have been developed and produced in a baculovirus expression system in our laboratory. These HIV-VLPs have been shown to induce, at systemic as well as mucosal (vaginal and intestinal) sites, HIV-1-specific CTLs as well as significant levels of cross-clade Nabs against HIV-1 primary field isolates, by intraperitoneal and intranasal administration in immunised Balb/c mice (Buonaguro 2002, 2005 and 2007). Intra-nasal immunization has been performed in a innovative mucosal adjuvanted formulation. Such results have been obtained in A pre-clinical study in non-human primates, funded by the NIH, is going to evaluate the systemic and mucosal immunization induced in higher animals by mucosal immunization with our HIV-VLPs.

**Conclusions**: The induction of immune response at systemic and mucosal sites by i.p. as well as i.n. administration of VLPs, shows the efficacy as biological vehicle for antigen delivery. Strategies to use VLPs in a broader array of applications, as antigen and/or bioactive-molecule delivery system, are currently under evaluation.

**Blocking cell migration with a synthetic urokinase receptor-derived peptide**

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Metastatic cells exhibit spontaneous rates of migration higher than their primary counterparts. Thus, the control of cell motility may be considered as a new and attractive approach for the clinical management of tumor progression. *In vivo* three-dimensional migration requires a constant regulation of speed and directionality during tumor cell dissemination. The receptor of the urokinase (uPAR) appears to be a key molecule in the coordination of these different events. Ligand-activated uPAR, exposing the specific $Ser^{88}$-Arg-Ser-Arg-$Tyr^{92}$ sequence, retain a potent chemotactic activity. Previous work directed to the functional characterization of the uPAR Ser-Arg-Ser-Arg-Tyr sequence, resulted in the finding that any position is indeed relevant to the chemotactic activity. With the hypothesis that specific mutations in the Ser-Arg-Ser-Arg-Tyr-sequence may generate cell migration inhibitors, we developed a number of new compounds named ICMs (Inhibitors of Cell Migration), which were tested for their ability to inhibit the Ser-Arg-Ser-Arg-Tyr-dependent cell migration. Among ICMs, we selected a short peptide named ICM32 which revealed to be a potent inhibitor of *in vitro* basal and chemoattractant-directed cell migration. Inhibitory effects, occurring at picomolar concentrations, are species- and tissue-independent, as FBS-directed cell migration was inhibited by ICM32 to an extent comprised between 40% and 60% in a variety of human and mouse cell lines. ICM32 prevents dynamic remodelling of cytoskeleton, inhibits wound healing and *in vitro* cell invasion of HT1080 human fibrosarcoma cells. Finally, we found that intravenous administration of ICM32 causes a three fold reduction of lung metastasis areas following intravenous injection of HT1080 cells in nude mice. Overall, these results encourage us to consider ICM32 a promising type of peptide-based anti-cancer drug.

**Intracellular zinc increase selectively inhibits p53$^{-/-}$ pancreatic adenocarcinoma cell growth by ROS/AIF-mediated apoptosis**

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Cellular treatment with non-toxic doses of zinc in association to the ionophore compound pyrrolidine dithiocarbamate (PDTC) inhibits p53$^{-/-}$ pancreatic cancer cell growth much more efficiently than gemcitabine, the gold standard chemotherapeutic agent for pancreatic cancer. In contrast, normal primary fibroblasts are less sensitive to the same treatment. Both the metal chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine and the radical scavenger *N*-acetyl-$_L$-cysteine are able to recover cell growth inhibition by Zn/PDTC, demonstrating that this effect depends on the increased levels of intracellular zinc and reactive oxygen species (ROS). We also report that pancreatic cancer cell growth inhibition is due to both cell cycle arrest and apoptosis. Interestingly, mitochondrial injury and apoptosis are associated to ROS-dependent nuclear translocation of the mitochondrial factor AIF, but not to the regulation of the expression of apoptotic genes and caspase activation. Finally, we show that resistance of normal fibroblasts to Zn/PDTC-induced cytotoxicity correlates with: 1) a lower level of basal and PDTC-mediated increase of intracellular zinc, compared to pancreatic cancer cells, and 2) a strong inhibition of *TP53* expression following Zn/PDTC treatment. These events may play a role in the protection of normal cells from Zn/PDTC induced apoptosis.

**FK 506 binding protein (FKBP51) gene silencing potentiates ionising radiation induced cell killing in an *in vitro* human melanoma model.**

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Malignant melanoma is an aggressive neoplasm. The prognosis is bad in advanced stages of disease due to the high metastatic potential of this tumour and resistance to conventional anticancer treatments. At the present, no efficient therapeutic strategies to control advanced disease are available.
A body of evidence supports a key role for nuclear factor kappa B (NF-κB) in resistance of melanoma cell to both chemo- and radiotherapy. The NF-κB transcriptional activity is normally inhibited by IκB proteins that sequester it in the cytoplasm. In response to different stimuli, he IKK complex phosphorylates two critical serine residues in IκBs, triggering events that lead to proteolitic degradation of these inhibitors thus allowing nuclear translocation of NF-κB proteins. Active NF-κB factors modulate the expression of a number of genes that sustain cell survival and it is widely reported that inhibition of this transcriptional activity sensitizes many tumour cells to death-inducing stimuli. We showed in a previous work that the isomerase activity of the immunophilin FK506 binding protein (FKBP51) is essential for activation of IKK in response to genotoxic insults,in melanoma.
Aim of this work is to investigate the effect of FKBP51 gene silencing, by small interference RNA (siRNA) methodology, on cell killing induced by ionising radiation, in a melanoma cell line established by a patient.
The siRNA for FKBP51 was shown to suppress NF-κB activity.
Cells, treated or not with FKBP51 siRNA for 48 h, were exposed at a 4 Gy dose of 6 MV X ray of a linear accelerator. Cell death was measured by annexin V staining and flow cytometry, after a further 48 h incubation. Both the ionising radiation treatment and exposure to FKBP51siRNA, significantly augmented the amount of cells positive to annexin V, by 50% and 65% respectively. In fact the mean percentages of annexin V positive cells were 20.5%$\pm$4.6, 30.1%$\pm$8.0 ($p=0.01$) and 33.4%$\pm$4.6 ($p<0.001$) for untreated cells, irradiated-untreated cells and FKBP51 siRNA-treated cells. However, the effect of the combination treatment was more than additive resulting in an increase of 150% of cells positive to annexin V. Indeed, the proportion of cell death was 50.0%$\pm$9.1 after irradiation of FKBP51 siRNA-treated cells ($p<0.00$). In conclusion, our study shows that FKBP51 gene silencing enhances ionising radiation-induced cell killing in melanoma, thereby providing a possible useful target for radiosensitizing strategies.

