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Technical Unit of

Radiation Biology and Human Health (UTBIORAD)

ANNUAL REPORT 2010

TECHNICAL UNIT OF
RADIATION BIOLOGY AND HUMAN HEALTH

ANNUAL REPORT 2010

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TECHNICAL UNIT OF
RADIATION BIOLOGY AND HUMAN HEALTH

Introduction

Carmela Marino*, Technical Unit Director

The main goal of the Technical Unit "Radiation Biology and Human Health" is the development of methods and models to evaluate the effects and the mechanisms of ionizing and non-ionizing radiation, as well as of other environmental toxic agents; of producing biomolecules through genetic modification of plant and organelle genomes; of promoting ENEA's technological and experimental activities related to human health and medicine.

The Unit has more than sixty scientific and technical employees working on a vast spectrum of fundamental and applied biological topics related to human health. This staff provides a critical mass for carrying out interdisciplinary researches with a considerable scientific output.

The Unit has an outstanding yield in publications and it is granted at national and European level. The strong and close collaborations with Universities of Rome and Viterbo allow us to guest more than twenty students for degree and doctorate, and postdocs. The presence of young investigators is encouraged in the research program, although the financial budget is often not adequate to support for long periods the most worthy of them. In the framework of long standing collaborations with Academia, Institutions and Research Laboratories in Europe, our scientists are able to participate to European Commission funded projects. Collaborations with other Technical Units within ENEA are in progress. Essential for information exchanges and innovation among scientists, a program of seminars by internal staff and invited guest speakers has been performed. Successful conference activities by staff members have included keynote and plenary presentations at international and national conferences.

The Technical Unit is divided into three labs:

- the Laboratory of Radiation Biology and Biomedicine, whose activities are focused on classical and molecular radiation biology, including ionizing and non-ionizing radiation, and experimental biomedicine;
- the Laboratory of Toxicology is mainly devoted to environmental toxicology and epidemiology, conducting laboratory investigations, human biomonitoring and epidemiological studies;
- the Laboratory of Biotechnology exploits the potential use of plants for human and animal health, adopting molecular biology and "omic" sciences basically in the area of plant-derived vaccines, antibody engineering and pharming.

A detailed description of the research activities and the main results obtained in the course of 2010 are presented in this Annual Report.

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*Radiobiologist, involved in research activity on biological effects of ionizing and non-ionizing radiation
President of EBEA (European BioElectromagnetics Association)
Associate Editor of the journal: *Bioelectromagnetics*, Wiley and Blackwell
National delegate in COST BM0704 Action

LABORATORY OF RADIATION BIOLOGY AND BIOMEDICINE

Introduction

Anna Saran, Lab Director

The activities of the Laboratory of Radiation Biology and Biomedicine are broad and multidisciplinary, with competences in the fields of classical and molecular radiation biology, including ionizing and non-ionizing radiation, carcinogenesis, experimental oncology, molecular immunology, cytogenetics, mechanisms of chronic inflammatory disorders, and a recently integrated line of research investigating the efficacy of natural compounds for human and animal health.

Studies on human health risks from noxious physical agents, like radiation, are required to set the limits on all potential exposures for environmental, occupational or therapeutic reasons. In this context, radiobiological studies providing quantitative and mechanistic information on the biology of low dose ionizing radiation responses are needed for accurate risk estimates in the low dose range, where considerable uncertainties persist due to insufficient statistical power of epidemiological studies. In vivo studies with animal models are required for improved understanding of such risks. A main aim of the laboratory is the development of suitable radiosensitive - phenotypically selected or genetically manipulated - mouse models, with focus on the mechanisms and genetics of radiation-induced cancer. Both “targeted” and “non-targeted” effects of radiation are analyzed using cellular and animal systems. The risk from exposure to low doses cosmic radiation, relevant for future manned space missions, is also an active area of investigation. Finally, work preliminary to the Regione Lazio-financed TOP IMPLART Project (Intensity Modulated Proton Linear Accelerator for RadioTherapy) is now in progress.

Linked to radiation work, research is carried out to identify genetic markers of predisposition to spontaneous and radiation-induced cancer. The genetic bases of normal cell/tissue radiosensitivity are investigated through the analysis of polymorphisms in genes involved in the processing of DNA damage in peripheral blood lymphocytes from cancer patients and healthy individuals. Ongoing work also explores the protective role of antioxidant treatment in cellular systems, with a view to developing new strategies to prevent oxidative damage to irradiated cells/tissues.

The laboratory is also actively involved in the study of thermal and non-thermal effects of electromagnetic fields using cellular and animal models. This area of study includes the design and implementation of experimental models and exposure systems, and the development of applications of electromagnetic fields for therapy and diagnostics. Analysis and evaluation of standards for the protection of workers and general public is also carried out. Laboratory members participate in EU expert networks to inform about the possible health risks of exposure to electromagnetic sources in the working, domestic or general environment.

In vitro and in vivo work in the laboratory investigates the effects of exposure to ionizing and RF/microwave radiation on the functions of the immune system, which plays a crucial role in protection from pathogens and homeostatic maintenance of the organism. This is accomplished through a complex network of cellular interactions, soluble factors and mechanisms of control. Toxic compounds and radiation, as well as

some pathogens, can perturb these regulatory mechanisms and favor the onset of pathologies. Alterations in immune functions may result in ineffective responses against pathogens, reduction in tumor surveillance, and development of immune-mediated diseases.

A central line of investigation in the laboratory is the study of tumor biology, and the mechanisms and genetics of cancer in cellular and animal models. These are helpful to test the complex biological processes driving tumor growth. Childhood nervous system tumors, such as neuroblastoma and medulloblastoma, and the mechanisms underlying their development, are actively investigated. Because the main cause of cancer deaths is metastasis, the formation of secondary tumors in organs distant from the site of the original cancer, the laboratory develops experimental models for understanding the process of metastasis at the molecular level, and the regulators of the genes activated or repressed during the epithelial-mesenchymal transition occurring at the onset of metastatic invasion. The laboratory is also focusing on the role of polyamines in cancer cell proliferation and ways to exploit their metabolism and function as antiproliferative and chemopreventive targets.

Non-cancer diseases, in particular chronic inflammatory diseases, also represent a significant portion of human morbidity. Among these, inflammatory bowel diseases are relatively common chronic disorders resulting from deregulated activation of intestinal mucosa immune system due to genetic and environmental factors. The molecular bases of chronic inflammatory disorders are explored by genomic, gene expression, and epidemiological approaches.

A newly integrated area of research in the laboratory further explores the role of the ENEA-patented remedy of natural extracts in oil from plants (MIX577), which has the capacity to properly regulate most of the complex events of wound healing processes. This product holds great promise in the treatment of wounds and very extensive burns, and important applications are foreseen in human and veterinary medicine.

Protective role of 17β-estradiol on medulloblastoma development in *Patched1* heterozygous mice

Mariateresa Mancuso, Simona Leonardi, Manuela Ceccarelli*, Emanuela Pasquali*, Ilaria De Stefano^, Simonetta Rebessi, Mirella Tanori, Vincenzo Di Majo, Simonetta Pazzaglia, Anna Saran
*Graduate student, ^Pre-doc student

Medulloblastoma (MB) is the most common pediatric tumor of the CNS, representing approximately 20% of all childhood CNS tumors. Although in recent years many molecular mechanisms that control MB development have been clarified, the effects of biological factors such as gender on this tumor remain to be explained. Epidemiological data, in fact, indicate a significant difference in the incidence of MB between the two sexes, with considerably higher susceptibility of males than females. Besides this different susceptibility, female gender is also a significant favorable prognostic factor in MB, with girls having a much better outcome. Despite these literature data, there has been little investigation into estrogen influence on MB development. In this study, we evaluated how hormone deficiency resulting from ovariectomy, as well as hormone replacement, influence the development of early and advanced MB stages in *Patched1* heterozygous mice, a well characterized mouse model of radiation-induced MB. Susceptibility to MB development was significantly increased in ovariectomized *Ptch1*$^{+/-}$ females, and restored to levels observed in control mice after estrogen replacement (Fig. 1). We next investigated the molecular mechanisms by which estrogen might influence tumor progression, and show that ERβ, but not ERα, is involved in modulation of MB development by estrogens (Fig. 2). Finally, this study shows that a functional interaction between estrogen- and IGF-I-mediated pathways may be responsible for the effects observed (Fig. 3).

This activity has been carried out in collaboration with Maria Grazia Prisco, Giovanni Scambia and Daniela Gallo (Catholic University of the Sacred Heart, Rome).

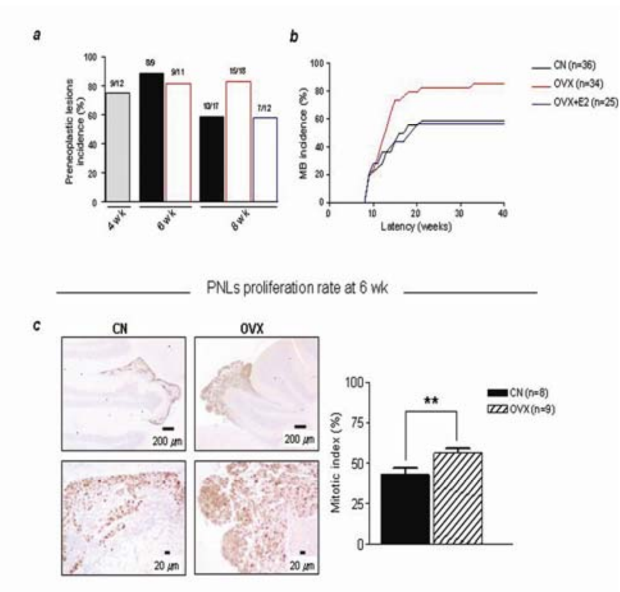
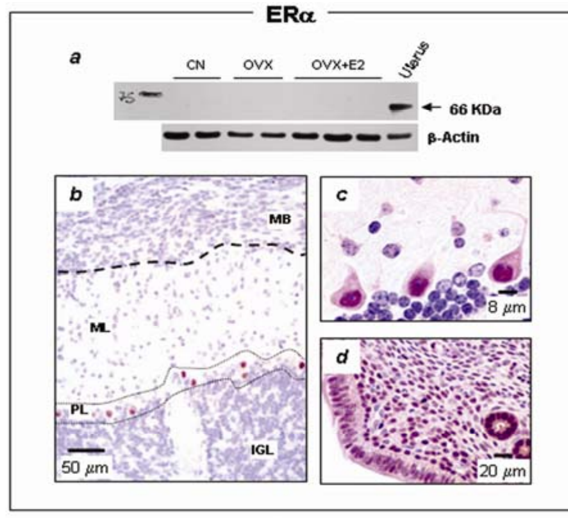


Fig. 1 - Effect of estrogen modulation on MB development. (*a*) Incidence of preneoplastic lesions from *Ptc1*$^{+/-}$ mice at different age. ■ 4 wk of age (the time of ovariectomy); ■ intact mice (CN) at 6 or 8 wk of age; □ OVX mice at 6 or 8 wk of age; □ OVX mice after estrogen replacement (OVX+E2) at 8 wk of age. (*b*) Estrogen deprivation caused a significant enhancement in MB development compared with CN group (85% vs 58%; *P* = 0.0173). Estrogen replacement restored tumor incidence to a value comparable to that of CN mice (56%; *P* = 0.0181 *vs* OVX). (*c*) Images of PNLs from CN and OVX groups showing analysis with anti-Ki67 at 6 wk of age and rate of cell proliferation.



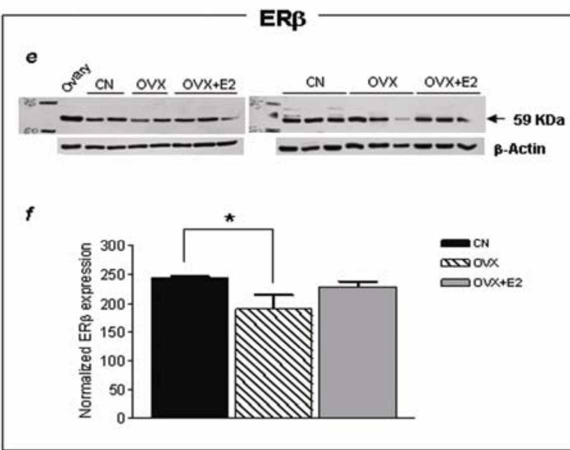


Fig. 2 - ERα and ERβ expression in MBs from the different experimental groups. (*a*) Representative immunoblot analysis of ERα in tumors from CN (n=5), OVX (n=5) and OVX+E2 (n=6) mice showing undetectable protein expression. Protein extract from uterus was used as positive control for ERα. (*b*,*c*) Representative image of immunostaining with anti-ERα antibody. No detectable ERα expression was observed in MBs from CN, OVX and OVX+E2 mice. All brains examined, regardless of the experimental group, showed positivity only in Purkinje cells. (*d*) Uterus immunostaining was performed as positive control.
(*e*) Immunoblot analysis of ERβ in tumors from CN (n=5), OVX (n=5) and OVX+E2 (n=6) mice; protein extract from ovary was used as positive control for ERβ. (*f*) Densitometric analysis of normalized ERβ protein levels; columns represent the mean ± SE for each group. Difference was statistically significant between tumors from CN and OVX groups (P=0.05). After estrogen replacement, ERβ expression was similar to that observed in CN group.

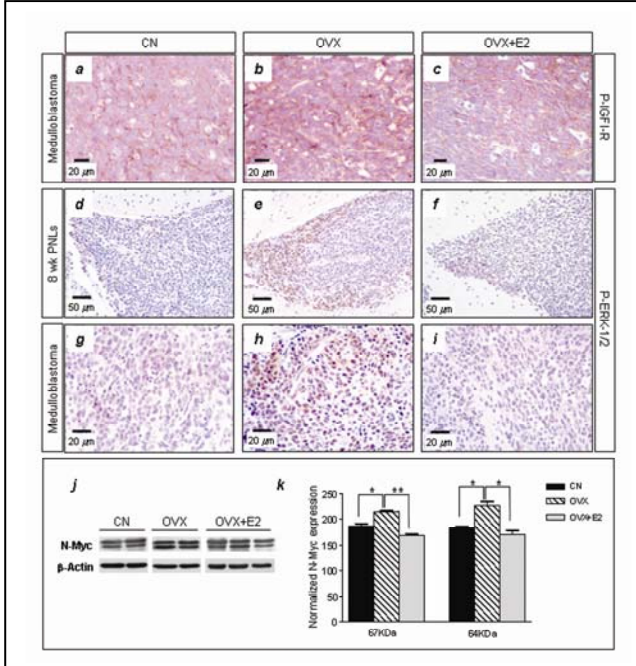


Fig. 3 - Effects of estrogen modulation on IGF-I signaling. (*a-c*) Representative images of MBs and (*d-f*) of PNLs at 8 wk of age from all experimental groups, showing analysis with anti-P-ERK-1/2. (*j*,*k*) Densitometric analysis of normalized N-Myc expression in tumors from CN, OVX and OVX+E2. Data are reported as mean ± SE.
**P*=0.0138 OVX *vs* CN, 67 kDa isoform; ***P* = 0.0033 OVX *vs* OVX + E2, 67 kDa isoform; **P* = 0.0359 OVX *vs* CN, 64 kDa isoform. **P* = 0.0235 OVX *vs* OVX + E2, 64 kDa isoform. PNLs = preneoplastic lesions.

Developmental and oncogenic effects of insulin-like growth factor-I in Ptc1$^{+/-}$ mouse cerebellum

Mirella Tanori, Melissa Santone*, Mariateresa Mancuso, Emanuela Pasquali, Simona Leonardi, Vincenzo Di Majo, Simonetta Rebessi, Anna Saran, Simonetta Pazzaglia
*Graduate student

Medulloblastoma (MB) arises from neoplastic transformation of granule neuron precursors (GNPs) of the cerebellum via deregulation of pathways involved in cerebellar development. Deregulation of the Sonic hedgehog/Patched1 (Shh/Ptc1) signaling pathway predisposes humans and mice to medulloblastoma. In the brain, insulin-like growth factor (IGF-I) plays a critical role during development as a neurotrophic and neuroprotective factor, and in tumorigenesis, as IGF-I receptor is often activated in MB. To investigate the mechanisms of genetic interactions between Shh and IGF signaling in the cerebellum, we crossed nestin/IGF-I transgenic (IGF-I Tg) mice, in which transgene expression occurs in neuron precursors, with Ptc1$^{+/-}$ knockout mice, a model of medulloblastoma in which cancer develops in a multistage process. The IGF-I transgene produced a marked brain overgrowth, and significantly accelerated tumor development, increasing the frequency of pre-neoplastic lesions as well as full MB in Ptc1$^{+/-}$/IGF-I Tg mice (Fig. 4). Mechanistically, tumor promotion by IGF-I mainly affected preneoplastic stages through de novo formation of lesions, while not influencing progression rate to full tumors. We also identified a marked increase in survival and proliferation, and a strong suppression of differentiation in neural precursors (Figs. 5 and 6) .As a whole, our findings indicate that IGF-I overexpression in neural precursors leads to brain overgrowth and fosters external granular layer (EGL) proliferative lesions through a mechanism favoring proliferation over terminal differentiation, acting as a landscape for tumor growth. Understanding the molecular events responsible for cerebellum development and their alterations in tumorigenesis is critical for the identification of potential therapeutic targets.

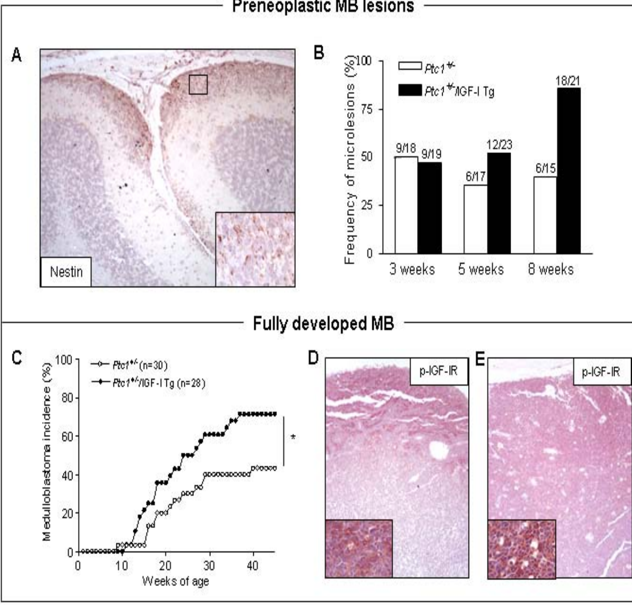


Fig. 4 - Acceleration of tumor development in the cerebellum of ***Ptc1$^{+/-}$*** **mice.** (A) Representative immunohistochemical analysis of nestin in preneoplastic lesions (PNLs) detected in *Ptc1$^{+/-}$* cerebellum at 3 ws of age. (B) Frequency of PNLs in cerebellum of *Ptc1$^{+/-}$* and *Ptc1$^{+/-}$*/IGF-I Tg mice of 3, 5 and 8 ws of age. (C) The IGF-I transgene caused a significant enhancement in MB development (71% in *Ptc1$^{+/-}$*/IGF-I Tg mice *vs*. 43% in *Ptc1$^{+/-}$* mice; $P < 0.05$).
(D and E) immunostaining for P-IGF-IR in MB from *Ptc1$^{+/-}$* mice and *Ptc1$^{+/-}$*/IGF-I Tg mice.