**A DAG-1 gene-promoter driven GFP expression vector as a tool for the in vivo evaluation of dystroglycan, a cancer and dystrophy-related gene**

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Dystroglycan (DG) is a widely expressed adhesion complex that anchors the cells to basement membrane and is involved in embryonic development and differentiation. DG protein expression is frequently reduced in human dystrophies although no primary mutations in DG gene have been identified. DG protein is also reduced in a variety of human cancers and its loss might promote tumor cells spreading and metastasis. DG protein expression is mainly regulated at a post-transcriptional level. However, changes in the expression levels of DG mRNA have been observed during cell growth and differentiation, thus suggesting a potential regulation at a transcriptional level.
In order to understand the mechanisms underlying this regulation and whether it might be important to regulate DG expression level in muscle as well as tumor cells, we aimed to identify and characterize the DG promoter region. DG gene (DAG1) presents a SP1-regulated minimal promoter that includes a CpG rich region which shows a variable degree of methylation in different cell lines. In order to be able to monitor promoter activity in vivo, we developed a GFP expression vector driven by DAG1 gene-promoter. This construct was successfully used to follow promoter activity during the differentiation of C2C12 myoblast cells in myotubes. We found that DG promoter activity is initially upregulated and then progressively decreases with differentiation. Promoter activity also increased when cells were cultured on gelatin-coated dishes and clearly correlated with cell density. We are now evaluating how different coating surfaces (i.e., laminin, matrigel and collagen) influence the GFP expression. Moreover, since DG protein expression was found to be downregulated by the antiandrogen flutamide in prostate cancer cells, we aim to use this tool to evaluate whether antiandrogen treatment can affect DAG1 promoter activity both in androgen-dependent and androgen-resistant cells. We believe we have developed a useful tool to evaluate DG promoter activity and plan to use it to further evaluate the role of DG gene and its regulation in the development of muscle as well as neoplastic disease using an in vivo imaging system in animal models.

**An activating point mutation of CXCR4 occurred in human melanoma and colon cancer cell lines.**

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CXCR4 is widely overexpressed in human cancers. CXCR4 function regulated cell invasion, proliferation and survival. Modulation of CXCR4 expression could reside on CXCR4 gene mutations. CXCR4 was analyzed for mutation in the coding region by gene sequencing in human melanoma and colon cancer cell lines. One somatic point mutation was detected in the CXCR4 coding region in the human colon cancer cell line, PD and in human melanoma cell line, LB. The CXCR4 transition (G574A) resulted in amino acid substitution, Valina 160 (Val 160) to Isoleucina (Ile160), in the CXCR4 fourth transmembrane region. PD and LB cell lines expressed CXCR4 protein as assessed through immunoblotting and immunocytochemistry. CXCR4 function was evaluated in PD and LB cell lines: SDF-1$\alpha$ -mediated extracellular signal – regulated kinase (Erk) activation and cell migration was not induced in the LB and PD cell lines compared to human melanoma cell lines bearing wild type CXCR4 receptor. To better define the function of the mutated receptor human A431, epidermoid cancer cell lines, were stably transfected with the mutated (G574A) CXCR4. The level of phosphorylated ERK is increased costitutively in the mutated cells compared to wild type transfected cells. SDF-1$\alpha$ was unable to induce migration in CXCR4-mut-A431 cells compared to CXCR4-WT-transduced A431 cells. In *in vivo* experiments CXCR4-WT and MUT were inoculated into the right flank of nude mice. CXCR4-WT A431 cells resulted in faster growing tumors compared to CXCR4-MUT A431 cell lines. Interestingly treatment with AMD3100, a well known CXCR4 antagonist, inhibited growth of CXCR4-WT– tumors but increased the growth of CXCR4-MUT- tumors such as an agonist.
This is the first report of a CXCR4 point mutation detected in human cancer cells affecting the receptor function.

**Differential expression of chemosensitivity pathways in BRCA1-defective breast cancer cells: an *in vitro* and *in vivo* study.**

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**Background**: Inherited germline mutations in BRCA1 is related to hereditary breast cancer. A feasible model to study BRCA1 function is HCC1937 BRCA1-defective breast cancer cell line and its derivative clone HCC1937/wtBRCA1 in which BRCA1 has been reconstituted by full-length gene transfection. Previously, we demonstrated the chemosensitivity to cisplatin (CDDP) in BRCA1-defective cells respect to BRCA1 reconstituted cells *in vitro*. In this study, we investigated the CDDP chemosensitivity in murine xenograft model and the differential whole gene expression profile by GeneChip microarray analysis in the defective and reconstituted cells.

**Methods**: SCID mice were xenografted into the interscapular area using 3 x $10^6$ HCC1937 or HCC1937/wt BRCA1 cells suspended in 100 µl RPMI-1640 medium, treated with CDDP 5 mg/kg or vehicle i.p., weekly, for 4 following weeks, after tumor detection. Tumor volume and mice survival were evaluated from the first day of treatment until day of death or sacrifice. Xenografts were retrieved from animals 12 hours after CDDP or vehicle treatment and immediately disaggregated. Cell suspension was used for cell cycle analysis by flow cytometry. Gene expression profile of HCC1937 and HCC1937/wtBRCA1 was evaluated using the HG-U133A 2.0 GeneChip array of Affymetrix. Array data were analyzed using the DNA-Chip Analyzer and the altered expression and the modulation of signaling pathways was analyzed using a network mapping tool, the Ingenuity Pathway Analysis Software (IPA).

**Results**: The *in vitro* evaluation of CDDP chemosensitivity resulted in a more pronounced dose-dependent reduction of HCC1937 cell growth as compared to HCC1937/wtBRCA1. Evaluation of the *in vivo* activity of CDDP disclosed a statistical significant reduction of the tumor volume in treated mice respect to controls ($p<0.005$). The antitumor activity of cisplatin in the HCC1937 xenografted mice was significantly higher than in HCC1937/wtBRCA1 xenografted mice ($p=0.043$). Cell cycle analysis of cancer cells obtained from retrieved tumors after CDDP treatment, showed almost complete abolition of S- and G2/M-phase in BRCA1-defective tumors cells, whereas BRCA1 reconstituted cells were not significantly affected by CDDP treatment and reacquired the G2/M block. Gene expression analysis revealed that 172 genes were modulated by BRCA1-reconstitution. With regard to CDDP related sensitivity genes, BRCA1-defective HCC1937 cells over-express RAD52 and XRCC4 mRNAs, and down-express ERCC1 and RRM1 mRNAs, when compared to HCC1937/wtBRCA1 cells. Overall gene modulation analyzed with IPA identified that the major perturbation of genes expression levels occur in proliferation and survival pathways, in G2/M and G1/S-phase checkpoints and in several signaling pathways as well as Insulin-like Growth Factor, Vascular Endothelial Growth Factor, estrogen receptor, PI3K/AKT, Epidermal Growth Factor.

**Discussion**: Our data are the first evidence of in vivo increased sensitivity to CDDP of BRCA1-defective breast cancer cells xenografted mice. We identified a clear modulation of DNA damage repair genes. Moreover, the loss of S- and G2/M-phase checkpoints correlates to antiproliferative effects induced by CDDP which is further supported by the finding of low levels of RRM1 expression in HCC1937 tumor cells. In conclusion, we support the concept of "BRCAness" as a specific status in the carcinogenesis of highly undifferentiated breast tumors, which is an emerging issue involving both hereditary and sporadic tumors. The "BRCAness" which is linked to drug sensitivity could be the specific target for new therapeutic approaches.

**HMGA2 mRNA expression correlates with the malignant phenotype in human thyroid neoplasias**

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The high mobility group proteins (HMG) A, are architectural chromatin proteins. They bind the minor groove of AT-rich DNA sequences through three short basic repeats, called "AT-hooks", located at the N-terminal region of the proteins. By interacting with the transcription machinery, HMGA proteins alter the chromatin structure and, thereby, regulate the transcriptional activity of several genes either enhancing or suppressing the ability of more usual transcriptional activators and repressors. The HMGA family is comprised of three proteins: HMGA1a, HMGA1b, and HMGA2. HMGA proteins are expressed at a high level during embryogenesis, while their expression becomes low to undetectable in adult tissues. HMGA proteins represent a unique example of genes being implicated in both benign and malignant neoplasias, but with different mechanisms. In fact, rearrangements of these genes represent a feature of most benign mesenchymal tumors. Conversely, HMGA overexpression is a common event in experimental and human malignancies and is also required for the appearance of the neoplastic phenotype.
Therefore, we have analysed the expression of the HMGA2 gene in a series of normal and neoplastic thyroid samples by immunohistochemistry and quantitative RT-PCR. HMGA2 protein was detectable in 4 of 21 follicular carcinomas (FTCs), 30 of 45 papillary carcinomas (PTCs), and 10 of 12 undifferentiated (anaplastic) carcinomas. As far as follicolar adenomas are concerned, only three cases of the 31 analyzed, showed HMGA2 protein expression whereas it was absent in 7 normal thyroid tissues and in 12 hyperplastic nodules. Quantitative RT-PCR essentially confirmed the immunohistochemical data: only a discrepancy was observed for the follicular and papillary carcinomas since most of them express higher HMGA2 specific mRNA levels in comparison to normal thyroids and adenomas. Independently from the factors responsible for the discrepancy of mRNA and protein levels in PTCs and FTCs the results shown here indicate that the evaluation of the HMGA2 specìfic mRNA level might be a good tool for the discrimination between follicular adenoma and carcinoma that represents one of the main problems in the diagnosis of thyroid neoplasias. In fact, the statistical evaluation of the HMGA2 mRNA levels indicates a significant difference in HMGA2 expression in benign and malignant neoplasias ($p=0.000141$).

**Expression profiling of highly degraded RNA extracted from formalin-fixed, paraffin-embedded breast tumor biopsies by oligonucleotide microarrays**

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Gene expression profiling of tumour biopsies promises to help improve clinical management of breast and other cancer patients by providing new ways to classify tumours and to predict disease outcome and responsiveness to therapy, but its application to large-scale studies or in clinical settings has been, so far, seriously hampered by the fact that in most cases RNA extracted from these biopsies is degraded and thus unsuitable for molecular analysis.
This is particularly true for formalin-fixed, paraffin-embedded (FFPE) samples, that are of great potential usefulness for translational cancer research but provide only highly fragmented RNA.
This represents a serious drawback, since FFPE tumor tissue banks are available with clinical and prognostic annotations, which could be exploited for molecular profiling studies, provided that reliable analytical technologies are found.
We applied and evaluated a microarray-based DASL (cDNA-mediated annealing, selection, extension and ligation) assay for analysis of 502 mRNAs in highly degraded total RNA extracted from cultured cells or FFPE cancer biopsies.
The DASL-based expression profiling assay applied to RNA extracted from MCF-7 cells before or after 24hrs stimulation with a mitogenic dose of E2 consistently allowed to detect hormone-induced gene expression changes also following extensive RNA degradation ‘in vitro’. Comparable results where obtained with tumor RNA extracted from FFPE breast cancer biopsies.
We implemented here a technology for parallel quantitative analysis by oligonucleotide microarrays of a large number of transcripts in highly degraded RNAs extracted from FFPE breast and bladder tumor biopsies which overcomes previously encountered limitations, and describe a pre-analytical RNA sample screening protocol. Accuracy and reproducibility of the DASL assay applied to FFPE cancer specimens were also evaluated using as source of RNA 17 paraffin blocks, corresponding to 15 distinct biopsies. Finally, to evaluate sensitivity of the DASL array method implemented here for detection of differential gene expression between FFPE tissue samples, we exploited the differences in RNA profiles between two different cancer types: bladder and breast cancer.
The results of this study provide a practical method for carrying out gene expression profiling analyses in FFPE breast tumor samples by DASL oligonucleotide microarrays, which is applicable also to normal and pathological samples of other origin. Sensitivity, reproducibility and accuracy of the assay indicate that it is well suited for retrospective clinical studies aiming at identifying prognostic and predictive gene profiles in archival FFPE tissue banks and, predictably, also on laser-capture micro dissected tissue samples, following an appropriate set up.
Research supported by: MUR (PRIN 2005063915_003 and 2006069030_003), UE (IP CRESCENDO, contract n.er LSHM-CT2005-018652) and AIRC.

**Pegylated liposomal doxorubicin in elderly patients (≥70 years) with HER negative metastatic breast cancer: a phase II trial**

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**Background.** Few chemotherapy regimens are suitable for the treatment of elderly patients with advanced breast cancer. Pegylated liposomal doxorubicin is active single agent in metastatic breast cancer (MBC) and lack overlapping toxicity. The possibility of combining these two drugs therefore seems attractive.
**Methods** Twenty-nine patients with MBC entered a phase II study of pegylated liposomal doxorubicin 25 mg/m2 intravenously (i.v.) on day 1 and 14, every 4 weeks for at least eight cicles. A two-staged Simon accrual design was adopted for this phase II trial. Patients were required to have measurable disease, previous chemotherapy with or without an anthracycline-containing regimen, and a normal left ventricular ejection fraction (LVEF).
**Results**. All patients with MBC were eligible, assessable for response and toxicity. The overall response rate (on an intent-to-treat basis) was 40% (11 of 28; 95% CI, 20%-54%). Two complete response and 9 partial responses were noted. In addition, 11 patients (40%) had stable disease of > 4 months duration, and 6 patients (20 %) had disease progression. Median time to disease progression was 10 months (range, 3-38 months) and median overall survival was 15 months (range, 4 to > 62 months). Neutropenia was the most frequent toxicity (grade 3 in 30% of patients and 19% of cycles), but neutropenic fever was seen in only 3 cases. No septic deaths occurred. Nonhematologic grade 3 side effects included skin toxicity (palmar-plantar erythrodysesthesia syndrome, 8%) and mucositis (14%). Late alopecia was seen in 45% of patients (grade 1 in 33%, and grade 2 in 12%). The median LVEFs were 64% (range, 50%-81%) at baseline and 62% (range, 37%-70%) after treatment. Only one patient presented an LVEF decrease to < 50%; however, no clinical heart failure was noted, and this patient recovered normal values after cessation of therapy.
**Conclusions.** Pegylated liposomal doxorubicin can be safely administered to patients with anthracycline-pretreated MBC and is active in this population. Final data analysis including geriatric evaluation will be presented.

**UROKINASE RECEPTOR-DERIVED CHEMOTACTIC PEPTIDE STIMULATES *IN VITRO* NEO-ANGIOGENESIS TROUGH THE VITRONECTIN RECEPTOR**

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Angiogenesis is a highly coordinated process required for normal development, in response to injury and for tumor growth. This process is sustained by a tightly regulated motility of endothelial cells. Angiogenesis is regulated by chemotactic stimuli and requires the activation of several signalling pathways that converge on cytoskeletal remodelling. The $Ser^{88}$-Arg-Ser-Arg-$Tyr^{92}$ chemotactic sequence of the urokinase receptor binds to FPR or FPR-L1 G protein–coupled cell receptors. We have recently found that the Ser-Arg-Ser-Arg-Tyr (SRSRY) peptide binds to the high affinity formyl peptide receptor (FPR), and specifically promotes cytoskeletal rearrangements and directional cell migration in a vitronectin receptor-dependent manner. Since Human Umbelical Vein Endothelial Cells (HUVEC) express a considerable amount of FPR as well as alphavbeta3 vitronectin receptor, we investigated on the possibility that SRSRY may triggers neo-angiogenesis. First, by conventional Boyden chamber assays, we found that SRSRY promotes directional cell migration of HUVEC in a dose-dependent manner, with an extent similar to VEGF. Maximal chemotactic effect is reached at 10 nM SRSRY concentration. In a tube-formation assay, SRSRY shows a remarkable ability to promote angiogenesis of endothelial cells. According to cell migration data, pro-angiogenic effect is dose-dependent and peaks at 10 nM SRSRY. Both endothelial cell migration and *in vitro* angiogenesis promoted by SRSRY are inhibited by cell pre-incubation with blocking anti-alphavbeta3 monoclonal antibodies, thus indicating that SRSRY exhibits an alphavbeta3-dependent pro-angiogenetic effect. These data suggest that pharmacological compounds able to specifically inhibit the $Ser^{88}$-Arg-Ser-Arg-$Tyr^{92}$ chemotactic sequence of the urokinase receptor may be considered useful for the control of tumor progression.