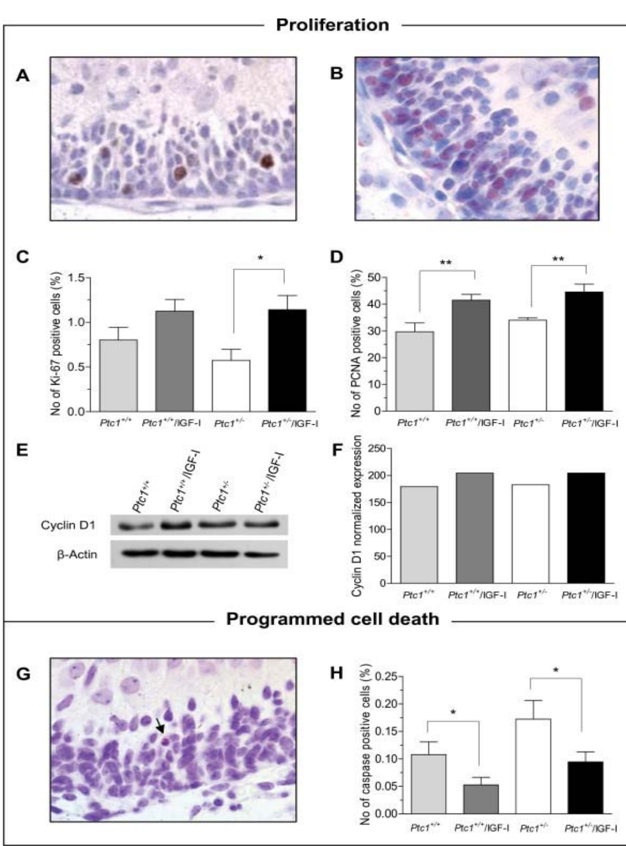


Fig. 5 - Analysis of proliferation, programmed cell death and IGF-I signaling in P5 cerebellum of *Ptc1* $^{+/+}$, *Ptc1*$^{+/-}$, *Ptc1*$^{+/+}$**/IGF-I Tg and** *Ptc1*$^{+/-}$**/IGF-I Tg mice**. (A) Representative image of Ki-67 and (B) PCNA immunostaining. (C) Graphic representation of frequency of Ki-67, and (D) PCNA positive cells in the EGL. (E) Immunoblot analysis of expression of cyclin D1 with relative β-actin to control protein loading, and (F) relative graphic representation of densitometric analysis. (G) Representative image of caspase-3 positive cells in the EGL. (H) Graphic representation showing the frequency of caspase-3 positive cells in the EGL.

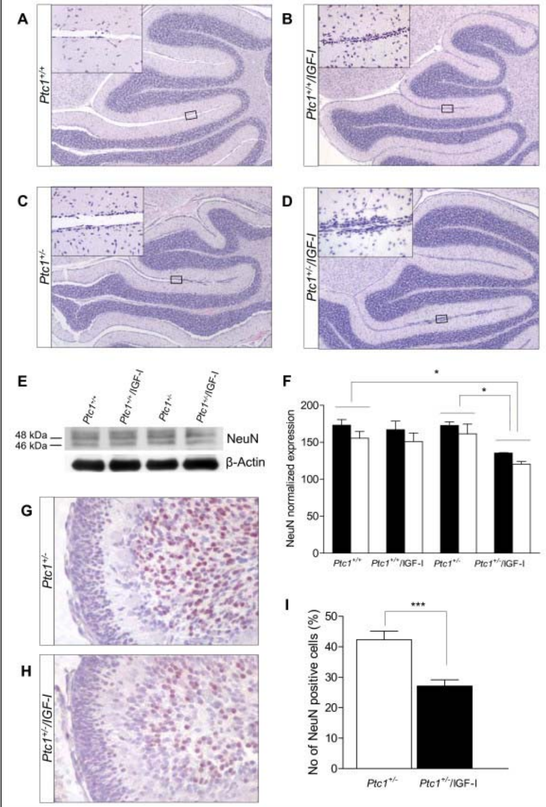


Fig. 6 - Delayed differentiation in neural precursors caused by IGF-I altered expression. (A) H&E-stained sagittal sections of mouse cerebellum at P15, showing physiological absence of EGL in the cerebellum of *Ptc1*$^{+/+}$ mice. A thin 1-cell layer of EGL was present in the cerebellum of *Ptc1*$^{+/+}$/IGF-I Tg (B), and *Ptc1*$^{+/-}$ mice (C). (D) A thicker 2-3-cells layer was observed in the EGL of *Ptc1*$^{+/-}$/IGF-I Tg mice. (E) Western blot analysis showing the level of NeuN (48 and 46 kDa, solid and open square, respectively) expression in cerebellum from *Ptc1*$^{+/+}$, *Ptc1*$^{+/+}$/IGF-I Tg, *Ptc1*$^{+/-}$, and *Ptc1*$^{+/-}$/IGF-I Tg mice at P5, with relative β-actin to control protein loading. (F) Graphic representation of densitometric analysis. (G and H) Immunohistochemical analysis showing a decrease in the expression of NeuN in the IGL of the cerebellum of *Ptc1*$^{+/-}$/IGF-I Tg mice (H) compared to *Ptc1*$^{+/-}$ mice (G). (I) Frequency of NeuN positive neurons in the IGL of *Ptc1*$^{+/-}$ and *Ptc1*$^{+/-}$/IGF-I Tg mice.

Genetic heterogeneity of inflammatory response and skin tumorigenesis in phenotypically selected mouse lines

Simonetta Pazzaglia, Mariateresa Mancuso, Anna Saran

Non-inbred AIR (AIRmax, AIRmin) and Car (Car-S, Car-R) mouse lines were generated from the same eight inbred mice through bidirectional selective breeding for acute inflammatory response and for susceptibility to two-stage skin tumorigenesis, respectively. Because AIR lines also showed a differential predisposition to skin tumorigenesis and Car lines differed in the extent of inflammatory response (Fig. 7), we carried out genome-wide association studies using SNP arrays to identify the genetic elements affecting skin tumor susceptibility and inflammatory response in AIR and Car lines. We found that the phenotypic outcome reflects a specific genetic profile in each mouse line, suggesting that distinct genetic elements, selected by differential genetic drifts, and exerting pleiotropic effects in each mouse population, control the skin tumor susceptibility and inflammatory response phenotypes (Fig. 8). These findings point to the complex link between skin tumor susceptibility and inflammatory response in mice. This activity has been carried out in collaboration with Wafa Hanna Koury Cabrera (Universidad Nacional de Tucumán, Argentina); Orlando Garcia Ribeiro, Olga M. Ibañez and Francisca Vorraro (Instituto Butantan, Brasil); Antonella Galvan and Tommaso A. Dragani (Istituto Nazionale Tumori, Milan); Anna Zolin and Silvano Milani (Università di Milano).

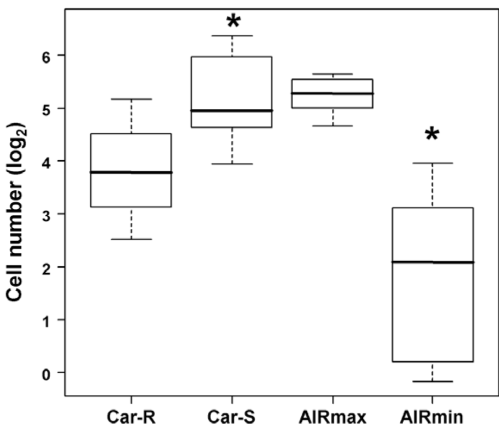


Fig. 7 - Differences in acute inflammatory response at 48 h after s.c. injection of Biogel 100 in AIRmax and AIRmin mice (P = $3.1e^{-06}$) or Car-R and Car-S mice (P = $3.1e^{-05}$). The line within each box represents the median number of 106 cells in the exudates (log_2 values); upper and lower edges of each box represent the 75th and 25th percentile, respectively; upper and lower bars indicate the highest and lowest values determined, respectively. Asterisks mean statistically significant differences between the mouse lines.

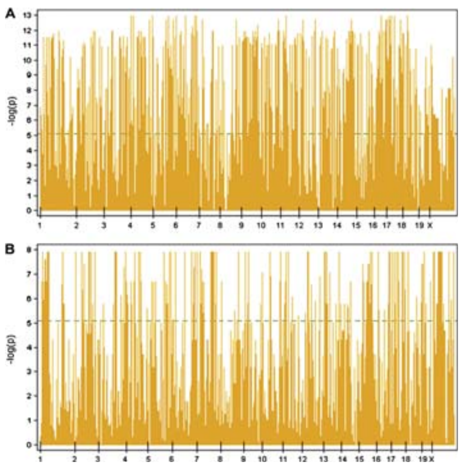


Fig. 8 - Genome-wide scans in (A) AIRmax and AIRmin or (B) Car-R and Car-S mouse lines, showing the differential segregation of informative SNPs against the marker map plot. Threshold (in dotted green line) P-value (a = 0.01) was calculated according to Bonferroni's criterion.

Microsatellite instability in radiation-induced murine tumours: influence of tumour type and radiation quality

Mariateresa Mancuso, Simonetta Pazzaglia

To investigate microsatellite instability (MSI) in radiation-induced murine tumours, its dependence on tissue (haemopoietic, intestinal, mammary, brain and skin) and radiation type, DNA from spontaneous, X-ray or neutron-induced mouse tumours were used in Polymerase Chain Reactions (PCR) with mono- or di-nucleotide repeat markers. Deviations from expected allele size caused by insertion/deletion events were assessed by capillary electrophoresis.

Tumours showing MSI increased from 16% in spontaneously arising tumours to 23% (P = 0.014) in X-ray-induced tumours and rising again to 83% (P << 0.001) in neutron-induced tumours. X-ray-induced Acute Myeloid Leukaemias (AML) had a higher level of mono-nucleotide instability (45%) than di-nucleotide instability (37%) (Fig. 9). Fifty percent of neutron-induced tumours were classified as MSI-high for mono-nucleotide markers and 10% for di-nucleotide markers. Distribution of MSI varied in the different tumour types and did not appear random.

In conclusion, exposure to ionising radiation, especially neutrons, promotes the development of MSI in mouse tumours. MSI may therefore play a role in mouse radiation tumourigenesis, particularly following high Linear Energy Transfer (LET) exposures. MSI events, for a comparable panel of genome-wide markers in different tissue types, were not randomly distributed throughout the genome. This activity has been carried out in collaboration with Jackie Haines, Margaret Coster, and Simon Bouffle (Health Protection Agency UK); Rene Huiskamp and Emmy Meijne (Nuclear Research and consultancy Group, NL); Jeff Bacher (Promega Corporation, US).

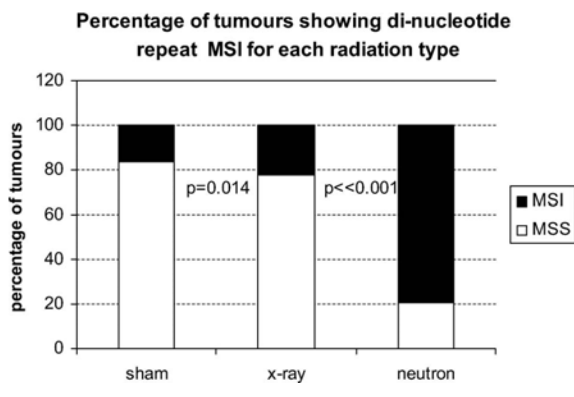


Fig. 9 - Summary of di-nucleotide repeat MSI in sham, X-ray and neutron-induced tumours showing the percentage of microsatellites stable (MSS) tumours and the percentage showing MSI. The statistical significance of the mutation frequency is also shown (difference between X-ray-induced tumours and associated sham controls P=0.014; difference between sham corrected x-ray and neutron-induced tumours P<0.001).

Basal cell carcinoma and the carcinogenic role of aberrant Hedgehog signaling

Anna Saran

Basal cell carcinoma (BCC) is the most frequent cancer in the white population and its incidence appears to be increasing worldwide. While the majority of BCCs arise sporadically, many cases are attributable to basal cell nevus syndrome, or Gorlin

syndrome, an autosomal dominantly inherited disorder characterized by the occurrence of multiple BCCs and by extracutaneous tumors. Genetic studies on patients with basal cell nevus syndrome indicate deregulation of the Hedgehog (Hh) pathway (Fig. 10) in epidermal keratinocytes as the primary event in the pathogenesis of BCC. This review summarizes the recent progress in understanding Hh-dependent BCC tumorigenesis, as well as evidence for deregulation of other molecular pathways, primarily the Wnt developmental pathway. Understanding the molecular genetics of BCC development has provided new opportunities for molecular therapy of this cancer by targeting Hh and other signaling pathways.

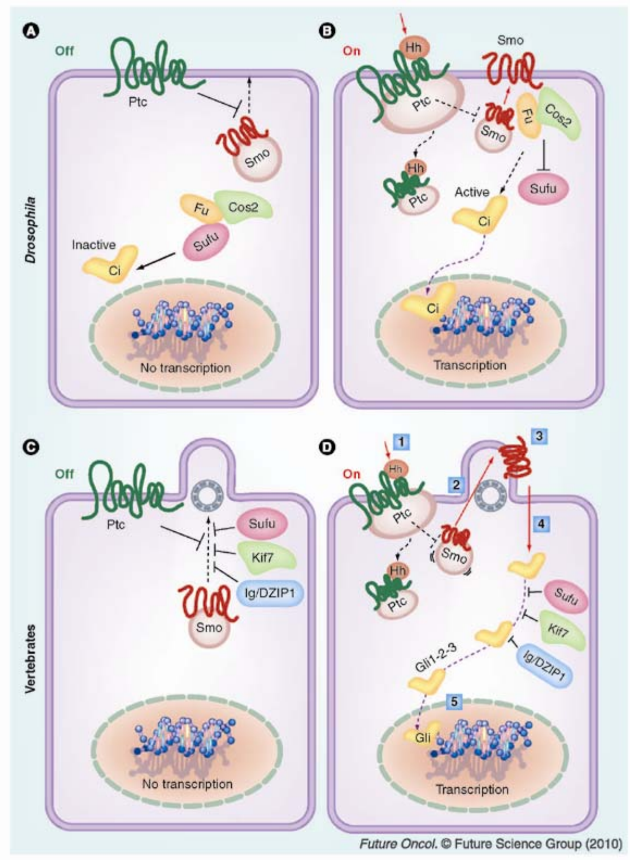


Fig. 10 - Hedgehog pathway. (A & B) *Drosophila* and (C & D) vertebrates. Ci: Cubitus interruptus; Cos2: Costal 2; Fu: Fused; Hh: Hedgehog; Ptc: Patched; Smo: Smoothened; Sufu: Suppressor of fused.

SCIENTIFIC COLLABORATIONS

- Dr Michael J. Atkinson (Helmholtz Center Munich)
- Dr Simon Bouffler (Health Protection Agency, Chilton, UK)
- Dr Tommaso A. Dragani (Istituto Nazionale Tumori, Milan)
- Dr. Daniela Gallo (Università Cattolica del Sacro Cuore, Rome)
- Dr Alberto Gulino (Università La Sapienza, Rome)
- Dr Heidi Hahn (University of Göettingen)
- Dr Roland Kanaar (Erasmus Medical Center, Rotterdam)
- Dr Leon Mullenders (Leiden University Medical Center)
- Dr Kevin Prise (Queen's University Belfast, UK)
- Dr Laure Sabatier (Commissariat à l'Energie Atomique, FR)
- Dr Paolo Salomoni (UCL Cancer Institute, London, UK)

- Dr Giovanni Scambia (Università Cattolica del Sacro Cuore, Rome)
- Dr Guillermo Taccioli (Boston University School of Medicine)
- Prof. Giovanni Briganti (Università degli Studi Guglielmo Marconi, Roma)

GRANTS

FIRB Italia-Israele RBINO4P4ET, 01/01/2006-25/07/2010. Principal investigator: Simonetta Pazzaglia.

Contract with SienaBiotech S.p.A for execution of the experiment: SEN0070826: Effect in tumor bearing (s.c. PTC+/- medulloblastoma xenograft) female nude mice after repente oral administration. Duration October 2009-March 2010. Principal investigator: Mariateresa Mancuso.

Contract with SienaBiotech S.p.A for execution of the experiment: "Effect of a hedgehog pathway inhibitor (Smo antagonist) on medulloblastoma growth in nude mice". Duration June-October 2010. Principal investigator: Mariateresa Mancuso.

Prenatal exposure to non-ionizing radiation: effects of WiFi signals on pregnancy outcome, peripheral B-cell compartment and antibody production

Manolo Sambucci*, Federica Laudisi*, Francesca Nasta**, Rosanna Pinto, Rossella Lodato**, Pierluigi Altavista, Giorgio A. Lovisolo, Carmela Marino and Claudio Pioli
*PhD student **Post-doc

During embryogenesis, the development of tissues, organs and systems, including the immune system, is particularly susceptible to the effects of noxious agents. We examined the effects of prenatal (*in utero*) exposure to WiFi signals on pregnancy outcome and the immune B-cell compartment, including antibody production. Sixteen mated (plug-positive) female mice were assigned to each of the following groups: cage control, sham-exposed and microwave-exposed (WiFi signals at 2.45 GHz, whole body, SAR 4 W/kg, 2 h/day, 14 consecutive days starting 5 days after mating).

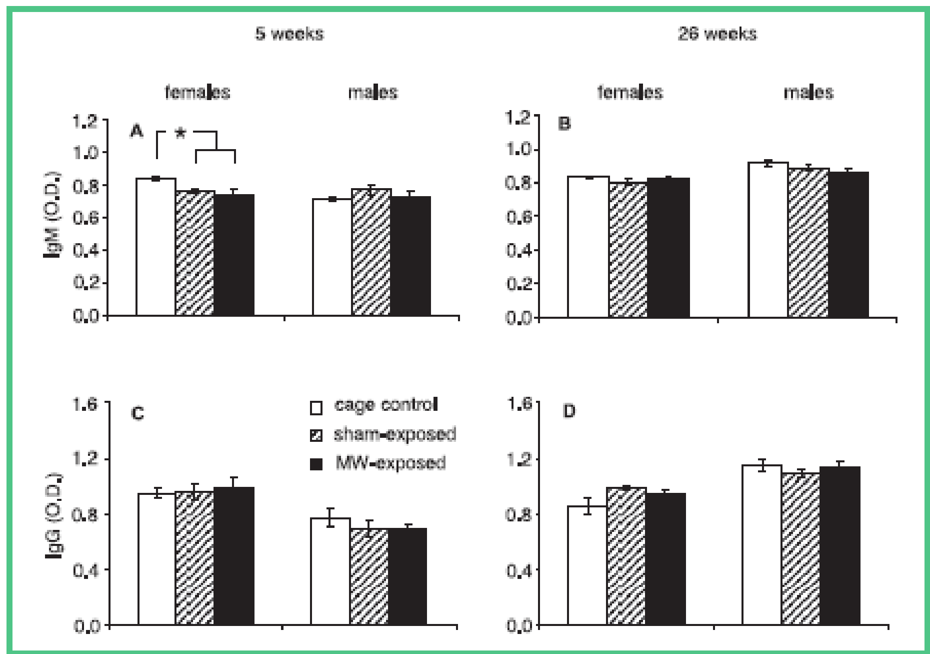


Fig.1 - Early and late effects of prenatal exposure to WiFi signals on antibody serum levels. Serum samples from 5- (left panels) and 26 (right panels)-week-old male and female mice, born to cage control (white bars), sham-exposed (hatched bars) and microwave (MW)-exposed (black bars) pregnant mice were analyzed for the presence of IgM (upper panels) and IgG (lower panels). ELISAs were performed on serially diluted samples. Values falling in the linear part of the OD vs. dilution sigmoid curve were used for comparisons. Results are OD values (means ± SEM) obtained after diluting sera 1:200 and 1:10,000 for IgM and IgG assays, respectively. *P<0.05 for sham-exposed compared to cage control and microwave-exposed compared to cage control.

No effects due to exposure to WiFi signals during pregnancy on mating success, number of newborns/mother and body weight at birth were found. Newborn mice were

left to grow until 5 or 26 weeks of age, when immunological analyses were performed. No differences due to exposure were found in spleen cell number, B-cell frequency or antibody serum levels (Fig. 1).
When challenged *in vitro* with LPS, B cells from all groups produced comparable amounts of IgM and IgG, and proliferated at a similar level. All these findings were consistently observed in the female and male offspring at both juvenile (5 weeks) and adult (26 weeks) ages. Stress-associated effects as well as age- and/or gender-related differences were observed for several parameters, confirming the sensitivity of the analyzed parameters. In conclusion, our results do not show any effect on pregnancy outcome or any early or late effects on B cell differentiation and function due to prenatal exposure to WiFi signals.