**Baculovirus-expressed HIV-1 Virus-Like Particles (HIV-VLP) activate a selctive gene transcription profile in *ex vivo* monocyte-derived dendritic cells (MDDCs) as well as in uncultured PBMCs.**

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**Aim:** A candidate HIV-1 vaccine model based on HIV-1 Pr55gag Virus-Like Particles (HIV-VLPs), produced in a baculovirus expression system and presenting a gp120 molecule from an Ugandan HIV-1 isolate of the clade A (HIV-$VLP_A$s) has been developed in our laboratory. The present study has been performed to evaluate the ability of the HIV-$VLP_A$ to induce the maturation and activation of monocyte-derived dendritic cells (MDDCs), and to analyze the pathway involved.
**Results:** The baculovirus-expressed HIV-VLPs efficiently induce maturation and activation of MDDCs and are incorporated into MDDCs preferentially via an actin-dependent macropinocytosis and endocytosis. The HIV-VLP-activated MDDCs show an enhanced Th1- and Th2-specific cytokine production, in particular, IL-12 p70, IL-6, IL-10 and TNF . The effects of VLPs on MDDCs are, to some extent, mediated through intra-cellular Toll-like receptors (TLR 3-5-7-9) signaling. The HIV-VLP-loaded MDDCs are able to induce a primary and secondary response in autologous human CD4+ T cells, in an *ex vivo* immunization assay. Moreover, the genomic transcriptional profile of VLP-activated DCs, by gene microarray analysis, show the upregulation of several genes involved in the immune response.
**Conclusions:** Our results show that baculovirus VLPs activate MDDCs and stimulate the production of cytokines involved in the Th-1 and Th-2 pathways, elucidating the mechanisms triggering the cell-mediated immunity observed in VLP-immunized animals. The intra-cellular Toll-like receptors appear to be involved in this process and additional signaling pathways induced by VLPs in the MDDCs are currently under evaluation by gene profiling analysis. These data give an insight into the mechanisms of the cellular immunity induced *in vivo* by VLPs, which may be extremely useful to optimize and modulate the immune response.

**HOX D13 expression across 79 Human Tumor Types in a Tissue Microarray parallels molecular analysis.**

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Hox genes represents a family of homeobox genes characterized by an unique genomic network organization: four compact chromosomal loci where 39 sequence corresponding genes can be aligned with each other in 13 antero-posterior paralogous groups. Hox genes regulates normal development and controls primary cellular processes (cell identity, cell division and differentiation) recently enriched by the discovery of their interaction with miRNAs and ncRNAs. Besides the HOX D13 gene involvement in both prostate morphogenesis defects of mutant mice and neuroendocrine differentiation of human advanced prostate cancers, HOX D13 generate a fusion protein, with Nup98 nucleoporin, involved in acute myelogenous leukaemia. Furthermore dysregulated HOX D13 expression has been detected in different tumor types such as breast cancer, melanoma, cervical cancer and atrocytomas. Here, to detect by immunohistochemistry the expression of HOX D13 homeoprotein in normal tissues and its potential dysregulation along the evolution of specific tumor types, we utilized tissue microarrays including >5,000 different normal tissue and cancer samples types from 79 different tumour categories. Results highlight significant differences in HOX D13 expression between specific normal tissues and corresponding tumor types (adrenal gland, brain, breast, colon, endometrium, lung, pancreas, myometrium, prostate, submandibular gland, skin, small intestine, smooth muscle, soft tissue, stomach, testis, urinary bladder, uterus cervix). HOX D13 gene expression has been further detected at mRNA level, through real-time quantification, for selected tumor types (colon, skin, lung, pancreas, prostate, salivary glands). The correspondence of HOX D13 expression at both mRNA and protein level represents a validation system which supports the potential diagnostic use of HOX D13 homeoprotein for immuno-histochemical determinations.

**Tissue microarray analysis reveals significant correlation between neurod1 and chromogranin a expression in human prostate cancer**

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BACKGROUND. Understanding the mechanisms through which prostate cancer acquires neuroendocrine differentiation is diagnostically and therapeutically relevant. Tissue microarrays (TMA) are powerful tools to analyze the clinical significance of new molecular markers in human cancers. Here, we have tested neuro-endocrine related markers on a prostate TMA containing 658 different specimens.
METHODS. The specimens were derived from patients treated for clinically localized prostate cancer by radical prostatectomy or transurethral resection. Expression of NeuroD1, ChromograninA (ChrA) and Androgen Receptor (AR) was analyzed by immunohistochemistry. Survival analysis by Kaplan-Meier curves and univariate and multivariate analysis were performed in order to assess the role and the impact of each marker on prognosis.
RESULTS. Staining for NeuroD1, ChrA and AR were positive in 73%, 49% and 77% of the available cases, respectively. As far as the overall survival is concerned, there were 87deaths and 295patients alive/censored (median follow-up 6y). For disease progression 77 events occurred at the median follow-up 5.4y. A significant correlation between NeuroD1 and ChrA and AR expression was recorded ($p<0.001$ and $p<0.03$, respectively). Additionally ChrA was strongly associated in multivariate analysis to Gleason score and Ki67 expression ($p<0.009$ and $p<0.0052$, respectively). Prognostic analyses reveal that none of the markers appear to be associated with either overall survival or disease-specific survival.
CONCLUSIONS. NeuroD1, ChrA, AR were evaluated on a large TMA of prostate cancer for the first time. Our results show that these markers are strongly associated but their expression does not correlate with overall or disease progression, suggesting a possible use as diagnostic markers.

**Evaluation of molecular and genetic markers involved in the progression from HCV-associated pre-neoplastic lesions toward hepatocellular carcinoma by genomic cDNA microarray analysis.**

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**Background.** Liver cancer is the fifth most frequent cancer of the world, affecting both men and women and represents about 6% of all new cancer cases diagnosed worldwide. Hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) worldwide and in the Campania Region the HCV seroprevalence is about 10-15% in the general population, with peaks above 40% in the over-45 age group. The molecular mechanisms of HCV-induced hepatocarcinogenesis are not yet fully understood. Besides the indirect effects of tissue inflammation and regeneration, a more direct oncogenic activity of HCV can be envisaged. In particular, perturbation of expression of cellular genes by early viral oncoproteins might be implicated in early steps of HCV-associated carcinogenesis. In this framework, the aim of this study have been the identification of molecular mechanisms involved in the progression from HCV-associated pre-neoplastic lesions (Chronic Active Hepatitis - CAH and Cirrhosis) toward hepatocellular carcinoma (HCC) for innovative diagnostic as well as therapeutic approaches.
**Results.** Tissues from HCV-associated pre-neoplastic and primary HCCs lesions (with their corresponding non-neoplasitc sites) have been obtained from patients referring to the Surgery Division "D" of the INT in Naples and matched for age, sex and social-economic conditions. A cDNA microarray analysis has been performed using chips displaying >30'000 cDNA clones, representing >20'000 genes. An unsupervised Eisen's clustering analysis, performed on >4'000 filtered genes, shows a significant clustering of the normal tissues from HCV and HCV-associated HCC. Preliminary evaluations of the global gene expression patterns show distinct expressions of cellular genes involved in liver functions, inflammation and cell cycle. Evaluation of specific genes differentially expressed in the stages of HCV-associated disease progression are currently ongoing by surpevised Eisen's clustering analysis.
**Conclusions.** The thorough analysis of gene expression patterns in HCV-associated lesions, compared to normal liver tissue counterpart, will significantly help in shedding lights on the HCV role in the etiopathogenesis of hepatocellular carcinoma. Moreover, it should allow the identification of possible prognostic/progression markers and the further characterization of molecular targets for innovative biotechnological therapeutic strategies (e.g. vaccines, monoclonal antibodies, gene therapy).

**Role of the antiapoptotic protein Bag-3 in Breast cancer**

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Breast cancer is the most common cancer and the second-leading cause of cancer mortality in women with approximately one in nine being affected in their life time. Breast neoplastic diseases comprise a broad spectrum of tumors ranging from benign fibroadenoma to the very aggressive undifferentiated carcinoma that it is lethal in a few months . Even though the knowledge of the molecular event underlying the generation of human breast carcinomas has made enormous progress, other information are required. In fact, diagnosis may be improved according to the genetic abnormalities that they contain. Moreover, information about genetic alterations occurring in breast carcinomas may suggest other therapeutic approaches.
Proteins that share the BAG (Bcl-2- Associated Athanogene) domain bind to the ATPase domain of the heat shock proteins (Hsp) and modulate their chaperone activity in protein folding. BAG family includes BAG3 protein, expressed in muscle, heart, brain and other tissues. In some cell types, BAG3 expression can be induced by heat shock or cell trasformation. We recently showed that BAG3 expression can be stimulated in human leukocytes by oxidative stress and the increase of its levels results in reducing stress- induced apoptosis. BAG3 therefore appeared to exert an anti-apoptotic property in some cell types. Furthermore, we found that downmodulation of BAG3 protein levels by a specific small interfering (si)RNA enhanced basal apoptosis and the apoptotic response to Tumor necrosis factor- Related Apoptosis Inducer (TRAIL) in thyroid carcinoma cell lines. In the present study, we investigated the expression of BAG3 in a range of breast cancer to evaluate whether this molecule can represent a candidate marker in the study of breast tumours. We show that no BAG3 expression is detectable in normal breast tissue. Conversely, BAG3 protein was abundant in all of the carcinoma samples with an intense staining in the cytoplasm. Positive staining was also found in fibroadenomas and hyperplasias, but in these cases the staining was in the nucleus and the number of positive cells was lower than carcinomas. Conversely, *In Situ* ductal carcinomas showed positivity restricted to the nuclei and the cytoplasm . Our goal is to evaluate if this different behavior of BAG3 in these samples might be dependent on different genetic alterations and if the different BAG3 immunolocalization might represent a useful tool for differentiating breast carcinomas for the aggressiveness and prognosis.

**Effects of docetaxel and tipifarnib on the apoptosis of human epidermoid cancer cells.**

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Docetaxel (Taxotere, DTX), a semisynthetic analog of Paclitaxel, is potent promoter of apoptosis in cancer cells. Cytotoxic mechanisms of antimitotic taxoids are not yet fully understood, but it has been demonstrated that docetaxel increases tubulin polymerisation, promotes microtubule assembly and also inhibits tubulin depolymerisation. We have previously demonstrated that DTX induces growth inhibition and apoptosis in human epidermoid KB cancer cells and a protective effect from apoptosis through the hyperactivation of a ras->erk-dependent survival pathway. In order to increase the anti-tumour potential of DTX we have used TIPIFARNIB (Zarnestra), a non peptidomimetic farnesyl transferase inhibitor that inhibits the farnesylation and consequently the activity of ras, in combination with DTX.
We have studied the effects of contemporary treatment of KB, HEP-2, CAL-33 and CAL-27 cells with TIPIFARNIB and DTX by the combination index-isobologram method, which is based on the median-effect principle developed by Chou and Talalay. The combined treatment resulted in a strong synergism when cells were exposed for 48 hours at the IC50 DTX IC50 TIPIFARNIB combination CI50 = 0.25 for KB and after 72 h of exposure the others cell lines, CI50 = 0.26 for HEP-2, CI50 = 0.52 for CAL-33 and CI50 = 0.69 for CAL-27. We have also demonstrated that the TIPIFARNIB was capable to inhibit the DTX-induced upregulation of ras, phosphorylated/activated-ERK1/2 and Akt-dependent signallings thus antagonizing a critical survival pathway elicited on tumor cells by the drug. Recently studies showed that the Heat Shock protein 90 (Hsp90) forms a multi-molecular complex with other Hsps and interactors. The binding of Hsp90 multichaperone complex with ATP prevents client proteins ubiquitination and proteasomal degradation. Most of client proteins of Hsp90 are key regulators of signal transduction, cell cycle, differentiation and survival. The Hsp70 binding maintain the multi-chaperone complex in the ADP bound status that is inactive and therefore unable to protect client proteins from ubiquitin-dependent degradation. Therefore we have evaluated effects induced by DTX and TIPIFARNIB alone or in combination on the expression of Hsp90 and Hsp70 in KB, HEP-2, CAL-33 and CAL-27 cancer cells. We have found that DTX/TIPIFARNIB reduces the expression of Hsp90 in HEP-2, CAL-33 and CAL-27cells, while increases in KB cells. The Hsp70 expression increases in all cells lines. On the basis of these data it can be hypothesized that the combined treatment enhances the multichaperone complex in the Hsp70-bound conformation that results pro-apoptotic. The Geldanamycin (GA) Hsp90 inhibitor can block proliferative mechanisms activated by cancer cells, that usually counteract the anti-tumor effect of conventional therapy. In order for potentiate the DTX-growth inhibition we have treated the parental and HSP90 transfected KB cells for 72 h with DTX and GA alone or in combination. We have showed that transfected cells was more resistant to anti-proliferative effect induced by drugs of than parental KB cells.
These data suggest the use of the combination in these therapeutic settings even if further experiments are required for the clinical translation.

**The combination Spermine/BSAO and Docetaxel synergize in the induction of apoptosis in human epidermoid cancer cells.**