An international project to confirm Soviet-era results on immunological and teratological effects of RF field exposure

Michael Repacholi*, Jochen Buschmann*, Claudio Pioli, and Roza Sypniewska*
(International Oversight Committee members for the Franco-Russian Project)
*Other Institutions' Collaborators

Results of key Soviet-era studies, dealing with effects on the immune system and teratological consequences in rats exposed to radiofrequency (RF) fields of around 2375 MHz (0.1–10 W/m^2) served, in part, as a basis for setting exposure limits in the USSR and the current RF standards in Russia. None of these Soviet studies were published in major international journals. However, their results raised sufficient concerns that needed to be verified by more recent modern laboratory methods. While admitting that the results of these studies are not entirely consistent, the Russian group suggested they indicated that semi-chronic exposure to microwaves at 5 W/m^2 evoked a pronounced autoimmune response compared with sham-exposed animals. Teratological effects were also noted in offspring of pregnant rats injected with blood serum from the exposed or sham-exposed animals. The World Health Organization's (WHO) International EMF Project considered important to confirm the results of the earlier Soviet studies [WHO Research Agenda, 2006], especially because they formed a basis for the current Russian RF standard. Industry sponsors (Mobile Manufacturers Forum and the GSM Association) agreed to fund this study provided WHO managed the ‘‘firewall committee’’ and that the final results were provided to WHO as a contribution to their RF health risk assessment process. WHO nominated the members of the International Oversight Committee (IOC) to provide advice and oversight for the studies. It was agreed the IOC was also acting as a firewall committee that dealt with the sponsors and researchers. It was agreed that the Soviet-era studies had to be replicated in the laboratory of Yuri Grigoriev in Moscow and of Bernard Veyret in Bordeaux. At the end of the study, each group published its results reaching different conclusions. The IOC published a commentary paper [Repacholi et al, 2010] attempting to explain the contrasting conclusions. When viewed as a whole, the results of French [Poulletier de Gannes et al, Radiat Res 172: 617-624, 2009] and Russian [Grigoriev et al, Bioelectromagnetics 31: 588–601, 2010] groups did not provide support for the original Soviet study results. Although Grigoriev et al. reported that they did confirm some of the immunological and teratological findings of the Soviet studies, following a very detailed analysis of both studies, the IOC concluded that this was not convincing. According to the current WHO RF research agenda, there should be more studies

conducted that relate to RF effects in children. Given the possible importance of RF-induced autoimmune responses in children and their mothers, as raised in the hypothesis of the original Soviet studies, it is recommended that further studies be conducted to properly test this hypothesis using well-established modern methods.

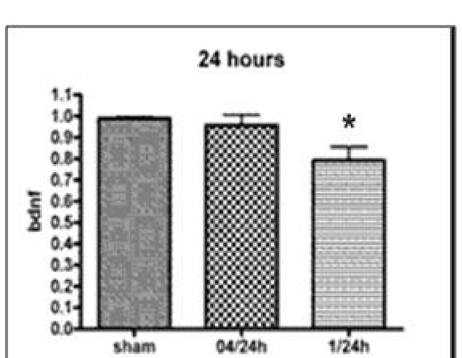
In vitro effects of radiofrequency electromagnetic fields on human fibroblasts primary cell cultures

Paolo Galloni, Rossella Lodato, Rosanna Pinto, Carmela Marino

The central nervous system (CNS) is among the biological targets of cell phones emission; fibroblasts represent a fine peripheral cell type for the study of RF fields on the CNS, due to their analogy with neuronal cells in many molecular and biochemical aspects. The aim of this study was the evaluation of possible effects of exposure to 900 MHz GSM RF fields on gene expression in human primary fibroblasts. A wire patch cell (WPC) operating in the frequency band of GSM 900 MHz was utilized as exposure system (Laval *et al,* Bioelectromagnetics 21:255–263, 2000). Eight different fibroblast cell lines, coming from 8 healthy volunteers yet available in the BioBank of I.R.C.C.S. Institute, Brescia, Italy, were used. Cells were exposed to 0.4 W/kg, 1 W/kg or sham exposed for 24 or 72 hours. After the exposure, samples were frozen and stored up to the gene expression analysis for the following genes: B cell lymphoma gene-2, BCL2; Bcl-2-associated X protein, BAX; P38 mitogen-activated protein kinases, MAPK-P38, Brain-derived neurotrophic factor, BDNF; cAMP response element binding 1, CREB1 and Extracellular Regulated Kinase 1, ERK1.

Effect of 24h exposure: a down regulation in the expression levels of p38 Map kinase ($F=8.536$, $p<0.05$) and BDNF, ($F=5.288$, $p<0.05$) was observed after 24h of exposure. In particular a Newman-Keuls Multiple Comparison Test indicated a significant reduction in the levels of p38 Map kinase with the dose of 0.4 W/kg ($p<0.05$), but not at the dose of 1 W/kg. Conversely, the 24h exposure caused a reduction in BDNF levels only with 1 W/kg dose ($p<0.05$) (Fig. 2a). No effect was observed on the expression levels of CREB, ERK, BAX and Bcl-2.

a



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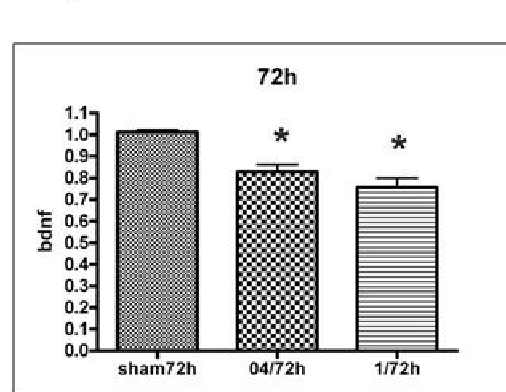

Fig 2 – a) effects of 24-hours exposure to GSM signals on BDNF expression in fibroblast; data are presented as mean ± SEM of relative expression level. Asterisks indicate p>0.05. 04 = 0.4 W/kg, 1 = 1 W/kg; **b) effects of 72-hours exposure to GSM signals on BDNF expression**

in fibroblast; data are presented as mean ± SEM of relative expression level. Asterisks indicate $p>0.05$. 04 = 0.4 W/kg, 1 = 1 W/kg.

Effect of 72h of exposure: an effect only on the levels of BCL-2 ($F=3.710$, $p<0.05$), and BDNF ($F=16.99$, $p<0.05$) was pointed out. However, the Newman-Keuls Multiple Comparison Test indicated that only the 0.4 W/kg dose significantly reduced the expression levels of BCL-2 as compared to sham condition ($p<0.05$), whereas both doses reduced the expression levels of BDNF (Fig. 2b). The study has been performed in collaboration with Anna Maria Cattaneo of the Genetics Lab, IRCCS, Brescia.

In vitro effects of radiofrequency electromagnetic fields on rat pheochromocytoma cells

Paolo Galloni, Barbara Benassi, Elisabetta Bennici, Rossella Lodato, Rosanna Pinto, Claudio Pioli, Carmela Marino

This study was carried out in the framework of the Italian National Project (PRIN) “Theoretical-experimental studies on neuronal cells exposed to low and high-frequency fields”, investigating possible effects caused by EMF exposure of different frequencies on various biological endpoints. In our investigation, neuronal-like cell cultures were exposed for 24 hours to WIFi fields in order to explore possible effects on proliferation and induction of apoptosis in vitro.
A wire patch cell (WPC) operating in the frequency band of WiFi (2450 MHz) was utilized as exposure system. Cultures of PC12 cells (rat pheocromocytoma), around the 10th culture passage, from CNR-IREA labs in Naples (Dr Scarfi) were utilized. Three doses were scheduled: 0.0 W/kg (sham), 0.4 W/kg and 4.0 W/kg of SAR, exposed for 24 hours at the frequency of 2450 MHz (WiFi); in some cases an additional group maintained in another incubator was included (control).
Five different replications of the procedures were performed. At the end of exposure, cells were immediately harvested for the evaluation of cell-cycle status, proliferation, viability and flow cytometric tests; an aliquot was frozen and stored until processing for Real-time PCR.
Cellular density in the cultures was measured counting cells in a Burker chamber immediately after the 24 hours of exposure; no differences in the proliferation index was reported comparing controls, sham, 0.4 W/kg and 4 W/kg groups.
Cells viability was evaluated by incorporation of Trypan blue dye observed under optical microscopy during the cell count; dead cells percentage was around 5% in all the groups, again failing to evidence any effect of exposure to the electromagnetic field.
Cell-cycle status was evaluated by flow cytometric analysis to establish the percentage of cells in the different phases sub G1, G1, S, G2-M; the majority of cells was in the G1phase in all groups, showing no effects of exposure to WiFi (Fig. 3).
Apoptotic status of cells was evaluated by Annexin V / Propidium Iodie staining, and flow cytometry observation after incorporation of the dyes. No differences in the percentage of alive, early-apoptotic and late-apoptotic or necrotic cells was reported in dependence of the exposure conditions.
In Fig. 4 the relative expression levels of the pro-apoptotic Bax and anti-apoptotic Bcl-2 genes are shown, in comparison to the housekeeping gene β-actine, evaluated by real-

time PCR in the cells following 24 hours of exposure. Statistical analyses (ANOVA) did not reveal any significant difference.

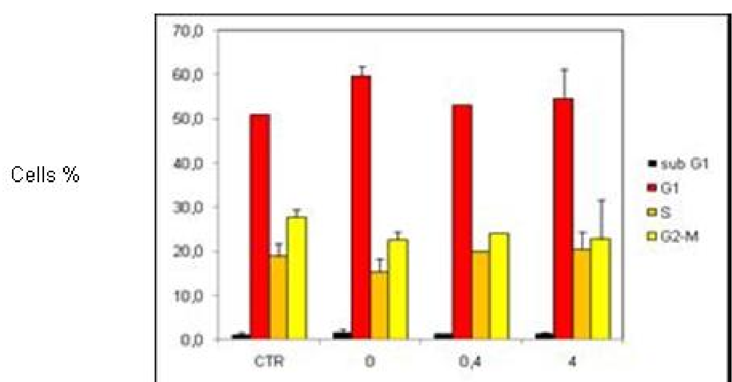

Fig. 3 - Effects of exposure to WiFi signals on cell cycle progression as assessed by flow cytometry. Values represent mean percentages ± standards deviation (five replicates) of cells in different phases of the cycle (Ctr, control; 0, sham; 0.4 and 4, exposed to 0.4.and 4 W/kg).

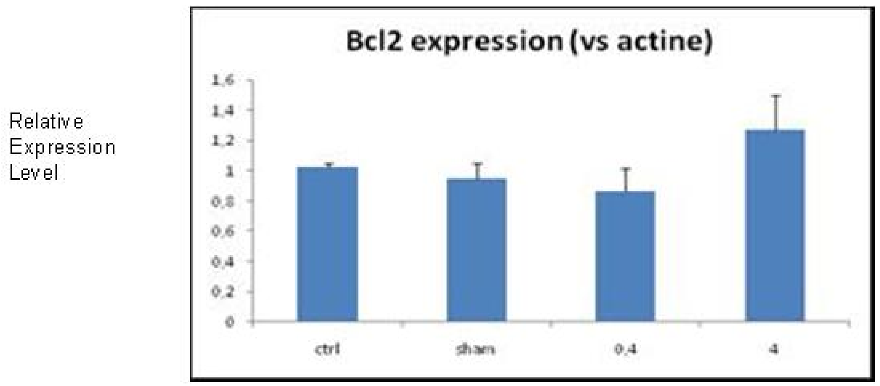

Fig. 4 - Effects of exposure to WiFi signals on Bcl-2 gene expression as evaluated by retro-transcription and real-time amplification. Values, after normalization to β-actin, were referred to the control group. Means ± standard deviation from 5 independent experiments are shown.

Possible Influence of GSM Exposure on Thyroid Morphology in Sprague-Dawley Rats

Paolo Galloni, Giorgio A. Lovisolo, Marta Piscitelli, Rosanna Pinto, Carmela Marino

Possible effects of radiofrequency fields on the endocrine systems is a primary research topic, since it plays a crucial role in human health status; the thyroid could be involved in interaction with electromagnetic fields emissions, due to his proximity to the cell phone during its normal use. This study is a replication of a recent (see report 2009)

protocol on effects of in vivo localized exposure to GSM electromagnetic fields in thyroid structure and function in female rats.
Thirty female Sprague-Dawley rats, weighting 200 g at the beginning of the experiment, were used. They were locally exposed, in proximity of the right ear, simulating the use of a cellular phone, by 3 different sets of 4 loop antennas (Lopresto *et al*, Radiation Protection Dosimetry 123: 473-482, 2007), 2 h per day, 5 days per week, for 4 weeks, 1.22 +/-0.1 W/kg of SAR at the frequency of 900 or 1800 MHz GSM modulated, in blind mode. Four groups of rats were scheduled: a 900 MHz-exposed (n=8), an 1800 MHz-exposed (n=8), a sham-exposed (n=8), a cage-control untreated (n=6); during the exposure, rats were kept in plastic restrainers. Body weight was periodically checked. Animals were sacrificed at the end of exposure and thyroid glands collected, 10% formalin fixed, processed to wax, cut and H&E stained and histopathologically evaluated. In addition, blood samples were collected for successive tests of serum levels of thyroid-related hormones.
The histopathological examination of gland explants is still in progress. The study was performed in collaboration with Maurizio Bossola (Institute of Histopathology and Cytodiagnosis, Catholic University, Rome) and Anna Lanzoni (Pathology Department, GlaxoSmithKline S.p.A, and the Aptuit Company, Verona).

A loop antenna for localized *in vivo* exposure at Wi-Fi frequencies: design and dosimetry

Caterina Merla*, Rosanna Pinto, Sergio Mancini, Giorgio A. Lovisolo
*Post-doc

I*n vivo* investigations were accomplished to assess possible health risks due to mobile phone exposures (GSM 900-1800, UMTS) localized on head organs.
These exposures were realized using printed loop antennas, designed to work at such frequencies. These antennas provided an efficient and confined exposure of the head of restrained animals (e.g. rats) placed in special Perspex cages.
Recently, the presence in public environments of new communication signals as the Wireless Fidelity (Wi-Fi) and the use of phones based on the Voice over IP (VoIP) protocol require novel *in vivo* experiments.
For these reasons, a loop antenna for localized exposure of small-restrained animals in Perspex cages was designed on the basis of previous systems. Optimal antenna feeding and stub positioning were numerically assessed. The optimized antenna was fabricated and S11 measurements were carried out with and without the biological load.
Numerical dosimetry was performed on a cubic phantom and compared with SAR measurements in the same condition, showing a good agreement.
SAR distribution, calculated in a realistic rat voxel model (Fig. 5), is highly confined near the antenna, presenting a good efficiency value (3.6 W/kg/W) inside the rat brain. Therefore the optimized antenna is suitable for localized *in vivo* exposure aiming at the evaluation of possible biological effects induced by the field emitted by Wi-Fi devices.
The study has been performed in collaboration with the Department of Information Engineering, Electronics and Telecommunications, Sapienza University of Rome.

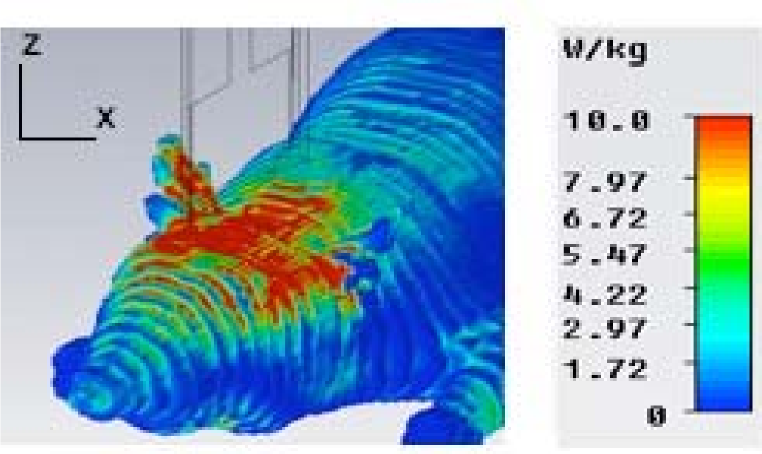

Fig. 5 - SAR distribution within the rat model

Microwave thermal ablation: changes in the dielectric parameters of *ex vivo* bovine liver during the treatment

Vanni Lopresto, Rosanna Pinto, Sergio Mancini, Rossella Lodato, Giorgio A. Lovisolo

Microwave thermal ablation (MTA) is a technique used for minimally invasive treatment of soft tissues pathologies, based on a very high temperature increase obtained through the electromagnetic field radiated by a microwave antenna. An accurate dosimetry, including an adequate model of the target tissue, should be performed to support the design of interstitial applicators. The heating process influences the dielectric properties of the tissue under treatment principally due to variation of water content. Aim of this work is to evaluate the changes of the dielectric parameters during a MTA treatment at 2.45 GHz in *ex vivo* bovine liver.
Performed measurements evidenced a significant change (reduction of about 50%) of both relative permittivity and electric conductivity in the tissue during the MTA treatment as temperature increased over 60°C, with a dramatic drop when temperature approached 100°C. Once the temperature raised over about 90°C the process was irreversible, even if the tissue was allowed to return toward the starting temperature.
In Fig. 6 the measured average patterns of the relative permittivity and the electric conductivity with respect to the temperature during MTA treatments are shown. The standard deviations of twenty repeated measurements were lower than 5%. The knowledge of changes of the dielectric properties in the target tissue can support the design of interstitial applicators and help the future development of MW treatment planning.
The study has been performed in collaboration with Department of Information Engineering, Electronics and Telecommunications, Sapienza University of Rome, R&D Unit, HS Hospital Service S.p.A., Rome, and Unit of Material Technologies, ENEA, Rome.

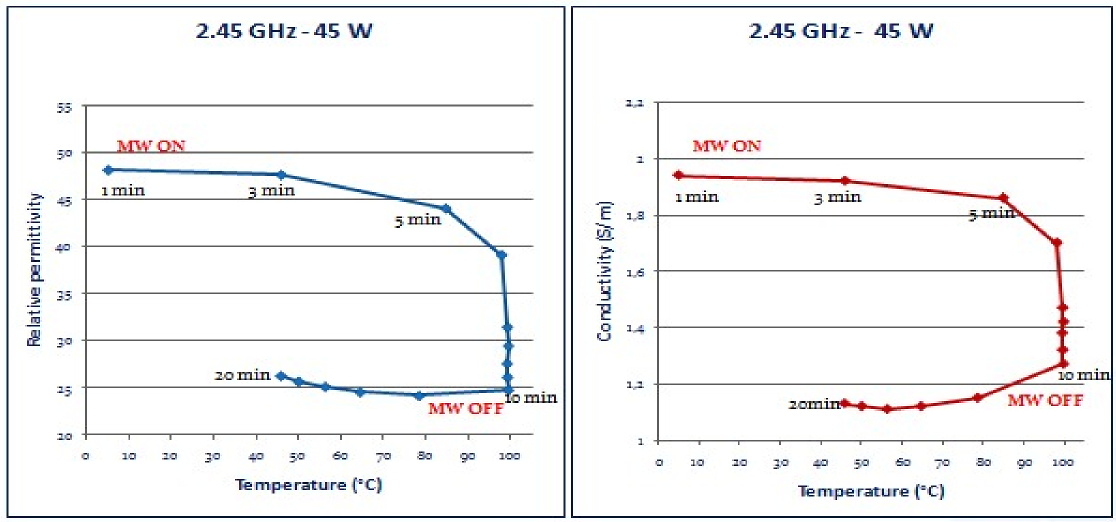

Fig. 6 - Relative permittivity and electric conductivity variations with temperature measured during MTA treatment at 2.45 GHz in *ex vivo* bovine liver. Changes were irreversible as tissue returned toward the starting temperature.

SCIENTIFIC COLLABORATIONS

- Dept. of Environmental Science, University of Eastern Finland (UEF), Kuopio (Prof. Jukka Juutilainen)
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GRANTS

SITES strategic project: Safety and health technology (founded by ISS-Ministry of Health), 2010-2012.

TAM project: MW termoablation (founded by FILAS-Regione Lazio), 2008-2011.

Exposure assessment in MRI (founded by ISPESL), 2008-2010.

MOLECULAR EPIDEMIOLOGY

Interaction between glutathione-S-transferase polymorphisms, smoking habit, and HPV infection in cervical cancer risk

Selena Palma[*], Flavia Novelli, Donatella Tirindelli, Antonella Testa
[*]Post-doc

Human papillomavirus (HPV) infection is considered the major cause of cervical cancer (CC), but a number of infected women do not develop invasive lesions, suggesting the role of genetic susceptibility and environmental co-factors for cancer outbreak.

The aim of this study was to investigate whether some glutathione-S-transferase (GST) polymorphisms could influence the risk to develop CC, either by themselves or in combination with smoking habit, in a cohort of high-risk HPV (HR-HPV) infected Italian women.