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Polyamines are ubiquitous molecules with a strong positive relationship to cell growth and to cancer development. However recent data reported that the addition of spermine to culture medium containing serum inhibits cellular proliferation. This effect is caused by polyamine oxidation products generated by bovine serum amine oxidase (BSAO).
In the present study we have assessed if the cytotoxicity of Docetaxel can be potentiated by treatment of tumour cells with BSAO and spermine. Docetaxel is an anti-neoplastic agent that increases tubulin polymerization, promotes microtubule assembly and inhibits tubulin depolymerization. The disruption of microtubules may induce apoptosis.
We have evaluated the growth inhibition induced by different concentrations of Spermine/BSAO and Docetaxel (DTX) in epidermoid head and neck carcinoma (KB) and breast adenocarcinoma (MCF-7) cells. We have performed these experiments with MTT assay and the resulting data were elaborated with a dedicated software (Calcusyn, by Chou and Talalay, Biosoft). Combination index (CI) values <1, 1, and >1 demonstrate synergy, additivity, and antagonism, respectively. Dose reduction index 50 (DRI:50) represents the order of magnitude (fold) of dose reduction obtained for $ED_{50}$ (50% growth inhibition) effect in combination setting as compared to each drug alone.
We have found that the combination Spermine/BSAO and Docetaxel (DTX) was highly synergistic when both cell lines were exposed for 48 hours to these drugs at a molar ratio 25/75, (combination index lower than 0.5). In the same experimental conditions, a synergism on the apoptosis induction is also observed by both fluorescence microscopy (after PI and anti-annexin V antibody) and FACS analysis. Moreover, we have found that the combination caused caspase 3 and 9 activation and PARP fragmentation. In addition, we have observed that the combination Spermine/BSAO/Docetaxel can reduce the activity of the anti-apoptotic Ras-Erk pathway, but at the same time it determined an increase of HSP90 expression and p-P38 MAPK activity, molecular markers involved in the pathways of oxidative stress. On the basis of these results and of the property of Spermine and BSAO to induce the oxidative stress through the formation of toxic aldehydes, we have evaluated the effects of the combination on the lipo-oxygenation and the production of the nitric oxide; we have found that the pharmacological combination increased both the intracellular levels of nitric oxide and the lipid peroxidative damage if compared with the cells treated with single agents. Moreover, the combination of Spermine/BSAO/DTX allows to reduce the anti-tumour active dose of the single agents without increasing toxic effects (Not overlapping toxicity). In conclusion, we think that the knowledge of the oxidative damage processes may represent an efficient way to design innovative anti-tumour therapeutic strategies.

**Whole-genome microarray analysis of estrogen action in hormone-responsive human breast cancer cells.**

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Estrogens (E2) are key regulators in many biological processes, along with a highly recognized role in breast cancer. Microarray experiments enable simultaneous measurement of the expression levels of virtually all transcripts present in cells, thereby providing a "molecular picture" of the cell state. Estrogen actions are particularly suited to be studied with microarray-based technologies since the key mediator, the estrogen receptor, is a transcription factor involved in many different processes within the cell. Thus, only a wide-scale technique can avoid the risk of loosing key aspects of its multi-purpose action. On the other hand, the genomic responses to a pharmacological or hormonal stimulus are dynamic molecular processes, where time influences gene activity and expression. The potential use of microarrays in time series has not been fully exploited so far, due to the shortage of analysis methods which take into proper account temporal relationships between samples.
We studied the effect of a stimulation with a mitogenic dose of E2 on two different hormone-responsive breast cancer cell lines (ZR-75.1 and MCF-7), in order to focus our attention primarily on a E2 "core response" and not on cell type specific responses. RNA was extracted from cells stimulated with E2 following starvation in steroid-free medium for 4 days; RNA samples were extracted before or after 1, 2, 4, 6, 8, 12, 16, 20, 24, 28 and 32 hours hormonal stimulation and the sample before stimulation was taken as reference.
Gene expression was monitored with the innovative Illumina BeadArray platform, which includes an average of 30-40 replicates for each probe sequence randomly distributed on the chip surface. This allows the signal to be more robust with respect to local fluctuations in hybridization efficiency or spatial effects. Automatic selection of genes significantly affected by the hormonal treatment over time was performed using BATS (Bayesian Analysis Time Series) software, a newly-developed tool with a truly functional approach which treats the gene expression temporal profile as a global signal instead of a collection of independent time-points (as commonly done in most of existing methods). The influence of the normalization algorithm applied on data and of different parameter or threshold choices for the selection of differentially expressed transcripts has also been evaluated. Selection was found robust with respect to changes in parameters and type of normalization.
We then identified a list of 630 differentially expressed genes over time in the two cell lines and we performed on them a cluster analysis to identify groups of genes with similar regulation dynamics. Finally, an analysis of the genes with a common expression profile, taking into account a slight difference in cell-cycle timing, was conducted. We show the amount of overlap between the profile clusters in the two cell lines, both as gene composition and expression profile over time.
Research supported by: MUR (PRIN 2005063915_003 and 2006069030_003), UE (IP CRESCENDO, contract n.er LSHM-CT2005-018652) and AIRC.

**Human Osteosarcoma U2OS Cell Growth Regulation by Leptin: Role of Adenylate Cyclase/cAMP Signaling Pathway**

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Several recent studies indicate that leptin can stimulate growth of many types of cancer cells, proposing leptin as a mitogen/survival factor. Moreover, leptin is considered a key regulator of bone growth. To better understand how leptin regulates cell growth and to investigate the potential role of leptin in development of bone tumors, we studied the leptin-induced signaling and proliferative response in human osteosarcoma cell lines. Here we report that leptin inhibits growth of human osteosarcoma U2OS cells by slowing down cell division cycle in a p53 mediated and cAMP dependent manner. Collectively, our data point out that the growth-promoting effect is not a widespread action by leptin, enforce the evidences of leptin as a cytokine with a relevant role on cell growth control, identify cAMP signaling as a critical pathway affected by leptin to ultimately modulate proliferation of human osteosarcoma cells. Targeting leptin and cAMP signaling pathways might be useful in prevention and/or therapy of bone proliferation diseases.

**Protease-mediated prodrug strategy in cancer and infectious diseases: a hypothesis for targeted activation of trivalent arsenic**

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A strategy for the selective in vivo activation of prodrugs by proteases is presented. The approach is based on the design of polythiol peptides that are able to neutralize the toxicity of As(III) through chelation and contemporarily to be recognized as a substrate of a disease-linked specific protease. Proteolytic cleavage implies conversion of such polythiol peptides into monothiol fragments with irreversible loss of the chelating effect, thus triggering the release of As(III) in the free and pharmacologically effective form. The proteases whose activity appears dramatically up-regulated in various pathologies, ranging from cancer to infectious diseases, can be conveniently employed as prodrug activators in the disease microenvironment.
The design of the representative peptide shown here (CSGKGPRQITACSGKGPRQITAC) has been assisted by molecular modeling in order to fulfill the dual characteristic to be an efficient As(III) chelator and simultaneously a substrate of the matrix metalloproteinase-9 (MMP-9), an enzyme whose hyper activation in cancer is often associated with enhanced extra cellular matrix degradation and cellular invasiveness. The prodrug can be formed by the spontaneous reaction between the peptide CSGKGPRQITACSGKGPRQITAC and $As(OH)_3$. This approach allows the possibility to release locally (in specific cancer tissues marked by abnormally high MMP-9 activity) the free and active form of $As(OH)_3$, which is a drug already used in the second-line therapy of patients with acute promyelocytic leukemia. We envisage that the protease-induced local delivery of $As(OH)_3$ might introduce new opportunities for the treatment of malignant melanoma and other cancers that are not curable with the currently available therapies.

**Overcoming glutathione-s-transferase-mediated cisplatin-resistance in human osteosarcoma cell lines**.

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Cisplatin (cis-diamminedichloroplatinum, CDDP) is one of the leader drugs for high-grade osteosarcoma (OS) chemotherapy. However, despite this, only few information about the mechanisms which are responsible for resistance to this drug in human OS cells have been reported so far. In this study we have evaluated the bases of CDDP resistance in a panel of eight human OS cell lines established from the U-2OS or Saos-2 parental cell lines. The increase in CDDP resistance compared to the parental cell line ranged from 4.0- to 62.5-fold for U-2OS variants, and from 7.4- to 112.1-fold for Saos-2 variants. Globally, CDDP-resistant variants did not show cross-resistance to doxorubicin (DX) or methotrexate (MTX), the other two most relevant drugs for OS chemotherapy. In our experimental models, CDDP resistance resulted to be mostly associated with the increase of both intracellular level and enzymatic activity of glutathione-S-transferase P1-1 (GSTP1-1), a phase II detoxification enzyme which contributes to chemoresistance in many human cancers. Differently from other tumor cell lines, reduced susceptibility to drug-induced apoptosis, decreased drug accumulation or increased drug efflux, decreased sensibility to drug-induced DNA fragmentation, and increased glutathione intracellular content did not result to play a significant role in the acquisition of CDDP resistance of our resistant variants. On the basis of these results, we also evaluated the clinical impact of GSTP1-1 in a series of 34 high-grade OS patients and we found that the increased expression of *GSTP1* gene was associated with a significantly higher relapse rate and a worse clinical outcome. All these indications prompted us to assess the *in vitro* effectiveness of *6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol* (NBDHEX), a new promising anticancer agent which is a very efficient inhibitor of GSTP1-1 and can accumulate in tumor cells by avoiding the extrusion mediated by multidrug resistance protein pumps. Moreover, the low concentrations which are necessary to exert cytotoxic effects on human tumor cells, together with the low toxicity exhibited in mice, have identified NBDHEX as new promising drug of potential clinical use. NBDHEX was tested on a panel of 10 human OS cell lines and 20 variants of the U-2OS or Saos-2 cell lines, which were resistant to CDDP, DX or MTX. NBDHEX resulted to be very active on the vast majority of these cell lines, including the drug-resistant variants with higher GSTP1 enzymatic activities. Drug combination studies further supported the indication of NBDHEX as new promising drug for OS treatment, showing that it can be used together with CDDP, DX or MTX although the modality of administration must be carefully defined on the basis of the evidence provided by this study. In conclusion, we showed that GSTP1-1 has a relevant impact for both CDDP resistance and clinical outcome of high-grade OS. Moreover, our findings indicated that targeting GSTP1-1 with NBDHEX may be considered a new promising therapeutic possibility for high-grade OS patients, which are unresponsive to conventional chemotherapy.

**Lovastatin induces apoptosis of k-ras-transformed thyroid cells via inhibition of ras farnesylation and by modulating redox state**

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Transformation of thyroid cells with either K-ras or H-ras viral oncogenes produces cell types with different phenotype and different response to the inhibition of the prenylation pathway by lovastatin or fanesyltransferase inhibitors. These inhibitors induce apoptosis in K-ras transformed FRTL-5 cells (FRTL-5-K-Ras ) whereas cell cycle arrest is induced in H-ras transformed FRTL-5 (FRTL-5-H-Ras).. In FRTL-5-K-Ras cells the product of K-ras gene is implicated in the scavenging of reactive oxygen species (ROS) through the activation of ERK1/2 kinases. We observed that lovastatin blocked ras activation through inhibition of farnesylation and induced apoptosis, increasing ROS levels through inhibition of ERK 1/2 signalling and Mn-SOD expression. Lovastatin-induced apoptosis was due to intracellular ROS increase since both, the anti-oxidant compound pyrrolidinedithiocarbamate (PDTC), or the SOD-mimetic compound antagonized apoptosis. Moreover, both p38 MAPK and NFkB pathways, activated as a consequence of high ROS levels, are involved in the apoptotic effect, indicating that cell death induced by Lovastatin was dependent on oxidative stress. Lovastatin anti-tumour efficacy in K-ras dependent thyroid tumours was further confirmed in vivo, proposing a new therapeutic strategy for those tumour diseases that are sustained by an inappropriate K-ras expression.

**Anandamide controls the migration of mda-mb 231 breast cancer cells through inhibition of rho/rho kinase pathway**

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The endocannabinoid system regulates cell proliferation and migration in human breast cancer cells. The stimulation of cannabinoid CB1 receptors induced a non-invasive phenotype in MDA-MB-231 cells, a highly invasive human breast cancer cell line. In a model of metastatic spreading *in vivo*, the metabolically stable anandamide analogue, 2-methyl-2'-F-anandamide (Met-F-AEA), significantly reduced the number and dimension of metastatic nodes. This effect was antagonized by the selective CB1 antagonist SR141716. We observed that Met-F-AEA inhibited adhesion and migration of MDA-MB-231 cells on type IV collagen by modulating FAK phosphorylation. It is well known that the small GTPase Rho and ROCK, one its effector molecules, regulate cytoskeleton and play a crucial role in cell adhesion and motility. In this study we showed that Met-F-AEA controls cell migration through inhibition of RhoA activity. To assess RhoA activation using the Rhotekin binding assay, we observed that the treatment of MDA-MB-231 cells with Met-F-AEA for a short time period reduced the level of GTP-bound Rho. We further observed that Met-F-AEA induced a decrease in actin stress fiber, and the appearance of a dense meshwork of actin filaments around the cell periphery. Moreover Met-F-AEA markedly blocked translocation of RhoA from cytosol to membrane. Similar effects were obtained transfecting the cells with a negative dominant mutant RhoA (N19RhoA) and treating cells with Rho-associated protein kinase (ROCK) inhibitor Y-27632.

**Effect of gastrokine 1 on apoptosis in gastric cancer cells**

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Gastric cancer is a leading cause of cancer death worldwide. Most patients with gastric cancer present locally advanced incurable disease and the overall survival is very poor. Considerable research efforts towards the epidemiology and pathogenesis of gastric cancer have not been translated into successful treatments. Prevention is likely to be the most effective mean for reducing the incidence and mortality of this disease. To be successful, this strategy depends upon the knowledge of the molecular changes underlying gastric carcinogenesis. The aggressive cancer-cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signalling pathways. Such alterations include an impaired ability of the cancer cell to undergo apoptosis. Despite aggressive therapies, resistance of many tumors to current treatment protocols due to apoptosis defects remains a major problem in cancer therapy. Thus, current attempts to improve cancer survival must include strategies that identify on tumor cell specific target of resistance to apoptosis. In this report, we show that the overexpression of gastrokine 1 (GKN1) (1) in gastric cancer cell lines (AGS and MKN28)) renders these cells susceptible to death by apoptosis. GKN1, know also as Antrum Mucosal Protein (AMP 18), is highly expressed in normal stomach tissue and drastically reduced or absent in gastric cancer (2). These cells, transfected with the full-length GKN1 cDNA cloned into the pcDNA 3.1 vector, showed an enhanced expression of Fas (CD95/APO-1) associated to a marked stimulation of apoptosis induced by treating the cells with monoclonal antibody (IgM) to Fas. Control cells exposed to IgM did not undergo apoptosis. Apoptosis was verified via transferase-mediated dUTP nick-end labelling TUNEL. Western blot and RT-PCR showed a mild increase of Fas both at protein and mRNA level. These findings suggest that the down-regulation of GKN1 expression in gastric tissues may play an important role in gastric carcinogenesis and that this protein could represent a promising target to improve cancer therapy.