The study population comprises 192 Italian women including 81 HR-HPV infected women bearing cervical lesions and 111 healthy controls.

The cases include: 26 low-grade squamous intraepithelial lesions (LSILs), 30 high-grade-SIL (HSILs), and 25 CCs, while controls were all negative for HPV.

Cases and controls were genotyped for GSTM1, GSTT1, and GSTP1 polymorphisms using PCR/RFLP technique.

Tab. 1 shows the distribution and the related frequencies of GSTs genotypes in cases and controls entirely, and in the different subgroups (smokers and non-smokers). Smoking habit, considered alone, was not found to be a risk factor for cervical lesions (CLs) [OR = 1.16, (0.59–2.27)]. The same result was obtained when we stratified for the different cervical lesions. On studying the association of GSTs gene polymorphisms with CLs, the combination of *GSTM1* null, *GSTT1* null and *GSTP1* AA genotypes, independently on smoking habit, seems to be related to a 5.7-fold increased risk of developing CLs with a considerable statistical significance ($p = 0.0091$).
We suggest that the investigation of multiple gene polymorphisms, versus single genes, could contribute to a better understanding of the effect of susceptibility genes on cancer risk. The present activity has been carried out in collaboration with L. Padua (Don Carlo Gnocchi Foundation, Rome); A. Venuti and L. Mariani (Regina Elena Cancer Institute); G. Prignano (IRCCS San Gallicano Institute); R. Cozzi (Roma 3 University)

Tab. 1 - Distribution of GSTM1, GSTT1 and GSTP1 genotypes in cases and controls

	GSTM1			*GSTT1*			*GSTP1*			
	Null	Present	Null frequency	Null	Present	Null frequency	AA	AG	GG	G frequency
Controls (n = 111)	58	53	0.52	22	89	0.20	55	53	3	0.27
Smokers (n = 32)	21	11	0.66	6	26	0.19	17	15	0	0.23
Non-smokers (n = 45)	21	24	0.47	10	35	0.22	20	23	2	0.30
Cases (n = 81)	49	32	0.60	23	58	0.28	40	35	6	0.29
Smokers (n = 28)	21	7	0.75	7	21	0.25	13	14	1	0.29
Non-smokers (n = 34)	16	18	0.47	10	24	0.29	15	15	4	0.34

DNA repair capacity and acute radiotherapy adverse effects in Italian breast cancer patients

Antonella Testa, Donatella Tirindelli

Therapeutic exposure to ionising radiation can induce normal tissue side effects which consistently differ among individuals suggesting a possible genetic control. One approach to elucidate the underlying mechanisms is to analyse the relation between genetic traits, biomarkers of in vitro DNA damage and side toxicity in vivo. 43 breast cancer (BC) patients receiving radiotherapy after a breast-conserving surgery were recruited together with 34 age- and sex-matched healthy controls. Adverse tissue reactions were recorded as indicators of radiotherapy susceptibility. All blood samples from both patients (35) and controls (34) were irradiated in vitro and DNA primary damage and repair kinetic were measured through Comet assay. All study subjects were genotyped for *XRCC1*, *OGG1* and *XRCC3* gene polymorphisms. In our small groups we found a positive association between *XRCC1* variant allele (399Gln) and the occurrence of BC [OR= 2.41, (1.24–4.66), $p = 0.01$]. BC patients exhibited a very significantly ($p < 0.001$) higher mean level of basal DNA damage when compared with healthy controls. Immediately after irradiation and 30’ later BC patients showed significantly higher ($p < 0.01$) level of DNA damage and this level persisted significantly higher ($p < 0.05$) 60’ later, when compared to healthy subjects (Fig. 1A). In Fig. 1B the residual DNA damage (RD) after 30’ and 60’ from irradiation is reported in three groups of patients showing G0, G1 and G2–G3 adverse reactions following Radiation Therapy Oncology Group (RTOG) scale. In patients showing no adverse reactions (G0) the RD at 60’ is significantly ($p = 0.0067$) lower than at 30 min. On the contrary, in patients showing G1 and G2–G3 adverse reactions no significant reduction of RD was observed comparing the values at 60’ and 30’ from irradiation. This activity has been carried out in collaboration with Silvia Sterpone, Valeria Mastellone, Tommaso Cornetta, and Renata Cozzi (University of “Roma TRE”); Luca Padua (Don Carlo Gnocchi” Foundation, Rome); Daniela Giammarino and Vittorio Donato “S. Camillo-Forlanini” Hospital, Rome).

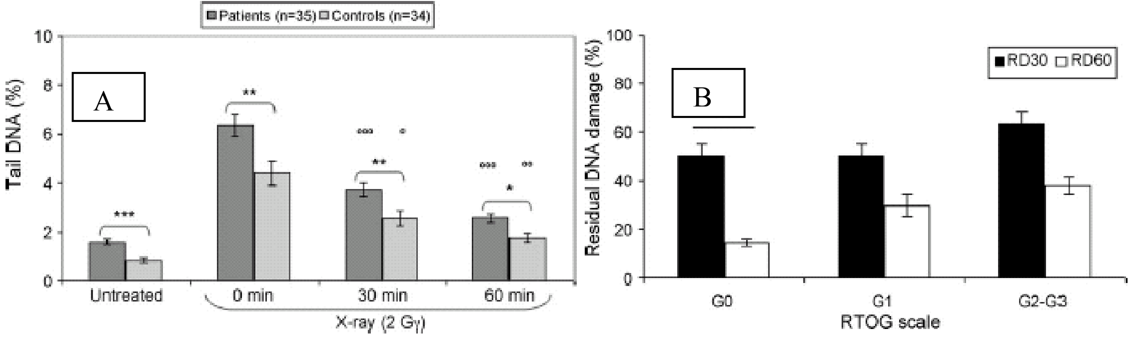

Fig. 1 – A. Basal and X-ray induced DNA damage expressed as Tail DNA (TD) in BC patients and controls. The Comet assay was performed in untreated condition, immediately after irradiation and 30’ and 60’ later. ***p < 0.001, **p < 0.01, *p < 0.05 at Mann–Withney U-test when comparing BC patients to controls; °°°p < 0.0001 at ANOVA nonparametric Friedman test when comparing TD values at 30’ and 60’ after irradiation with basal value in BC patients; °p = 0.008, °°p = 0.002 at ANOVA nonparametric Friedman test when comparing TD values at 30’ and 60’ after irradiation, respectively with basal value in controls. **B.** RD 30’ and 60’ after irradiation in BC patients classified on the basis of RTOG scale. *p = 0.0067.

Single-nucleotide polymorphisms in BER and HRR genes, XRCC1 haplotypes and breast cancer risk in Caucasian women

Flavia Novelli, Clarice Patrono, Antonella Testa

Breast cancer (BC) is worldwide the most common type of female cancer and is principally represented by sporadic cases. A substantial proportion of people may be predisposed to this pathology through genetic and environmental factors. Enhanced sensitivities and variability in processing the induced DNA damage can be responsible of both higher risk of developing cancer and elevated normal tissue adverse reactions. These complex traits are influenced by low penetrance genetic factors, in particular by single-nucleotide polymorphisms (SNPs) in genes, such as those genes involved in DNA repair. This study aimed to assess if SNPs of genes involved in different DNA repair pathways were associated with an increased risk of developing BC and early adverse reactions after radiotherapy (RT). In order to investigate the connection between gene variants and BC, we performed a genotype and haplotype study on 43 Italian BC patients and 31 healthy controls. Five SNPs (*XRCC1*-77 T>C, *XRCC1*-194, *XRCC1*-399, *OGG1*-326, *XRCC3*-241) were genotyped. *XRCC1, OGG1* and *XRCC3* genes are involved in base excision repair (BER) and homologous recombination repair (HRR). We studied the relationship between combined SNPs, *XRCC1* haplotypes and an increased risk of BC.

We found a significant association between BC occurrence and *XRCC1*-399 [OR = 4.67 (1.65-13.23), p=0.005]. On the contrary, the other SNPs weren't associated with BC. Furthermore, *XRCC1* haplotype H3, with the variant alleles in the promoter and at codon 399 and the wild-type allele at codon 194, was associated with higher BC risk [OR = 7.04 (1.63-30), p=0.009], (Tab. 2).

Tab. 2 - Association between haplotypes of *XRCC1* and BC risk

	Haplotypes[a]	Cases (%)	Controls (%)	OR (95% CI)
H1	C–C–G	5 (11.6)	11 (35.5)	1.00 (Ref.)
H2	T–C–A	15 (34.9)	9 (29)	3.67 (0.96–14)
H3	C–C–A	16 (37.3)	5 (16.1)	7.04 (1.63–30)[b]
H4	T–C–G	0 (0)	3 (9.7)	0.30 (0.01–6.86)
H5	T–T–G	1 (2.3)	2 (6.5)	1.1 (0.08–15.16)
H6	T–T–A	4 (9.3)	1 (3.2)	8.8 (0.77–100)
H7	C–T–G	2 (4.6)	0 (0)	10.45 (0.42–257)

[a] The haplotype is defined as the allele present at the position −77 (T→C); 194 (C→T); 399 (G→A), respectively

[b] Fisher's test $p = 0.009$

The probability for developing this tumor was also increased by the number of SNPs in different genes. We found a significantly higher BC risk in patient carrying ≥ 3 SNPs compared to those with < 3 variant alleles, [OR = 2.72 (0.99-7.39), p=0.04], (Fig. 2A). The probability for the development of Grade ≥ 2 toxicity (according to Radiation Morbidity Scoring Scheme EORTC/RTOG) was higher for patients with ≥ 3 SNPs [OR = 2.42 (0.26-22.5)] but p value was not significant (Fig. 2B).

We showed that the combination of SNPs in different genes involved in BER and HRR mechanisms affects BC risk. We can suppose that genetic variants in multiple repair genes may have an additive effect in cancer occurrence. Our results are preliminary and

further investigations are needed in order to explain the importance of SNPs in individual radiosensitivity. This activity has been carried out in collaboration with Silvia Sterpone, Valeria Mastellone, Tommaso Cornetta, and Renata Cozzi (University of "Roma TRE"); Luca Padua (Don Carlo Gnocchi Foundation, Rome); Daniela Giammarino and Vittorio Donato "S. Camillo-Forlanini" Hospital, Rome).

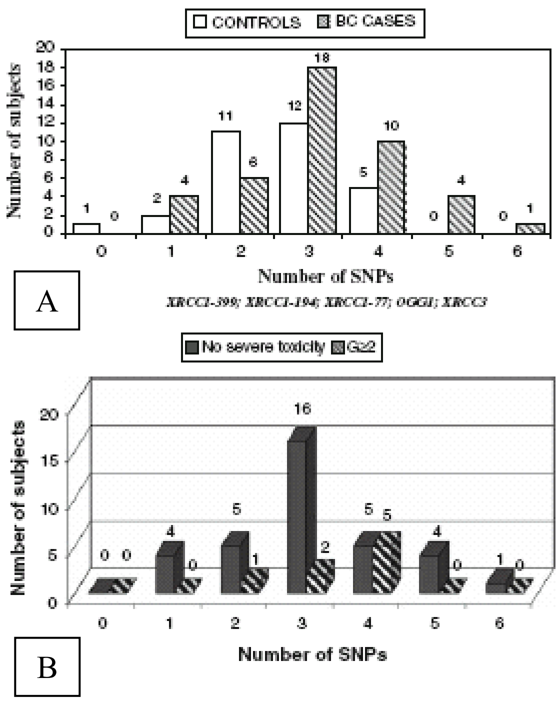

Fig. 2 – A) Distribution of the number of variants in controls and BC patients. 45% of controls carries <3 SNPs, 55% carries 3-4 variants. 23% of BC patients carries <3 SNPs, 77% showed $\geq$3 variants. **B**) Distribution of the number of variants according to radiation-induced early side effects in BC patients (Grade $\geq$ 2 vs. Grade $\leq$ 2).

G_0 and G_2 chromosomal assays in the evaluation of radiosensitivity in a cohort of Italian breast cancer patients

Tommaso Poggioli*, Selena Palma, Antonella Testa
*Graduate student

Breast cancer (BC) is the most common type of malignancy in female patients and radio-treatment is the conventional therapy even if a great number of studies reported that enhanced sensitivity to ionizing radiation as measured as chromosome effects is present in a significant proportion of cancer patients, including breast cancer ones. In this study we analysed whether peripheral blood lymphocytes from sporadic BC patients and healthy subjects showed a different sensitivity to ionizing radiation and whether cytogenetic radiosensitivity may serve as a breast cancer risk biomarker. To test this hypothesis, the in vitro radiation sensitivity was measured by using both G_0 and G_2 chromosome radiosensitivity assays, on 46 subjects (23 BC patients and 23 healthy subjects). The choice of BC patients as study population was due to published observation that deficiency or impairment in DNA repair capacity can be a predisposing factor in familial and in some sporadic BC cases. Genomic instability has also been described in various cancer diseases including breast cancer.

Results for both G_0 and G_2 assays obtained on healthy controls and BC patients are shown in Fig. 3. The mean basal frequency of aberration yield in BC patients was not significantly higher than that observed in healthy controls.

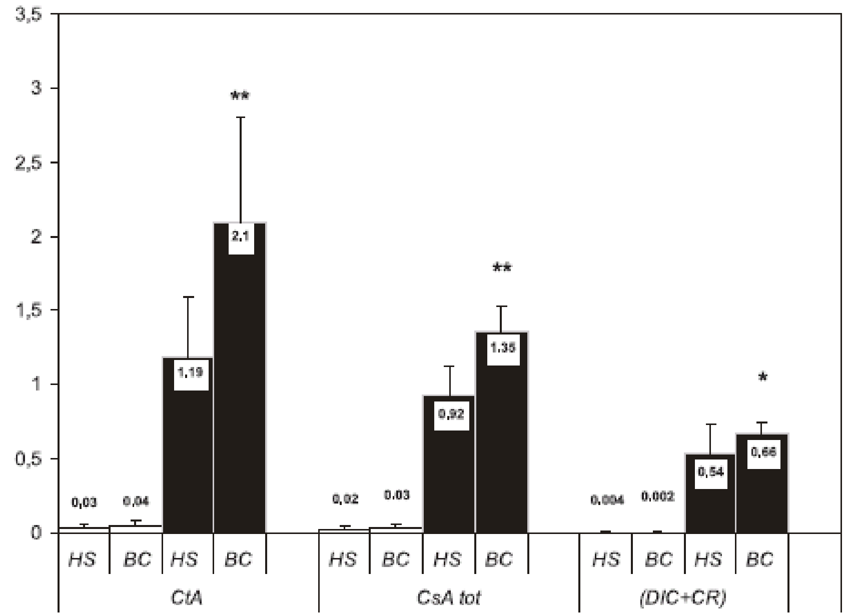

Fig. 3 - G_2 and G_0 mean frequencies (± SD) observed in lymphocytes of healthy subjects (HS) and BC patients before (white columns) and after X ray treatment (black columns). CtA = Chromatid Aberrations; CsA tot = sum of Dicentric + Rings + excess acentric fragments; DIC + CR = Dicentrics + Rings. $^{**}p < 0.0001$ $^{*}p = 0.02$ at Student's t test.

When we compare the results obtained after X ray treatment, we always found higher mean values in BC patients compared to healthy controls. In particular comparing the mean frequency of chromatid breaks we found a statistically significant higher mean value in BC patients (2.10 vs 1.19). A significant result was also found comparing the mean frequency of total amount of chromosome aberrations (CsA tot) between the two groups. As far as the mean frequency of dicentric chromosomes and centric rings, BC patients showed a significantly higher value.
These data indicate that lymphocytes of our BC patients are more sensitive to ionizing radiation compared to those of healthy subjects when we consider both G_2 and G_0 radiosensitivity assay values. This result is in agreement with our previous paper where the same subjects (both patients and healthy controls) were analysed by the Comet assay. This activity has been carried out in collaboration with Silvia Sterpone and Renata Cozzi (University of "Roma TRE").

SCIENTIFIC COLLABORATIONS

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GRANT

National Public Ministry of Health, Project: “Definizione biomolecolare delle fasce di rischio per management e terapia del cervicocarcinoma uterino”.

REGULATION OF IMMUNE RESPONSE: CELLULAR AND MOLECULAR ASPECTS

Claudio Pioli, Flavia Novelli, Federica Laudisi, Manolo Sambucci

The immune system is devoted to the protection of the organisms from infections. T cells, which represent a key component of an immune response, need to recognize and fight pathogens while remaining tolerant to host molecules, harmless commensal microorganism and food antigens. Upon activation naïve CD4 T cells differentiate in distinct types of effector cells, namely Th1, Th2 and Th17 cells. Through specific sets of cytokines and other soluble or cell-bound products, these cells drive the immune response leading to the eradication of different groups of pathogens. Differentiation and functions of effector Th cells are kept under control by regulatory T cells which prevent chronic inflammation and maintain tolerance. Alterations in regulatory T cell differentiation and functions lead to over-exuberant responses and are associated with immune-mediated diseases.

PolyADP-ribosylation and regulatory T cell differentiation: a new potential therapeutic target.

$CD4^+CD25^+$ regulatory T (Treg) cells are essential to maintain immunological self-tolerance and control immune homeostasis. Differentiation and function of Treg cells depend on the expression of the nuclear transcription factor Foxp3 (forkhead box p3). In mice, a deletion in the fork-head domain of Foxp3 induces the scurfy phenotype characterized by fatal lymphoproliferative disease and multi-organ inflammation. In humans, mutations of Foxp3 lead to the Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) disorder. Treg cells, which are produced in the thymus as functionally mature cells, can also differentiate from naïve CD4 T cells in peripheral tissues, under specific conditions. Foxp3-expressing inducible Treg (iTreg) cells can be obtained from $CD4^+CD25^-$ cells *in vitro* and *in vivo*. This conversion is depend on TGF-β_1 and, to a lower extent, on IL-2. Factors that peripherally generate $Foxp3^+$ iTreg cells *in vivo* are still unclear.

PolyADP-ribose polymerases (PARPs) synthesize and covalently bind branched polymers of ADP-ribose to acceptor proteins using NAD^+ as a substrate. PARP-1, initially characterized for its involvement in DNA damage detection and repair, plays a relevant role in inflammation and immune response. It regulates NFAT and NFκB activation, is involved in dendritic cell maturation and in B and T cell functions. Parp-1KO mice are resistant to several inflammatory/immune-mediated diseases.

We extended our previous studies on the effects of PARP-1 inactivation in Treg cell differentiation. Increased numbers of regulatory $CD4^+CD25^+/Foxp3^+$ T cells were found in thymus, spleen and lymph nodes of PARP-1KO mice as compared to WT controls. The increased Treg cell frequency at periphery resulted in impaired CD4 cell proliferation and IL-2 production, which could be restored by CD25 cell-depletion. Purified *naïve* CD4 cells from PARP-1KO mice stimulated *in vitro* expressed Foxp3 mRNA at higher level and generated a higher number of Foxp3 cells (inducible Treg cells) than the WT counterpart. These findings were confirmed under several conditions of stimulation.

Treg cells from WT or PARP-1 KO mice were challenged for their ability to inhibit cell proliferation and cytokine production in freshly isolated $CD4^+CD25^-$ Th cells or in Th1 and Th2 effector cells. PARP-1 KO and WT Treg cells were equally able to suppress cell proliferation in freshly isolated $CD4^+CD25^-$ cells, as shown by $[^3H]$TdR uptake and CFSE labeling of $CD4^+CD25^-$ cells (Fig. 1A-C). PARP-1 KO and WT Treg cells also inhibited IL-2 production to a similar level after 24 and 72 h of stimulation (Fig. 1D, 1G). Treg cells from PARP-1 KO and WT mice were also equally effective in the control of cell proliferation (Fig. 1E, 1F) and cytokine production (Fig. 1H, 1I) in Th1- and Th2-polarized cells. The proliferative responses of Th1 (Fig. 1E, maximum inhibition 55%) and Th2 (Fig. 1F, maximum inhibition 70%) cells were suppressed less than freshly isolated $CD4^+CD25^-$ cells (Fig. 1A, maximum inhibition 95%). Altogether, these data demonstrate that PARP-1 KO cells are functional *in vitro* and can control cell proliferation and cytokine production in effector T cells.