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**The CB1 receptor antagonist rimonabant (SR141716) inhibits colon cancer cell proliferation inducing mitotic cell death**

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Rimonabant is a highly selective antagonist/inverse agonist for the cannabinoid receptor type 1 (CB1). Due to its ability to block the CB1 receptor that controls food intake regulation at central and peripheral level, rimonabant is now in clinical development for the treatment of obesity and obesity-related co-morbidities. However, rimonabant shows a plethora of pharmacological effects in a number of physiopathological conditions including cancer. We have demonstrated that rimonabant exerts anti-tumour effects in thyroid and breast tumours. In the invasive and metastatic breast cancer cells MDA-MB-231 that are p53 negative, the anti-proliferative effect was characterized by a cell cycle arrest, without induction of apoptosis and was mediated by the inhibition of p42/44 MAPK phosphorylation and needed lipid rafts/caveolae integrity to occur. Starting from these observations we studied the anti-tumour activity of rimonabant in DLD1 colon cancer cells lacking a functional p53, in order to provide evidence on the potential anti-tumoural activity of rimonabant in cancer cells resistant to drugs that induce apoptosis in a p53-mediated manner. We treated DLD1 cancer cells with increasing concentrations of rimonabant for 24 and 48 hours, determining the most effective doses in reducing cell growth. Results showed a dose- and time-dependent inhibition of cell proliferation in the range from 2.5 to 10.0 micromolar, due to a mitotic cell cycle arrest. Moreover, in order to clarify the mechanism of action at the basis of the observed inhibition, we searched for mitotic catastrophe, which can be considered one form of p53-independent cell death occurring during, or close to the metaphase. Determination of the mitotic catastrophe was performed by observation of aberrant mitoses and cell cycle analysis. Results showed that rimonabant was able to significantly reduced cell growth and inhibited colony formation without inducing apoptosis. In addition, rimonabant produced a G2/M cell cycle arrest and a parallel enhancement of the number of mitoses associated to elevated DNA double strand breaks and chromosome misjoining events, hallmarks of mitotic catastrophe. Protein expression analyses of Cyclin B1, Aurora B, Survivin, PARP-1 and p38/MAPK demonstrated that rimonabant-induced mitotic catastrophe, is mediated by the suppression of DNA spindle assembly checkpoint and DNA damage checkpoint. In conclusion, our preliminary data indicate that rimonabant might represent a therapeutic tool in the treatment of solid tumours that, due to the absence of a functional p53, are resistant to chemotheraphy.

**The G-quadruplex ligand RHPS4 has antitumoral activity alone and in combination therapy with camptothecin *in vitro* and in xenografts.**

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We previously demonstrated that RHPS4, a pentacyclic acridine binding G4 DNA, when used a low concentration and for a long time, produced the arrest of cell growth and telomere erosion, thus indicating that this compound exerts its effect by inhibiting telomerase. More interstingly, the treatment of cells with RHPS4 at higher doses and for a shorter time, produced rapidly apoptosis and senescence in cells by damaging directly the telomere structure. Based on this background, the aim of this study has been to evaluate the potential use of this compound as potential antineoplastic agent alone or in combination therapy.
The antitumoral activity of RHPS4 given as single agent was evaluated on several human tumor lines xenografted in immunosuppressed mice. Moreover, toxicological analysis were performed by studying the effects of drug treatment on hematological parameters and on major organs. Finally, we studied the efficacy of RHPS4/antineoplastic drug combination both *in vitro* on culture cells and *in vivo* on xenografts.
Our results demonstrated that RHPS4 given alone was able to inhibit the growth of the different xenografts evaluated and exhibited a more favourable therapeutic index if compared with conventional chemotherapeutics. Moreover, RHPS4 showed activity in drug resistant human melanoma and reduced spontaneous metastases. It was well tolerated in mice as no toxic effects were observed, including no changes in red blood cells and platelet counts and no histological modifications were observed. The combination with campthotecin was strongly synergistic in reducing the survival of cells *in vitro*. Finally, this combination was highly effective *in vivo* in reducing the growth of tumors and in increasing the survival of mice.
These data indicate that RHPS4 has a good pharmacodynamic profile and in combination with campthotecin shows a good antitumoral activity, making this compound promising for clinical development.

**DNPQ-Pro induces differentiation, cell cycle arrest and apoptosis in human colon adenocarcinoma cell line.**

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Adenocarcinoma cells are remarkably resistant to injury by radiation and systemic, immunological, and chemotherapeutic agents. The mechanisms underlying the survival advantage may be in part related to the high endogenous expression of stress proteins, which include members of heat shock proteins family (HSP). These proteins are believed to bind and protect critical cellular proteins, preventing their denaturation by adverse factors or conditions. Although the expression of HSPs can be induced by a variety of stressful stimuli, certain neoplasms, including human intestinal T84, HT-29, and Caco2 adenocarcinoma cell lines, express constitutively high levels even under non- stress conditions. In particular Caco2 human colonic adenocarcinoma were significantly more sensitive to the injurious effects of oxidants and tumor necrosis factor (TNF) but were unresponsive to doxorubicin. The primary objective of the present study was to elucidate the underlying molecular mechanisms of the antiproliferative action of a quinone-based pentacyclic derivative (3*S*,3'*R*)spiro[(hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione)-6,3'-(2',3'-dihydrothieno[2,3-]naphtho-4',9'-dione (DTNQ-Pro), a new potential anticancer agent related to anthracyclines (daunorubicin and doxorubicin) and anthracenediones (mitoxantrone), identified in our laboratory.
Notably, this compound was 17-fold more active than doxorubicin on the SW620 human colon carcinoma cell line ($IC_{50}$ 10 *vs* 178nM). In addition no cross-resistance with this compound was observed in doxorubicin and cisplatin-resistant cell lines. However, even though this derivative had many of the characteristics similar to those of classical quinonic-based DNA intercalators, they were not able to inhibit topoisomerase II at equicytotoxic concentractions. This data seems indicated that other factors such as differences in cellular uptake, distribution within the cell, and/or an additional target within the cell might also affect the cytotoxicity of this derivative. In this study, we have determinated the biochemical events elicited by the DTNQ-Pro and further investigated the cytotoxic activity through mitochondrial superoxide anions modulation, the mechanism of cell cycle perturbation, and induction of apoptosis of this compound on Caco-2 human colon adenocarcinoma cell.

**Activating mutations of protein kinase B/AKT in epidermoid lung carcinoma**

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Somatic mutations of the AKT1 gene at a hot spot in exon 4 that results in the substitution at amino acid 17 (E17K) in the lipid-binding domain of AKT1 have been found in human cancer patients. The E17K mutation activates AKT1 by abnormal localization of the enzyme to the plasma membrane resulting in the stimulation of downstream signalling and cell transformation. The purpose of this study was to determine the AKT1 gene was mutated and activated in lung cancer. Here, we performed mutational analysis of the pleckstrin homology domain of AKT1 in lung cancer by direct sequencing of exons 1-4 in 102 NSCLC tissue samples (76 adenocarcinoma, 32 squamous cell carcinoma and 11 large cell carcinoma). We detected the missense mutations G->A mutation (E17K) in three squamous cell carcinoma but not in adenocarcinoma (9%). The activity of the endogenous kinase carrying the E17K mutation immunoprecipitated by tumour tissue was significantly higher compared with the wild-type kinase immunoprecipitated by adjacent normal tissue as determined both by *in vitro* kinase assay using a *consensus* peptide as substrate and by *in vivo* analysis of the phosphorylation status of AKT1 itself (pT308, pS473) or of known downstream substrates such as GSK3 (pS9/S22) and p27 (T198). Immunostaining analysis suggested that the increased activity exhibited by AKT1 carrying the E17K mutation was associated with preferential localization at the plasma membrane in comparison with the wild-type AKT1 in adjacent normal tissue. In conclusion, this is the first report of AKT1 mutation in lung cancer. Our data provide evidence that, although AKT1 mutations are apparently rare in lung cancer (3%), the oncogenic properties of AKT1 may contribute significantly to the development of lung carcinoma of the squamous histotype.