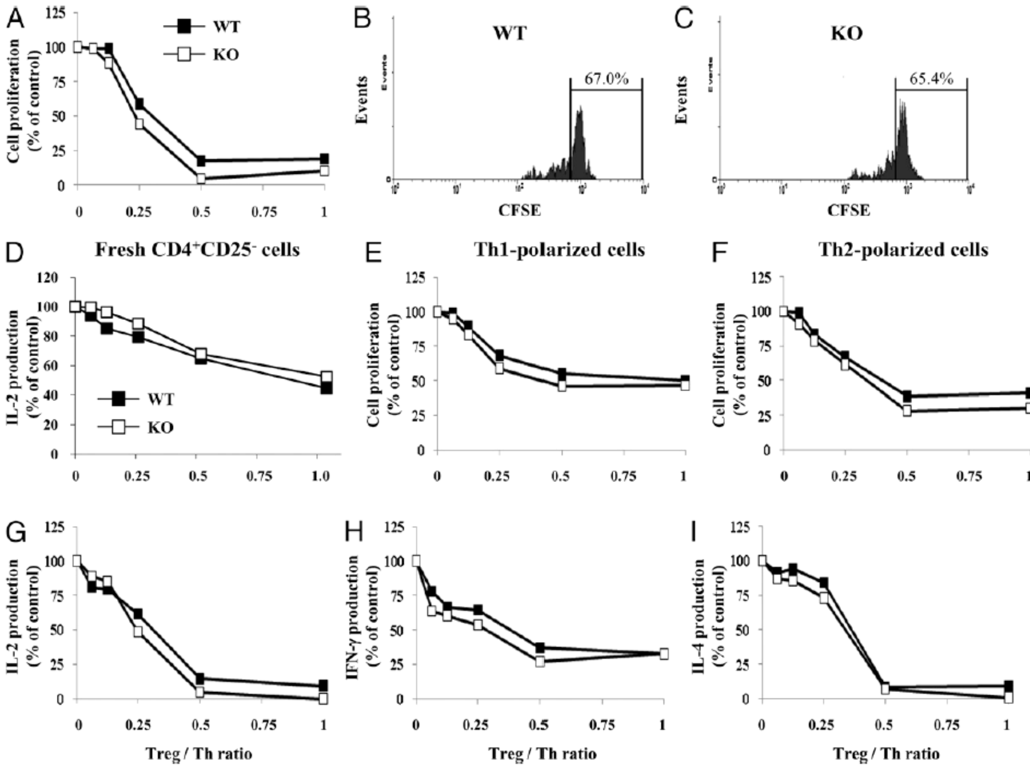

Figure 1 - *In vitro* functional characterization of regulatory T cells from PARP-1 KO mice. In vitro inhibitory assays: Increasing numbers of purified $CD4^+CD25^+$ Treg cells from C57BL/6Parp-1^{WT} (WT, black symbols) or C57BL/6Parp-1^{KO} (KO, white symbols) mice were cultured with freshly isolated WT $CD4^+CD25^-$ cells (A-D, G) or Th1- (E, H) or Th2-polarized (F, I) cells in the presence of WT T cell-depleted spleen cells as APCs. After 72 h of stimulation with anti-CD3 mAb, cell proliferation was assessed by $[^3H]$TdR uptake (A, E, F) or CFSE labeling of $CD4^+CD25^-$ target cells (B, C). Cell proliferation of WT $CD4^+CD25^-$ and Th1- and Th2-polarized target cells in the absence of Treg cells, measured as cpm ± SE, was 4751 ± 234, 8656 ± 461, and 7476 ± 388, respectively. Numbers in B and C represent the percentages of non-proliferating cells. Cytokine production was assessed after 72 h of stimulation (G-I). Cytokine concentrations in culture supernatants of WT $CD4^+CD25^-$ and Th1- and Th2-polarized target cells, expressed as pg/ml ± SE, were 613 ± 32, 13,300 ± 505, and 7,310 ± 446, respectively. IL-2 concentration was also evaluated after 24 h of stimulation (D).

To verify whether PARP-1 KO Treg cells were functional also *in vivo*, a Graft Versus Host Disease (GHVD) model was used. BALB/c host mice were given total body irradiation (8 Gy). All mice received T cell depleted (TCD) bone marrow (BM) cells alone or with C57Bl/6 WT T-helper cells or with T-helper cells plus C57Bl/6 WT or plus PARP-1 KO Treg cells. Survival was monitored daily (Fig. 2A) while body weight was measured every 3/7 days (Fig. 2B). Mice given only TCD BM cells appeared healthy and 80% of the animals survived for at least 120 days. Mice given TCD BM and allogenic T-helper cells developed a severe acute GVH and all died within 8 days.

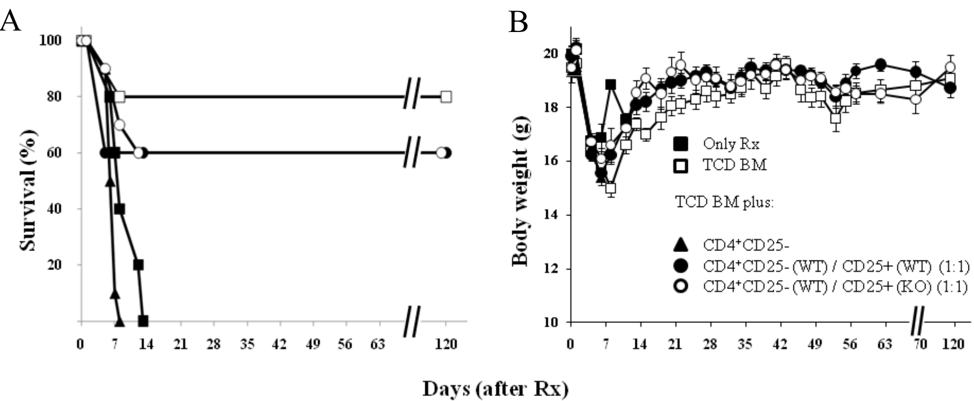

Figure 2 - Regulatory T cells from PARP-1 KO mice control graft versus host disease in mice transplanted with MHC-mismatched T helper cells. BALB/c hosts mice were given total body irradiation (8 Gy). Mice received T cell depleted (TCD) bone marrow (BM) cells alone (open squares) or with C57Bl/6 WT T-helper cells ($CD4^{+}CD25^{-}$; closed triangles) or T-helper cells plus C57Bl/6 WT (close circles) or plus PARP-1 KO Tregs (open circles). **A**, survival was monitored daily. **B**, body weight was measured every 3/7 days; values are means ± S.E. (10 mice/group).

Mice receiving PARP-1 KO Treg cells were protected from GHVD as well as those getting the C57Bl/6 WT Treg cells. Thus, PARP-1 KO Treg cells displayed their suppressive activity also *in vivo*. All together our results show that PARP-1 deficiency results in a higher number of Treg cells *in vivo* and in a higher rate of CD4 naïve to Treg cell conversion *in vitro*, as compared to WT controls. Moreover, PARP-1 KO Treg cells are functional as assessed *in vitro* and *in vivo*. Our findings represent the first evidence that PARP-1 affects Treg cell differentiation potentially opening new perspectives in the therapeutic modulation of immune responses.

COLLABORATIONS

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MOLECULAR MECHANISMS OF TUMOR INVASION AND METASTASIS

Expression of Slug is regulated by c-Myb and is required for invasion and bone marrow homing of cancer cells of different origin

Barbara Tanno, Fabiola Sesti*, Vincenzo Cesi, Donatella Tirindelli, Giuseppe Raschellà
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The main cause of death for cancer patients is metastasis, the formation of secondary tumors in organs distant from the site of the original cancer. A series of well-coordinated and interconnected biological processes must occur to drive tumor cells from the site of the primary neoplasm to a distant location. To acquire an invasive phenotype, tumor cells need to interact with components of the extracellular matrix (ECM), such as collagen and hyaluronic acid, and with soluble growth factors, such as members of the transforming growth factor β (TGF-β) and fibroblast growth factor (FGF) families, epidermal growth factor (EGF) and SF/HGF. During local invasion, the first step of the metastatic process, tumor cells of epithelial origin undergo a trans-differentiation process termed Epithelial Mesenchymal Transition (EMT). Throughout EMT, tumor cells lose cell–cell interactions and apico-basal polarity and acquire mesenchymal and migratory properties. EMT is a reversible process: once cancer cells localize to a site of metastasis undergo a Mesenchymal Epithelial Transition (MET) through which they reacquire many of the characteristics of the tumor cells at the primary site. In EMT, loss of epithelial properties occurs through down-regulation of the expression of epithelial-specific proteins (e.g., E-cadherin and cytokeratins), and the acquisition of mesenchymal proteins (e.g., N-cadherin and vimentin). In addition, cancer cells undergoing EMT activate proteinases that allow them to pass through the extracellular matrix and to become more resistant to anoikis, a form of cell death that occurs in cells detached from their stroma support. A central question for understanding the process of metastasis at the molecular level is: which are the regulators of the genes activated or repressed during EMT that occurs at the onset of invasion? Several transcription factors that strongly repress E-cadherin - such as members of the Snail, ZEB and basic helix-loop-helix (bHLH) families - are now thought to be inducers of the phenotypic changes required for EMT. Nevertheless, the specific role of these different repressors in tumorigenesis is not fully understood. In particular, the Snail family of transcription factors that in vertebrates includes Snail (SNAI1, Snail1), Slug (SNAI2, Snail2) and SNAI3, is involved in physiological and pathological EMT. In this work we demonstrated that the proto-oncogene c-Myb activates Slug expression in embryonic kidney, colon carcinoma, chronic myeloid leukemia and neuroblastoma cells (Fig. 1). c-Myb control over Slug expression is actuated through a transcriptional mechanism (Fig. 2) c-Myb-activated Slug expression is associated with increased *in vitro* invasion and is required for bone marrow homing of K562 cells in mice (Fig. 3). Together, these data support the existence of a functional relationship between the proto-oncogene *c-myb* and metastasis-associated *slug* in hematopoietic and non-hematopoietic cancer cells. This activity has been carried out in collaboration with Gianluca Bossi (Regina Elena Institute of Rome); Giovanna Ferrari-Amorotti, Rita Bussolari and Bruno Calabretta (University of Modena).

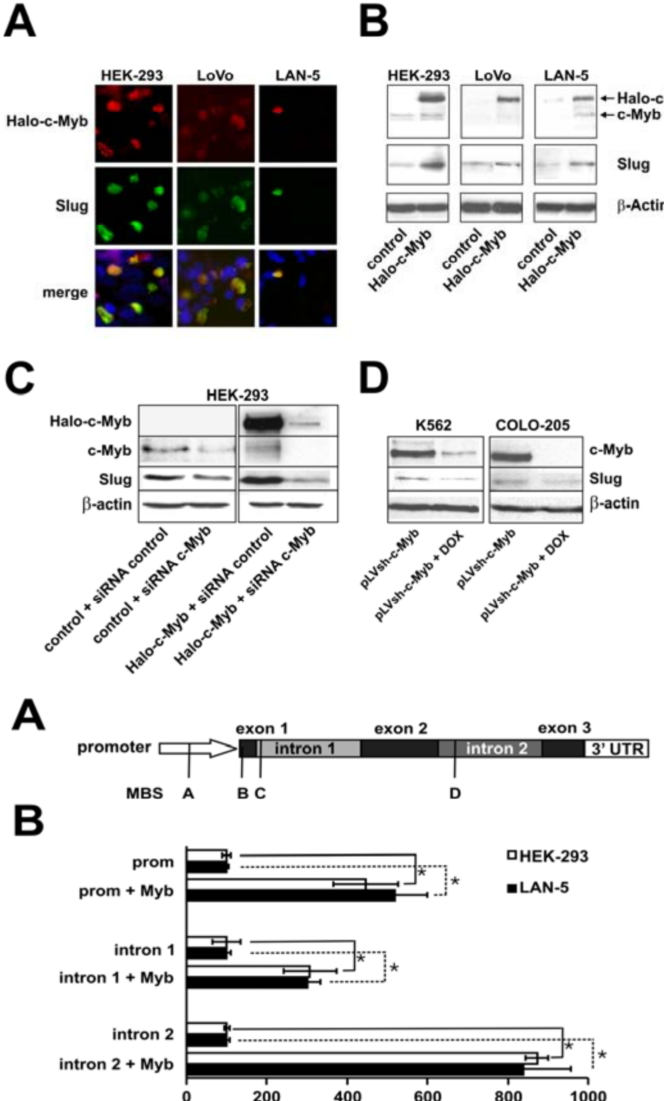

Fig. 1 - A: Slug is co-expressed in nuclei of cells ectopically expressing c-Myb. B: Western blot analysis that shows Slug protein expression in cells ectopically expressing c-Myb. C: c-Myb silencing by siRNA down-regulates Slug expression in cells transfected with an expression vector encoding c-Myb. D: c-Myb silencing by shRNA expression down-regulates Slug in cells endogenously expressing c-Myb. HEK-293: embryonic carcinoma; LoVo and COLO-205: colon neuroblastoma; K562: cronic myelogenous leukemia.

Fig. 2 - A: *S*cheme of the human slug gene. Potential Myb binding sites in the promoter region and in intron 1 and 2 are indicated. B: Functional assays were carried out by transfection of HEK-293 and LAN-5 cells with luciferase reporter plasmids containing the slug promoter (prom), or intron 1 (intron 1) or intron 2 (intron 2) alone or with an expression plasmid encoding c-Myb (Myb).

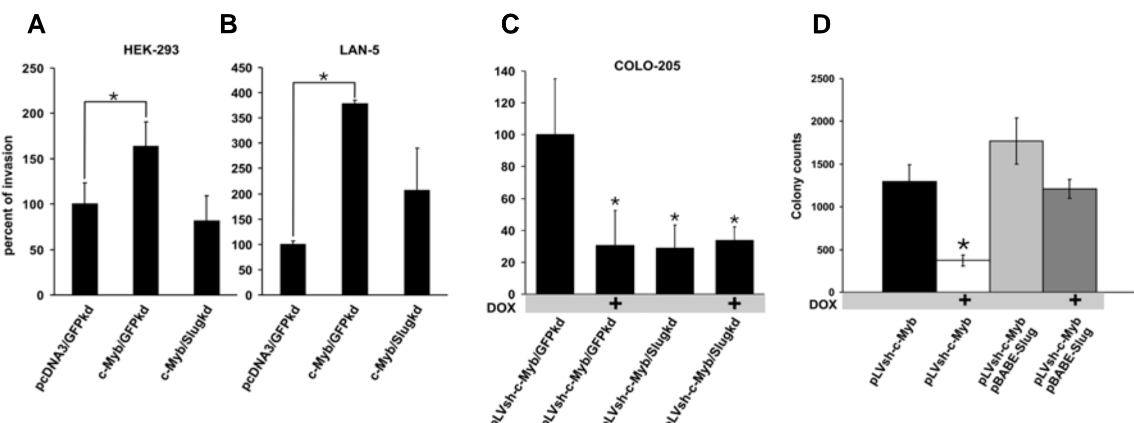

Fig. 3 - A and B: invasion increases in cells expressing c-Myb (c-Myb/GFPkd) and decreases upon Slug silencing (c-Myb/Slugkd). C: invasion decreases upon c-Myb (pLVsh-c-Myb/GFPkd + DOX) and Slug (pLVsh-c-Myb/Slugkd) silencing. D: Bone marrow homing in SCID mice injected with K562 cells decreases upon c-Myb silencing (pLVsh-c-Myb + DOX) and increases upon ectopic Slug expression (pLVsh-cMyb/pBABE-Slug).

Addiction of MYCN amplified tumours to b-myb underscores a reciprocal regulatory loop

Barbara Tanno, Giuseppe Raschellà

Neuroblastoma (NB) is the most common childhood extra-cranial tumour. NB derives from precursor cells of neuroectodermal origin and it can develop anywhere in the sympathetic system. About 40% of NBs at diagnosis are localized tumours which, in general, respond to chemotherapy and have an outcome that spans from favorable (stages 1, 2 and 3 with no MYCN amplification) to intermediate/poor (stages 2 and 3 with MYCN amplification). However, the greatest clinical challenge is represented by stage 4 which accounts for approximately 50% of NB cases at diagnosis. Stage 4 NB is characterized by metastases at distant sites such as cortical bone, bone marrow, and lymph nodes non-contiguous to the primary tumour. Outcome of these patients is generally very poor, with survival rates at 5 years not exceeding 30%. MYCN is a member of the MYC family of oncoproteins frequently amplified or overexpressed in aggressive NB. In this study we have identified the gene B-MYB, encoding the transcription factor also known as MYBL2, as a downstream target of MYCN. Using multiple in silico databases we show that expression of B-MYB significantly correlates with that of MYCN in neuroblastoma patients. MYCN binds to (Fig. 4) and activates (Fig. 5) the B-MYB gene in vivo and in vitro. Blunting B-MYB expression by RNA interference causes reduced proliferation of MYCN amplified, but not MYCN-non amplified, neuroblastoma cell lines, indicating that tumour cells are addicted to B-MYB in a MYCN dependent manner. Notably, B-MYB binds in vivo to the MYCN amplicon

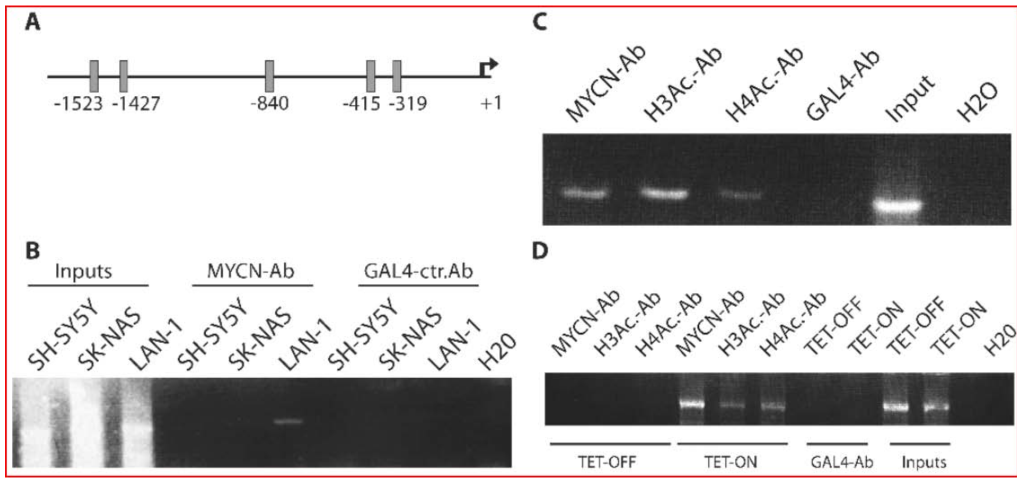

Fig. 4 - MYCN binds to the B-MYB promoter in vivo and induces histones acetylation. A) distribution of the five putative E-BOXes on the B-MYB promoter. B) Chromatin IP analysis of the MYCN protein bound onto the B-MYB promoter in SHSY5Y and SKNAS (non MYCN amplified) and LAN-1 (MYCN amplified) neuroblastoma cell lines. Cross-linked chromatin was immuno-precipitated with MYCN or GAL4, used as a negative control, antibodies as indicated. C) Chromatin-IP analysis to assess the presence of acetylated histones H3 and H4 on the B-MYB promoter region containing the putative MYCN binding sites. D) Chromatin-IP analysis of factors bound onto the B-MYB promoter in the presence or absence of inducible MYCN. Antibodies used and conditions are shown in the top of the panel.

and is required for its expression. We conclude that MYCN and B-MYB are engaged in a reciprocal regulatory loop whose pharmacological targeting could be beneficial to patients with the aggressive forms of cancer in which MYCN is amplified.
This activity has been carried out in collaboration with Francesco Gualdrini, Daisy Corvetta, Sandra Cantilena, Olesya Chayka, Arturo Sala (University College of London).

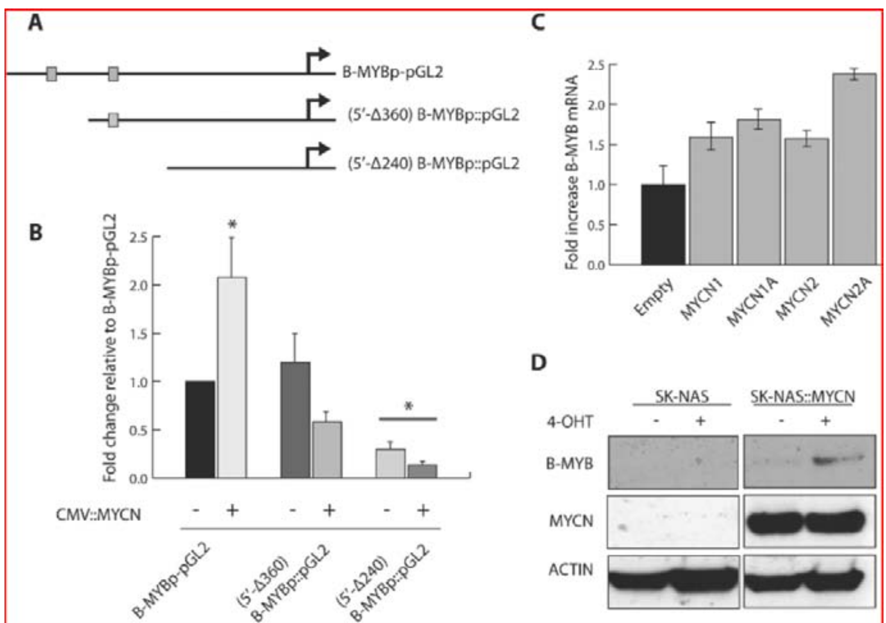

Fig. 5 - MYCN transcriptionally activates B-MYB mRNA and protein. A) Schematic representation of three reporter vectors containing fragments of the BMYB promoter: B-MYBp-pGL2 (from position -102 to -709, relative to the transcription start site); 5'-Δ360 (from position -102 to position -360); 5'-Δ240 (from position -102 to -240). B) Quantifications of luciferase assays showing the activity of the B-MYB promoter segments in the presence or absence of exogenously expressed MYCN. Error bars indicate standard deviations and the asterisk indicates statistically significant differences (*=$p<0.001$) with respect to the levels of B-MYBp-pGL2 promoter activity, which was arbitrarily set to 1. C) Real time PCR analysis of BMYB expression in 293 cells transiently transfected with a MYCN expression vector. The expression levels of four independent MYCN transfectants relative to cells transfected with empty vector are shown. Error bars indicate standard deviations obtained from triplicate assays. D) Western blot analysis showing enhanced expression of B-MYB caused by activation of MYCN in SKANAS::MYCN(ER) cells. (+ or -) 4-OHT indicates that the cells were cultured in the presence or absence of 4-hydroxytamoxifen, respectively.

c-Myb expression is regulated by TGF-beta signaling and contributes to epithelial mesenchymal transition (EMT) in ER^+ breast cancer cells

Vincenzo Cesi, Arianna Casciati, Fabiola Sesti, Barbara Tanno, Giuseppe Raschellà

Breast tumors can be subdivided in Estrogen Receptor positive (ER+) and ER negative (ER-). Although ER+ are, in general, less aggressive than ER- tumors, nevertheless the former can develop metastatic potential. Experimental evidence indicates that tumor cells exposed to specific cytokines can acquire invasive potential without further

mutation events in their genome. TGF-beta is a cytokine that suppresses proliferation and causes cell death in normal cells although it contributes to tumor progression in transformed cells that have become resistant to its suppressive effects. Breast cancer cells are frequently equipped to respond to TGF-beta stimulation. The oncogene c-myb plays an important function in differentiation and proliferation inside and outside the hematopoietic system. Recently, we demonstrated that c-Myb activates the expression of Slug in tumor cells of different origin. This finding expands the role of c-Myb to the acquisition of invasive properties of tumor cells and to the execution of the epithelial mesenchymal transition (EMT) since Slug plays a central part in both processes. In breast cancer, ER controls c-Myb expression through a transcriptional mechanism. In this work we demonstrate that in ER+ breast cancer cells c-Myb expression is activated by TGF-beta signaling through transcription activation and protein stabilization (Fig. 6). TGF-beta-induced c-Myb expression activates EMT-associated and anti-apoptotic genes such as Slug and Bcl-2 (Fig. 7) and it contributes to cytoskeleton reorganization stimulating F-actin fibers formation (Fig. 8). Together these data suggest that in breast cancer cells c-Myb is a downstream effector of pro-invasive changes driven by TGF-beta signaling. This study has been performed with Bruno Calabretta (University of Modena).

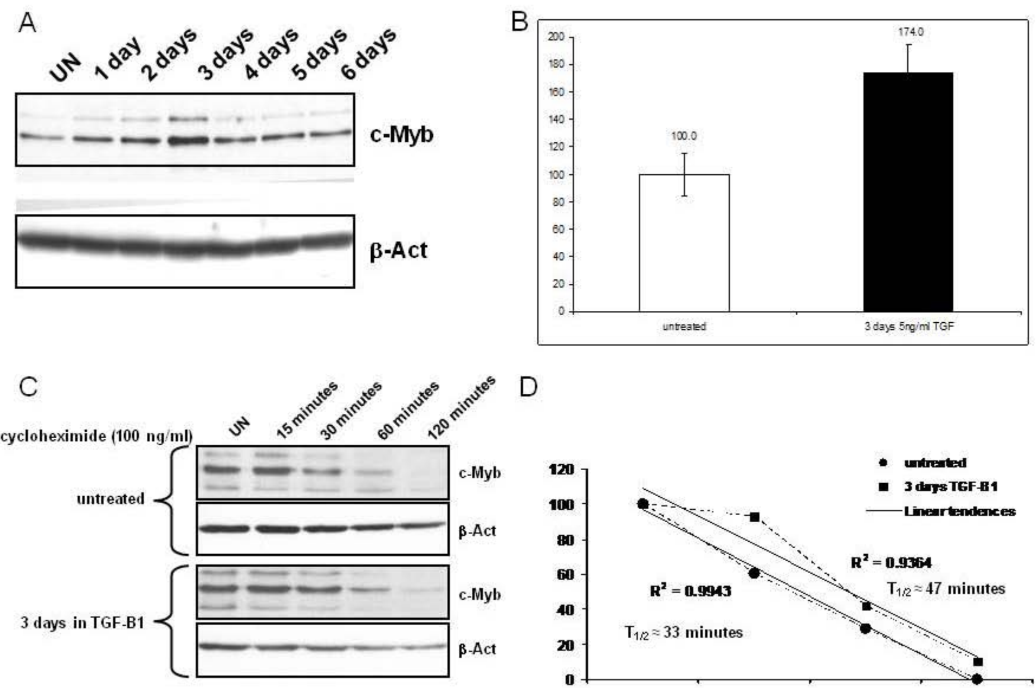

Fig. 6 - A: TGF-beta treatment (5 ng/ml) of MCF-7 breast cancer cells causes upregulation of c-Myb, that peaks at 3 days. B: TGF-beta stimulates transcription from c-Myb promoter (luciferase assay). C: Translational block realized by cycloheximide treatment was utilized to analyze c-Myb protein stability in the presence or in the absence of TGF-beta. D: After densitometric reading of the blots in C, c-Myb half life was calculated. The graph shows that c-Myb half life at 3 days of TGF-beta treatment is longer (47 min) compared to untreated cells (33 min).

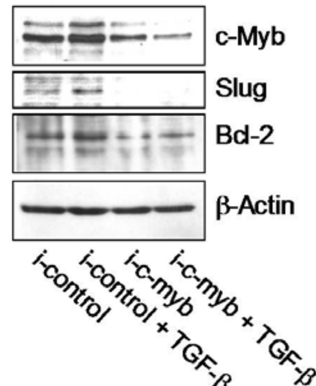

Fig. 7 - TGF-beta promotes Slug and Bcl-2 expression which are dependent on c-Myb since c-Myb silencing (i-c-myb) abrogates Slug and Bcl-2 expression. I-control: siRNA negative control.

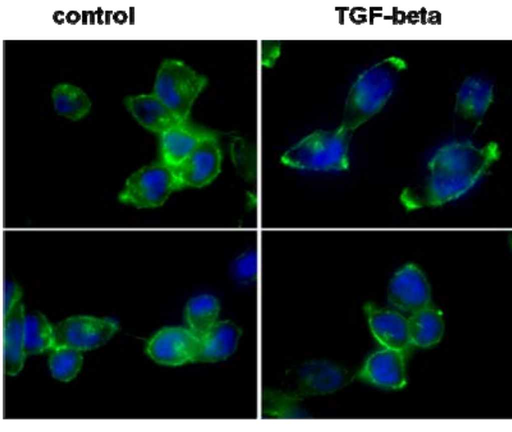

Fig. 8 - TGF-beta induces F-actin stress fibers and morphological changes which are, at least in part, dependent on c-Myb since c-Myb silencing (siRNA Myb) reverts these changes. Controls are MCF-7 cells grown in normal culture conditions.

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Fondazione Italiana per la lotta al Neuroblastoma, project title: "Identification and characterization of genes involved in the acquisition of the invasive phenotype in Neuroblastoma" 2008-2010. Principal Investigator: Giuseppe Raschellà.

MOLECULAR BASES OF CHRONIC INFLAMMATORY DISORDERS

Laura Stronati, Anna Negroni, Roberta Vitali, Maria Pierdomenico[*], Manuela Costanzo[^]
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The inflammatory bowel diseases (IBD), Crohn disease (CD) and ulcerative colitis (UC), are relatively common chronic disorders, thought to result from inappropriate and continual activation of intestinal mucosa immune system due to a complex interplay of genetic, environmental and microbial factors.

Interactions between gut microbiota and innate immune receptors in IBD

Clinical and experimental studies indicate that intestinal bacteria are involved in the initiation and amplification of IBD in genetically altered hosts. Recently, microbiological research has mainly focused on *E.coli* strains able to adhere and to invade (AIEC, Adherent Invasive *E.coli*) intestinal epithelial cells, surviving within macrophages and inducing secretion of high levels of TNFalpha. Adhesion of AIEC strains depends on the expression of a specific surface receptor, CEACAM6 (cell adhesion molecule 6 related to carcinoembryonic antigen), abnormally expressed by ileal epithelial cells in adult CD patients.
We previously demonstrated that gene expression of the AIEC specific receptor, CEACAM6, is in vitro and ex vivo inducible by the proinflammatory cytokines, TNFα and IFNγ, and by the prototype AIEC strain, LF82 (kindly provided by Dr. A. Darfeuille-Michaud, Universitè d'Auvergne, Clermont-Ferrand, France). In this year we tested the ability of the AIEC strain, LF82, to over-induce CEACAM6 in organ culture explants by RT-PCR. We further detected an increase of mRNA of the cytokines TNFalpha and IL-8 after 24 hours of incubation of tissue with LF82 (Fig. 1). By immunohistochemistry, an apical localization of CEACAM6 in intestinal epithelium of CD patients was evidenced (Fig. 2). We identified and characterized two adhesive/invasive bacterial strains present in the intestine of IBD pediatric patients, the first (EC10) found in biopsy specimens of a CD and the second one (EC15) in UC patient out of 34 IBD pts investigated.
They were biochemically characterized as belonging to Escherichia coli group and phylogenetically characterized as belonging to A and D groups, respectively. Their invasivity indexes were 1.4% for EC10 and 0.7% for EC15 (LF82: 1.29%). They were positive for the fimbrial adhesive gene (fimH) and negative for more pathogenicity genes (intimin, verocytotoxin (VTEC), enteroaggregant factor (EPEC). These two adhesive-invasive Escherichia coli strains, EC10 and EC15, have been found exhibiting a strong ability to upregulate in vitro CEACAM6 as well as proinflammatory cytokine (TNFalpha and IL-8) expression (Fig. 3). Three adhesive strains, biochemically characterized as belonging to Escherichia coli group, found in a control, a CD and a UC patient, respectively, were used as negative controls for invasivity.

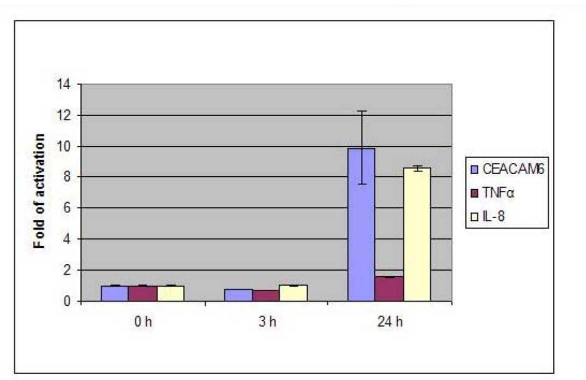

Fig. 1 - LF82 is able to increase CEACAM6, TNFα and IL-8 gene mRNA expression in organ cultures of CD patients after 24h of incubation.

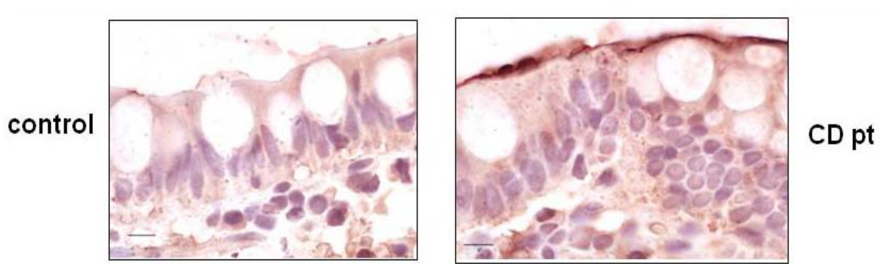

Fig. 2 - Immunohistochemistry showing CEACAM6 over-expression at the intestinal epithelium surface in CD patients compared to control.

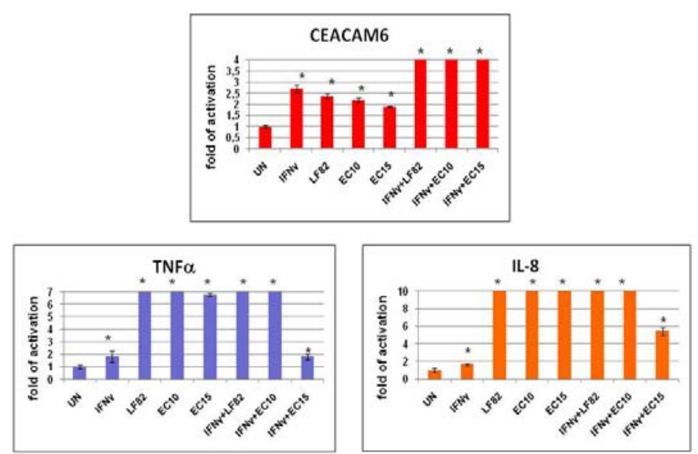

Fig. 3 - **EC10 and EC15 are able to increase in vitro gene expression of CEACAM6 and of the proinflammatory cytokines TNFα and IL-8 .**

Fecal HMGB1 is a novel marker of inflammatory bowel disease

High mobility group box 1 (HMGB1) is a nuclear protein with functions in the regulation of transcription. In inflammatory conditions, HMGB1 is actively secreted from immune cells in the extracellular matrix, where it behaviors as a proinflammatory cytokine. It is well established that HMGB1 is susceptible to a number of post-translational modifications before to be actively secreted, causing its accumulation in the cytosol. HMGB1 has been implicated in several inflammatory and autoimmune disorders, such as sepsis syndromes, rheumatoid arthritis, systemic lupus erythematosus and type 1 diabetes. In the gastrointestinal tract, increased levels of HMGB1 have been related to intestinal barrier dysfunction and, in mouse models, to colonic inflammation.

The aim of the present study was to investigate the role of HMGB1 in inflammatory bowel disease (IBD). In particular we assessed the presence of HMGB1 in the stools of patients with IBD in order to evaluate its role as non-invasive subclinical marker of intestinal inflammation.

We analyzed stools of 20 children with Crohn's disease (CD), 21 with ulcerative colitis (UC) and 13 healthy controls. Gene/protein expression levels of HMGB1 were also assessed in bioptic specimens of all children by real time PCR and western blot assay. Moreover, intracellular localization and post-translational modifications were analyzed by western blot, after nuclear-cytoplasmic separation, and by immunohistochemistry. Finally we evaluated the capability of intestinal epithelial cells and organ explant culture to secrete HMGB1 in response to inflammatory stimuli.

HMGB1 protein was significantly increased ($p<0.01$) in the stools of patients as compared to controls, where it was undetectable (Fig. 4). However, mRNA and protein expression was unchanged in bioptic tissues of patients as compared to controls (Fig. 5). Analyzing separately the nuclear and cytoplasmic fraction of bioptic specimen, HMGB1 was significantly enhanced ($p<0.01$) in the inflamed as compared to uninflamed tissue, only in the cytoplasmic fraction (Fig. 3). Furthermore only cytoplasmatic HMGB1 is methylated (Fig. 6). Immunohistochemistry from bioptic tissues of CD patients and controls, showed that HMGB1 is strictly confined in the nucleus of the uninflamed tissues of patients and controls, while it is largely found in the cytosol of patient inflamed cells (Fig. 7). Finally, the organ explant culture experiments show that HMGB1 is significantly enhanced in the medium of immunostimulated as compared to untreated specimens (Fig. 8).

Our study shows for the first time that HMGB1 is secreted by human inflamed intestinal tissues and it is abundantly found in the stools of IBD patients. Hence, it can be considered as a novel marker of intestinal inflammation. We also suggest that the large presence of HMGB1 in the fecal stream of patients mainly derives from the active secretion of the protein stored in the nucleus.

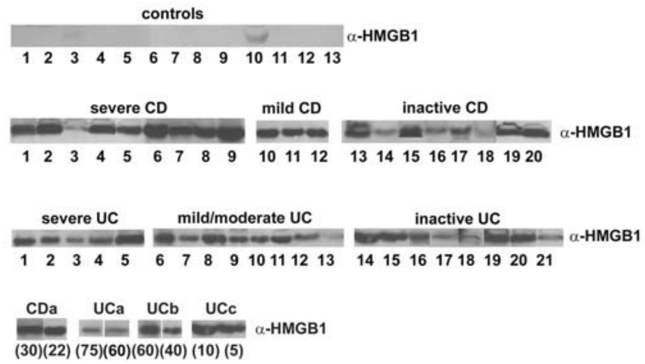

Fig. 4 - HMGB1 is abundantly found in fecal extract of IBD patients.

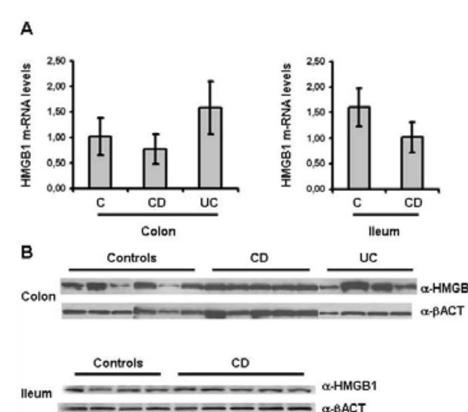

Fig. 5 - HMGB1 gene/protein expression is not increased in bioptic specimens of IBD patients.

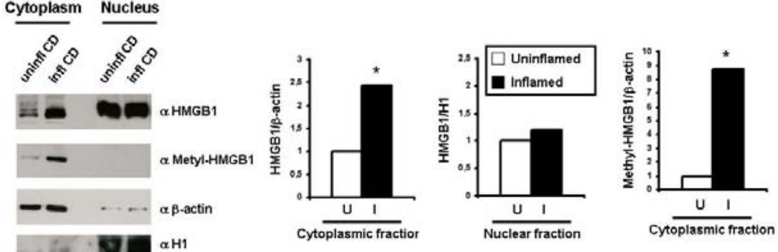

Fig. 6 - HMGB1 is translocated from the nucleus to the cytoplasm during inflammation. Methylated HMGB1 increases in cytoplasmic compartment of inflamed cells.

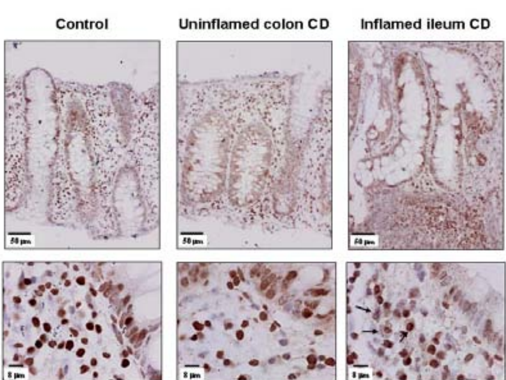

Fig. 7 - Immunohistochemical staining of HMGB1 intracellular localization in CD patients and controls.

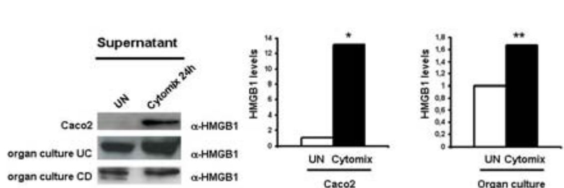

Fig. 8 - In vitro and ex vivo extracellular secretion of HMGB1 in response to pro-inflammatory stimuli.

SCIENTIFIC COLLABORATIONS

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- Dipartimento Scienze della Salute Pubblica, Università La Sapienza Roma

GRANT

Broad Medical Research Program (BMRP) No. IBD-0225R3 "Molecular characterization of mucosa-associated intestinal microbiota and intestinal innate immune response: searching for additional mechanisms in pediatric Crohn's Disease". 2008-2010.

NON-CARCINOGENIC EFFECTS OF COSMIC RADIATION, OXIDATIVE STRESS, AND POLYAMINES METABOLISM

Identification of specific biomarker for low doses of high LET radiation in mouse peripheral blood lymphocytes *in vivo*

Roberto Amendola, Teresa Bellissimo[*], Katarzyna Kobos[^], Emiliano Fratini°
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In the last decades, the studies on the space environment are intensifying, so it could be required a longer human presence in the space. The knowledge of the human risks is an increasing necessity to pursue, considering the longer periods away from the earth atmosphere. Very few information have been collected on the real risk of the exposure to low doses cosmic radiation, and how we can evaluate them under a biological profile. The interaction between cosmic ray and shielding materials of the spaceship produces neutrons as secondary radiation, so that neutrons are the main radiation inside the spaceship and it could be a risk factor for astronauts. Furthermore, neutron radiation is a risk factor for nuclear power station workers and long-range flight crew, and nowadays neutrons are increasingly used in radiotherapy becoming both a chance and a risk at the same time. In all cases of radiation exposure, skin reaction is a key diagnostic and prognostic factor to consider, and i*n vivo* experiments carried out in the past years by a 14 MeV Neutron irradiation, at Frascati Neutron Generator (FNG), evidenced a dose related onset of re-epithelialization program in charge of both keratin clusters and Ca-binding proteins. Since this expression profile resembles *psoriasis* acute phase, the hypothesis to perform experiments on biopsies from psoriatic patients is still under evaluation.
A screening of the transcriptional response of in vivo mouse peripheral blood lymphocytes to the two selected doses of high energy neutrons in function of the time from irradiation, have been performed on cDNA microarrays. Total RNA was prepared from lymphocytes (separated from whole blood) of 6 individual mice per experimental point. Five RNA pools are made from 6 individual mice's total RNA of each experimental point: RNA collected at 6 hours from 0.2 Gy irradiation, collected at 24 hours from 0.2 Gy irradiation, collected at 6 hours from 1 Gy irradiation, collected at 24 hours from the same dose irradiation, and sham-irradiated control group.
Statistical filtering and functional clustering of the data have been done using Gulphar and Multi Experiment Viewer (MEV) softwares.
Gene ontology analysis, by FatiGo+, does not show significant p-value for the association of modulated gene in biological process, molecular function, cellular component, and transcription factors. In many instances p-values have a quite low value, but they do not reach the significance threshold. Therefore, it is not possible to opine that neutron radiation modulates particular biological processes or functional classes of genes in peripheral blood lymphocytes.
Subsequently the genes, modulated in irradiated peripheral blood lymphocytes, have been compared with the data, obtained on peripheral blood mononuclear cells of both C57Bl/6 mice and human irradiated with 50 cGy, 200 cGy, or 1 Gy TBI as a single fraction from a Cs137 gamma source. This work, demonstrating that a mouse profile could predict human radiation exposure, suggests that further refinement of the mouse signature, including profiles derived from higher doses, could be effective in generating a dose-specific predictor of human radiation exposure.

In Fig. 1, the comparison that underlines some common data is shown.

0.2 Gy 6h

ID	Position	Chromosome	RefSeqID	GeneSymbol	Valore
uhnmcdna0000677	2B10	10	NM_009120	Sar1a	-0,746
uhnmcdna0012111	32D05	11	NM_008211	H3f3b	-1,517
uhnmcdna0016787	4N17	4	NM_027450	Glipr2	1.019
uhnmcdna0001450	4B20	16	NM_025800	Ppp1r2	-0.697
uhnmcdna0017022	5M12	7	NM_011247	Rbbp6	0.711
uhnmcdna0002272	6L08	1	NM_009418	Tpp2	-1.382
uhnmcdna0013779	36J06	6	NM_011777	Zyx	-0.645
uhnmcdna0001499	4J22	8	NM_011296	Rps18	-1.177
uhnmcdna0009398	25O04	11	NM_026428	Dcxr	0.66

0.2 Gy 24h

ID	Position	Chromosome	RefSeq	Gene Symbol	Valore
uhnmcdna0012016	32C08	17	NM_025963	Rps10	-3.452
uhnmcdna0001455	4D06	11	NM_011508	Eif1	1.0355
uhnmcdna0012111	32D05	11	NM_008211	H3f3b	-1.9925
uhnmcdna0021682	19K05	8	NM_133962	Arhgef18	0.787
uhnmcdna0009398	25O04	11	NM_026428	Dcxr	1.4035

1 Gy 6h

ID	Position	Chromosome	RefSeq	Gene Symbol	Valore
uhnmcdna0022050	20M07	11	NM_008211	H3f3b	-0.9775

1 Gy 24h

ID	Position	Chromosome	RefSeq	Gene Symbol	Valore
uhnmcdna0012016	32C08	17	NM_025963	Rps10	-1.311

Fig. 1 - Modulated genes that have been identified as hypothetical biomarker of irradiation in mouse blood. In the near future, these biomarker could have a diagnostic utility for neutron radiation exposure.

THESEUS: Towards Human Exploration of Space: a EUropean Strategy

Roberto Amendola

THESEUS is a 24 months coordination action funded by the European Commission Seventh Framework Programme (FP7). Past space missions in Earth orbit have

demonstrated that human beings can survive and work in space for long durations. However, there are pending technological, medical and psychological issues to be solved before adventuring in longer duration space missions (protection against ionizing radiation, psychological issues, behavior and performances, prevention of bone loss, etc). Furthermore technological breakthroughs, e.g. in life support systems and recycling technologies are required to reduce the costs of these expeditions to acceptable levels. The solution of these issues will need scientific and technological breakthroughs of interest for clinical and industrial applications and will also allow identifying the relevance of these questions to health issues on Earth. Despite existing ESA or NASA studies or roadmaps, Europe still has no roadmap approved by the European scientific and industrial communities.
The objective of THESEUS is to develop an integrated life sciences research roadmap enabling European human space exploration in synergy with the ESA strategy, taking advantage of the expertise available in Europe and identifying the potential of non space applications and dual research and development.

- Objective 1: Identify disciplinary research priorities;
- Objective 2: Focus on fields with high terrestrial application potential;
- Objective 3: Build a European network as the core of this strategy.

THESEUS comprises six Workpackages, and, in the first 12 months, a workshop has been taken to draw a consistent scheme of worldwide experts consulting relation on main topic and key-issues on different disciplines. At the end of the past year, questionnaires have been collected and harmonized to produce a first draft of experimental key. Past and up-coming activities are described and summarized at: http://theseus-eu.org.

Oxidative stress and polyamines metabolism

Roberto Amendola, Valerio Tanzi*, Marina Trippanera*, Katarzyna Kobos, Emiliano Fratini
*pre-graduate student

Role of chronic oxidative stress induced in the repair of defective cell lines by SMO
The aim of the project is to determine the existence of a hypothetical DNA damage threshold, which could trigger the cellular decision for DNA repair or apoptosis. Low level of constitutive DNA damage is accomplished by ectopical expression of murine Spermine Oxidase enzyme (mSMO), a component of the polyamines metabolism pathway. Low level of X-irradiations, as challenging doses of damage, will be evaluated in proficient and both transcription-coupled-repair (TCR), a sub-class of a nucleotide-excision-repair (NER) and base-excision-repair (BER) deficient cell lines.
Chinese hamster ovary parental cell line (AA8), the NER deficient counterpart (UV61), and the BER deficient counterpart (EM9) were transfected with mSMO, by retro-viral infection. Stable transfected colonies have been analysed for cell viability, apoptosis, gamma-H2AX and ROS over-production. Identical end-points have been evaluated after 1 and 10 cGy of low LET irradiation by X-Ray generator (Gilardoni, 250 KeV, 1.5 mA, dose-rate 0.1 Gy/min), performed at the ENEA Casaccia. Endpoints were determined at 6 and 24 hours after exposure.

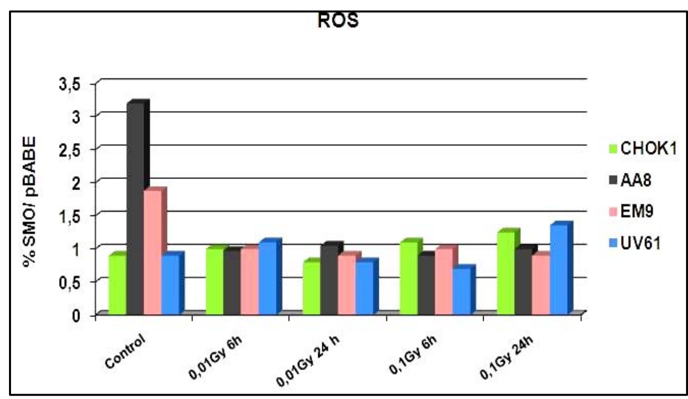
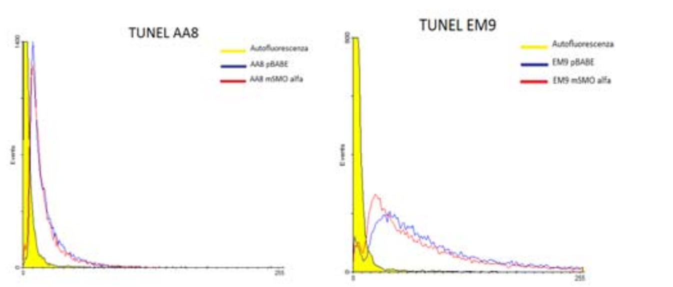


Fig. 2 - ROS ratio between mSMO and mock transfected cell lines evaluated by H2FDC-flow cytometry technique.



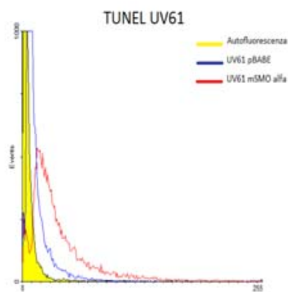


Fig. 3 - TUNEL ratio between mSMO and mock transfected cell lines evaluated by TUNEL-flow cytometry technique.

The priming dose of low level of ROS over exposure by mSMO did not provoke evaluable alteration on surviving capabilities on parental AA8, and, respectively, TCR (NER) and BER deficient UV61 and EM9 cell lines. Accordingly the mSMO over-expression increases the level of ROS, but below the threshold to induce DNA repair cellular commitment, mainly in the AA8 and EM9 cell lines (Fig. 2). Interestingly, only in the UV61 cell line, a higher level of apoptosis is present in parallel with mSMO over activity (Fig. 3). Since mammals SMO is differentially activated in a tissue specific manner, it could be of interest to gain more knowledge in determining if very low dose exposure of X rays could start DNA repair processes, taking advantage also of the experimental upcoming evidences in both NER and BER deficient cell line models.

Role of SMO and SSAT as breast cancer prognostic factors

Polyamine metabolism has a critical role in cell death and proliferation representing a potential target for intervention in breast cancer (BC). This study investigates the expression of spermine oxidase (SMO) and its prognostic significance in BC. Biochemical analysis of Spm analogues BENSpm and CPENSpm, utilized in anticancer therapy, was also carried out to test their property in silico and in vitro on the recombinant SMO enzyme. BC tissue samples were analyzed for SMO transcript level and SMO activity. Student's t test was applied to evaluate the significance of the differences in value observed in T and NT samples. The structure modeling analysis of BENSpm and CPENSpm complexes formed with the SMO enzyme and their inhibitory activity, assayed by in vitro experiments, were examined. Both the expression level of SMO mRNA and SMO enzyme activity were significantly lower in BC samples

compared to NT samples, as shown in Fig. 4. The modeling of BENSpm and CPENSpm complexes formed with SMO and their inhibition properties showed that both were good inhibitors. This study shows that underexpression of SMO is a negative marker in BC. The SMO induction is a remarkable chemotherapeutical target.

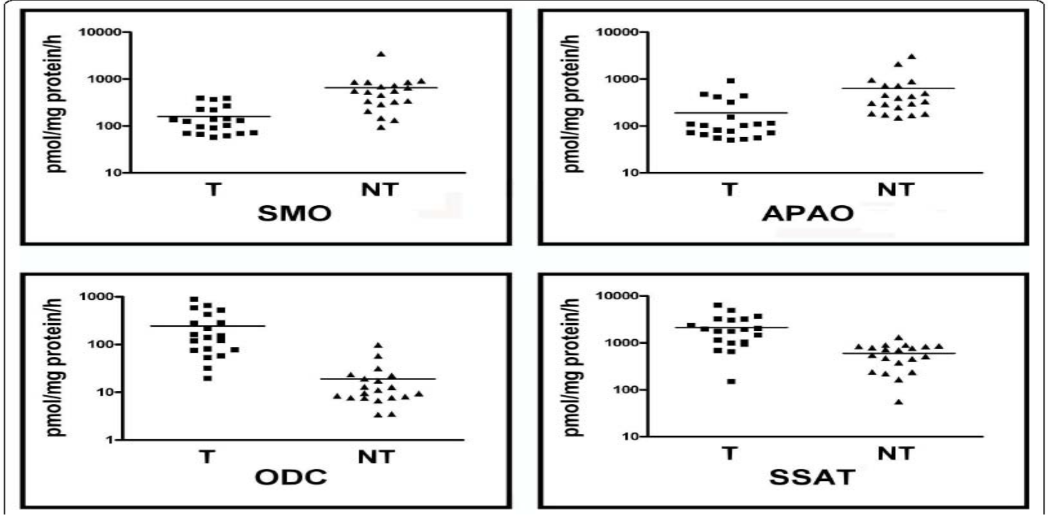


Fig. 4 - SMO, APAO, ODC and SSAT activities. Enzyme activities from tumor (T) and nontumor (NT) samples were assayed. Results are mean (SD) with n value of 20. The p values (<0.05) were measured with Wilcoxon matched pairs signed rank test.

SCIENTIFIC COLLABORATIONS

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GENETIC DAMAGE, OXIDATIVE STRESS AND PROTECTIVE ROLE OF ANTIOXIDANT TREATMENT IN IRRADIATED AND BYSTANDER CELLS

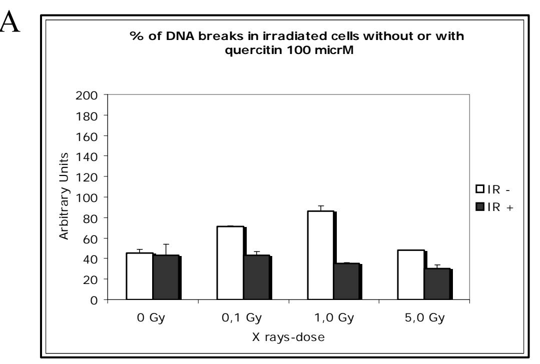
Anna Giovanetti, Tommaso Cornetta*, Akhilesh Datt Pandey^, Giorgia Aversa°, Giorgio Leter
*Post-doc, ^ENEA International fellowship, °pre-graduate student

These activiyties have been carried out in collaboration with Stefano Rufini, Tor Vergata University Rome, and Sandra Incerpi, University "Roma Tre" Rome.
Recent evidences have demonstrated that ionizing radiation (IR) can affect also cells not directly hit but in contact with the irradiated ones. This phenomenon was termed "bystander effect". A number of studies suggest that the irradiated cells transmit damage by direct intercellular communication or by indirect mediators such as long lived free radicals and cytokines released into the surrounding medium suggesting that bystander effect is associated with up-regulation of oxidative metabolism.
The occurrence of non-targeted effects may greatly modify the linear no-threshold concept (LNT), used to establish international rules of radiation protection, which states that the carcinogenic risk increases linearly with increasing dose; an eventual change of this concept has great potential impact in the managing of radiation exposure.
Due to the increased utilization of IR in human life, the development of efficacious radio-protector will be a fundamental contribution to radiation oncology and public health. IR induces ROS, which cause various DNA damages in directly irradiated or bystander cells, including strand breaks that lead to formation of chromosomal aberrations as well as increased number of apoptotic cells.
In order to individuate biomarkers specific for the bystander effect and the mechanisms underlying the transmission of the bystander signals, HaCat human keratinocytes, were irradiated with a Gilardoni 300 kV generator at increasing doses of X rays (0; 0.1; 1.0; 5.0; 15.0 Gy). One hour after their medium (ICM) was filtered (0,22 μm) and added to un-exposed cells, the bystander cells. Different experimental endpoints such as DNA fragmentation, ROS production, apoptotic index, were characterized in cells directly irradiated or treated with the conditioned medium. Finally, we have verified the protective effects of the antioxidant quercetin.
Results show that the ICM was able to induce DNA damage and increasing of ROS production and that treatment with quercetin induced a decrease of both the direct or bystander effects.

Evaluation of DNA fragmentation in IR and BE cells without and with the antioxidant quercetin

The alkaline Comet assay has been used to measure DNA fragmentation induced by X-Ray in cells directly irradiated or treated with ICM from the irradiated cells, with and without quercetin treatment. One hundred comets on each slide, coded and blindly scored, were acquired using an image analysis software or by visual scoring method.
The antioxidant quercetin was added to the HaCaT cells to be irradiated (IR) 1 h before treatment. In Fig. 1 is shown genetic damage in cells treated with X-rays and with the ICM from the irradiated cells, without or with quercetin treatment.

A



B

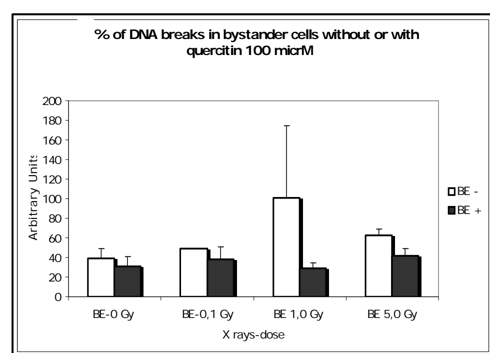


Fig. 1 – White and black bars represent cells treated without and with quercetin, respectively (100 μM). With both doses of quercetin, IR (A) and conditioned medium treated cells (B) show lower values of DNA damage.

In order to choose the quercetin concentration not inducing cytotoxic effects on HaCaT cells the MTT assay was performed. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay is a colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dye, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth.
Various enzymatic methods are used to detect oxidative DNA lesions. Fpg (formamido–pyrimidine–DNA–glycosylase) initiates the repair of oxidized bases (principally 8-oxoguanine from DNA, producing apurinic sites converted in breaks by the associated AP-endonuclease activity), by excising them and cutting the sugar-phosphate backbone of the DNA molecule. Introducing (Fpg) in Comet assay after the lysis step, makes it possible to detect oxidative DNA damage.

ROS generation in IR and BE cells

Reactive oxygen species (ROS) is a strong intracellular defense system against various pathogens including bacteria, virus, mycoplasma and generated naturally inside the cells. Evolution of ROS is a very complex and it intricates with several very important fabrics of cell physiology and cell signaling proteins/components.
Abnormal elevation of ROS level inside the cells is capable to kill the cells or it can trigger the apoptosis radiation-induced genomic instability (RIGI) due to high ROS level manifests as a heritable increased rate of genetic alterations in the progeny of irradiated cells generations after the initial insult. The progeny can show an increased frequency of chromosomal translocations, deletions, mutations, micronuclei, and decreased plating efficiency. What perpetuates RIGI is unclear; however, persistently increased levels of reactive oxygen species (ROS) are frequently associated with genomically unstable clones.
In this order first we tried to establish the basal level of ROS. We found that several factors affect the basal level of ROS like cell confluence, time period for cell culture medium incubation and contamination.
Any type of contamination could significantly increase the basal ROS level. Cell confluence also affects the ROS. When confluence level is more than 80%, it increases the ROS level and retards the cell growth.

In order to verify the capacity of generating ROS after irradiation or treatment with conditioned medium, HaCat cells were irradiated and ROS production was measured with FACS technique by using DCF dye. Interestingly we found that there is slightly dose dependent increase of ROS level due to treatment of direct ionizing radiation (IR) at 0 Gy, 0.1 Gy, 1 Gy and 5 Gy **(**Fig. 2**)**.

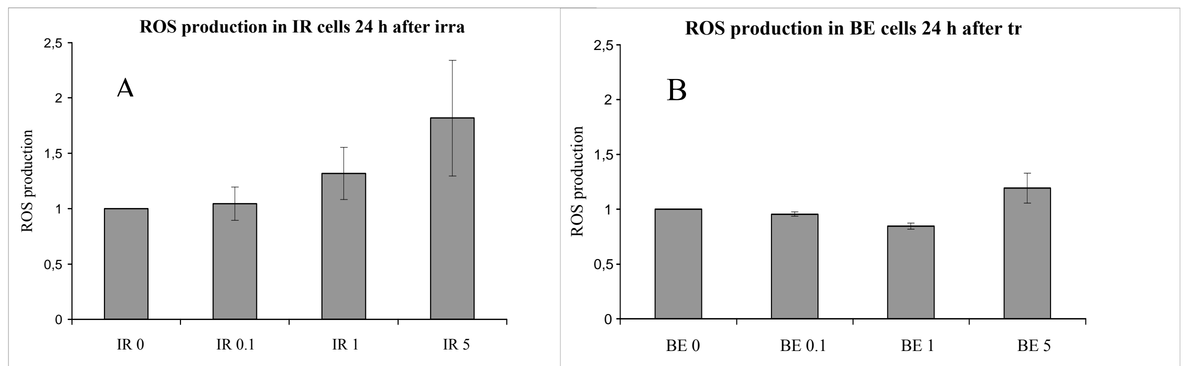


Fig. 2 – A) ROS levels in IR cells after direct ionizing radiation 0 Gy, 0.1 Gy, 1 Gy and 5 Gy. Data represent mean of three repeated independent experiments. **B)** ROS levels in BE cells from 0 Gy, 0.1 Gy, 1 Gy and 5 Gy.

In order to better discriminate between the different experimental points ROS generation was triggered by the Lipopolysaccharide (LPS) of gram negative bacteria (E.coli, Sigma) 5 μg/ml. Preliminary results show that LPS enhance the differences in ROS production between 0 and 1 Gy IR cells (data not shown).

Apoptotic index IR and BE cells

A main effect of ionizing radiation is the induction of apoptosis. Apoptotic index (AI) is a measure of the number of apoptotic events or cell deaths expressed as a ratio or percentage of all cells present or all cells counted. Cell apoptosis was measured in cells irradiated with 0; 0.1 and 1 Gy of X rays (IR) and in cells treated with medium from the irradiated ones (BE) by light microscopy and DAPI (diamidino-2-phenylindole) staining. DAPI is a DNA-specific dye that displays a blue fluorescence. This dye can pass through intact, living cell membranes, but apoptosis increases cell membrane permeability and uptake of DAPI, leaving a stronger blue stain. In addition, the nuclear morphology of normal cells is round, clear-edged, uniformly stained. Apoptotic cells show irregular edges around the nucleus, chromosome concentration in the nucleus, heavier coloring, and, with nuclear pyknosis, an increased number of nuclear body fragments. For these reasons, the intensity of the fluorescence can help to identify apoptotic cells.
Results showed a dose-dependent increasing of AI in the directly irradiated cells while no correlation with dose was observed in BE cells (data not shown).

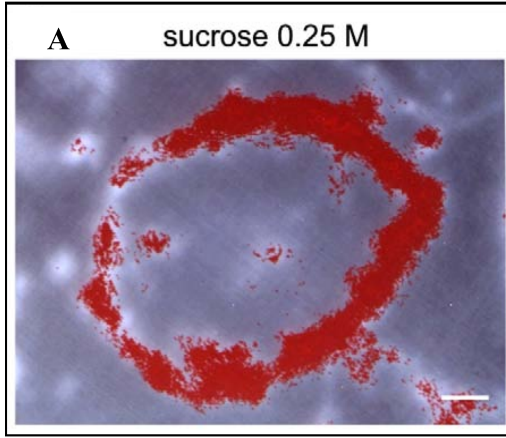
The role of cellular membrane's rafts in transmitting bystander signalling

Rafts are cell membrane's microdomains enriched in cholesterol and sphingolipids regulating signal transmission. Experiments have been carried out to assess the transmission of bystander effect after depletion of sphingolipids or cholesterol. In

particular irradiated cells were treated with cyclodextrin (CD) to obtain depletion of rafts cholesterol. Comet Assay was performed to evaluate differences in DNA damage in cells treated with medium from the irradiated ones, with and without CD. If CD is added to the cells to be irradiated the bystander damage decreased.

Nuclear Shield: a multi-enzyme task-force for nucleus protection

In eukaryotic cells the nuclear envelope isolates and protects DNA from various molecules that could accidentally damage its structure or interfere with its processing. Cytoplasm and nucleoplasm also contain several enzymes designed for nucleus preservation. The aim of this research work was to verify the existence of an additional protection machinery localized at the nuclear membrane and formed by an hypercrowding of cationic enzymes linked to the nuclear envelope by electrostatic interactions. Electron spectroscopic imaging, zeta potential measurements, isoelectrofocusing and mass spectrometry analysis were used to characterize this surprising structure that behaves as a sort of "Nuclear Shield" (Fig. 3A). In fact, key protection enzymes like glutathione transferase, catalase and also glutathione peroxidase are highly represented. In the nuclear shield the local concentration of these antioxidant enzymes is enhanced up to seven times with respect to the cytosol (Fig. 3B), and provides significant protection of the genetic material against oxidative damage. To assess if the depletion of this "nuclear shield" increased the sensitivity of DNA to oxidative damage, isolated nuclei with and without shield were exposed to hydrogen peroxide, DNA damage was analysed by Comet assay. As shown in Fig. 4, the depletion of the shield increased the amount of genetic damage measured as percentage of DNA breaks.



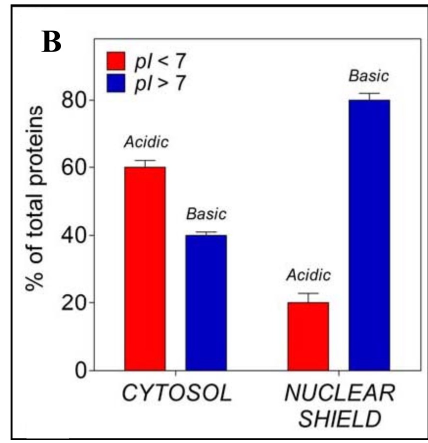


Fig. 3 – A) Quantification of membrane area and proteins of the nuclear shield in different tissues and ESI of isolated rat liver nuclei. The putative thickness was calculated assuming a strictly packed multilayer structure (density = 1 g/cm^3), a mean diameter of 5 nm and a mean molecular mass of 50 kDa for the shield proteins; **B)** Percent of total acidic and basic proteins in cytosol and nuclear shield from proteomic analysis.

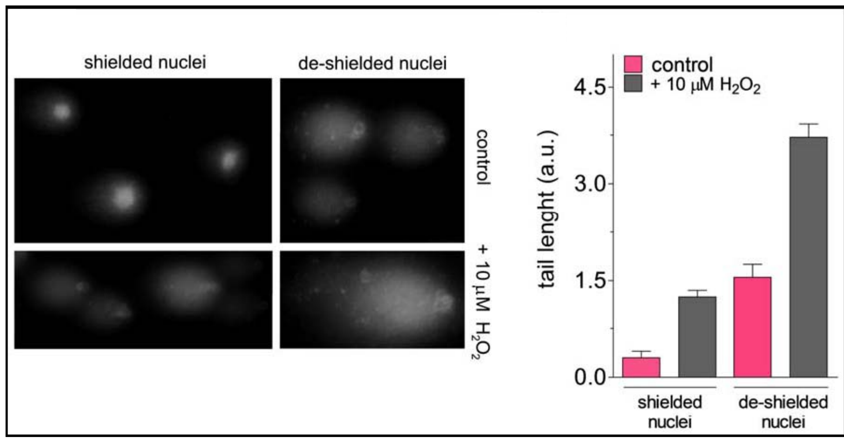


Fig. 4 - Comet assay on shielded and de-shielded nuclei exposed or not exposed to 10 µM H_2O_2

The next objective of this collaboration is to verify the effects on "nuclear shield" induced by ionizing radiation and if the shield plays a role in the low doses un-targeted effects such as bystander effect or adaptive response.

Use of Saccharomyces cerevisiae as system model to study human Topoisomerase Ib role in DNA damage induced by low doses ionizing radiation

Previous studies have shown that human Topoisomerase Ib plays a key role for the DNA single and double strand breaks formation and relegation as a response to various DNA damages in vivo but its role still remains unclear. In the eukaryotic *Saccharomyces cerevisiae* Topoisomerase Ib is not essential, thus this organism has been utilized for structural and functional studies on this enzyme.
Therefore we have decided to utilize this biological model to verify the possible interaction between radio-induced ROS, Topoisomerase Ib and DNA damage. On *Saccharomyces cerevisiae* cells exposed to 30 Gy of X rays are analysed: the extend of DNA damage by single cell gel electrophoresis (comet assay), cell viability, Topo Ib activity by the DNA Relaxation Assay and Gel Shift Assay. Our data indicate that the inactivation of Topo Ib increased the extent of DNA damage following ionizing radiation. Results of this collaborative research are still being processed.

SCIENTIFIC COLLABORATIONS

- Dr Silvia Castelli (Tor Vergata University, Rome, Italy)
- Dr Marco Chianelli (Regina Apostolorum Hospital, Albano, Italy)
- Prof Alessandro Desideri (Tor Vergata University, Rome, Italy)
- Prof Sandra Incerpi (Rome Tre University, Rome, Italy)
- Dr Valentina Pallottini (Rome Tre University, Rome, Italy)
- Prof Giorgio Ricci (Tor Vergata University, Rome, Italy)

- Prof Stefano Rufini (Tor Vergata University, Rome, Italy)
- Prof Antonella Sgura (Rome Tre University, Rome, Italy)
- Prof Alberto Signore (University of Rome La Sapienza, S Andrea Hospital)
- Prof Giuseppe Valacchi (Università di Siena)
- Dr Zeenat Jahan (Tor Vergata University, Rome, Italy)

Maria Balduzzi

Study on the contribution of human small intestine to the overall first-pass metabolism of Clorpyrifos

This study was conducted at the Department "Mechanisms of Toxicity" of the Environment and Primary Prevention Unit of the Istituto Superiore di Sanità (Roma), scientific leader Dr. E.Testai, and financed by the ISS.
In the framework of this research program, the objective of my specific aim section was to characterize the P450 profile of human microsomes from the duodenal (HDM)/jejunal (H2SM) portion of individual donors by means of quantitative western blotting (Fig. 1).
The cytochromes P450 catalyze the biotranformation of both endo- and xeno-biotics and are concentrated prominently in the liver. Although it has been demonstrated that enteric CYPs can contribute significantly to the metabolism of several drugs, the P450 expression in the small intestine mucosa has not yet fully characterized.
In the present study, human intestinal preparations were analyzed for cytochromes P450 3A4, 3A5, 2C9, 2C19, 2B6 and the average specific content, or lack of detection, of each P450 was determined. Moreover, the individual variation in CYPs contents has been systematically compared with their catalytic activity, as shown in the figure below, where microsomal samples from the same donor were analyzed for chlorpyrifos (CPF) bioactivation to the toxic metabolite oxon (CPFO), and for CYP3A4 expression.
Results show that large interindividual variations exist in the expression levels of individual P450s, CYP3A4 and CYP2C9 representing the most abundant isoforms expressed in the small intestine.
All the experimental work is completed and a journal paper is now under preparation.

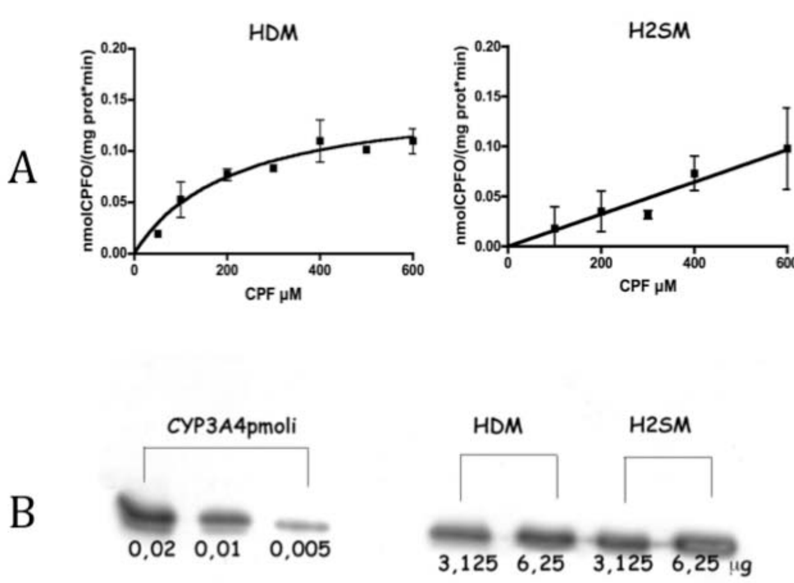


Fig. 1 - Kinetic curves (A) and western blot (B) of corresponding HDM/H2SM samples.

Evaluation of curcumin neuroprotective ability in primary retinal cultures

This study was conducted at the Department “Cell Biology and Neuroscience” of the Istituto Superiore di Sanità (Roma).
The aim of this study was to characterize the neuroprotective ability of curcumin, a phenolic compound extracted from the rhizome of Curcuma longa, and to analyze its effects on NMDA receptor (NMDAr). NMDAr modifications were observed in primary retinal cell cultures using immunocytochemistry, whole-cell patch-clamp recording and western blot analysis. Cell death was evaluated with the TUNEL assay in primary retinal and hippocampal cultures. Optical fluorimetric recordings with Fura 2-AM were utilized to monitor [Ca2+]i. Results show that curcumin dose- and time-dependently protected both retinal and hippocampal neurons against NMDA-induced cell death, possibly related to an increased level of NR2A, confirming its anti-excitotoxic property and a possible therapeutic use of curcumin based on neuromodulation of NMDArs.
Fig. 2 shows the immunocytochemical characterization of retinal cell cultures while in the lower panels the effects of curcumin on NR1 phosphorylation is shown as pNR1 immunoreactivity.

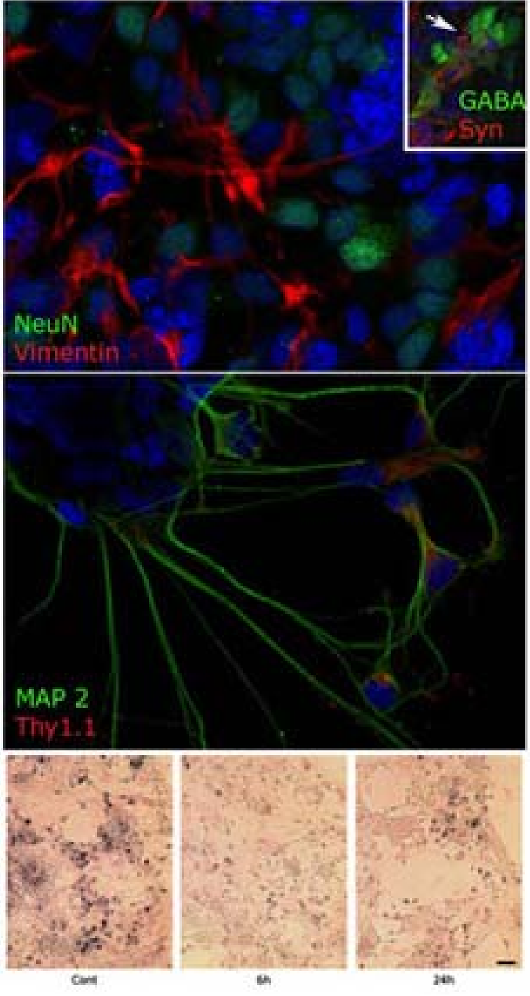


Fig. 2 - Immunocytochemical characterization of retinal cell cultures (upper panels). The effects of curcumin on NR1 phosphorylation is shown as pNR1 immunoreactivity in lower panels.

Research activities in the fields of in vitro radiobiology preliminary to the TOP IMPLART project

The TOP IMPLART (Oncological Therapy with Protons in Italian – Intensity Modulated Proton Linear Accelerator for RadioTherapy) is a large project to be developed over several years financed by the Regione Lazio and involving ENEA, ISS and IRE. The project requires a deep insight into basic and applied research topics, together with the comparison of the various acceleration techniques, the construction of prototypes and a thorough study of the clinical effectiveness and of the treatment planning techniques. Complementary to the TOP project is the implementation of research in the field of in vitro biological effects of protons irradiation taking advantage of the novel accelerator facility. The experimental work accomplished till now is related to the characterization of cell lines suitable for the purpose. Human fibroblast cell lines and primary human endothelial cells have been irradiated with α- and γ-rays and dose-related effects have been detected such as γ-H2AX and micronuclei formation. Experiments on adaptive response induced either by low dose of γ-rays and bystander factors released after a-irradiation are in progress.

SCIENTIFIC COLLABORATIONS

- Malchiodi Albedi F., Department Cell Biology and Neuroscience, Istituto Superiore di Sanità (Roma)
- Tabocchini M. A., Department of Technology and Health of the Istituto Superiore di Sanità (Roma)

NATURAL COMPOUNDS GROUP FOR HUMAN AND ANIMAL HEALTH

Fiorella Carnevali, Stephen Andrew van der Esch, Tiziana Coccioletti, Franca Mozzini

Antiviral activity against Orf virus of a medical device (MIX 577) based on Neem (*Azadirachta indica* (A. Juss)) and St John's Wort (*Hypericum perforatum* (L.)) extracts

Orf virus (OV) is the prototype of the *Parapoxvirus* genus and is the causative agent of an exanthemous disease in sheep and goats known as contagious pustular dermatitis or also *ecthyma contagiosum*, which is a zoonotic disease. Orf virus infection is a professional zoonotic disease that interests mainly those workers who come in direct contact with infected animals or their products. The virus penetrates through skin lesions and replicates in epidermal cells, the lesions become evident and progresses from a rash towards a pustular stadium ending up with crust formation. The crust, which contains millions of viral particles, dries up and detaches from the infected animal thus contaminating the surrounding environment. No authorised drugs exist for the treatment of *ecthyma contagiosum* in animal as in human although synthetic products from the acyclic nucleoside phosphonate family exist for which the efficacy *in vitro* has been demonstrated. In this study the antiviral efficacy of a medical device based on natural substances extracted from the neem tree (*Azdirachta indica* (A. Juss)) and *Hypericum perforatum* (L.) has been evaluated. The results demonstrate that the wound healing properties of this medical device might favour a faster healing of the treated animals while the demonstration that it has anti-viral properties deserves further attention.

The Bari Experiment (TBE)

Wound healing relies on a complex sequence of cellular and biochemical events. It is based on a regenerative system for restoring the anatomy and function of injured tissues. The regeneration is not complete because the resulting scar tissue does not have the same anatomy and function as the original one.
A combination of plant extracts of Neem and St John's wort in plant oil was extensively tested for its capacity to properly regulate the complex events of the wound healing process. The aim of this study was to further investigate the efficacy of the extract combination to promote the healing process on full thickness skin excision wounds performed on 5 adult sheep. The skin-excised areas were treated either with the extract combination (Mix of 50% neem oil and 50% hypericum oil), the two single extract components (NO, neem oil, 100 %; HO, hypericum oil, 100 %) or a disinfectant as control (0.5% NaOCl). The healing rate was calculated using the Gilman's equation, and the histological (HI) and immuno-histochemical (IHC) parameters were evaluated.
The results demonstrated that healing quality differed significantly depending on the treatment composition. The highest quality wound healing and the lowest extent of fibrosis was observed in the group treated with the extract combination (MIX), whereas the poorest wound healing and highest fibrosis was observed in the control group. The groups treated with the two single extract components (100% NO and 100% HO) gave

intermediate wound healing performances (van der Esch S.A., Carnevali F., The Bari Experiment – Discussion Document).

Medical Devices for the treatment of Pressure Ulcers: State of the Art

Aim of the document is to describe the state of the art on Pressure Ulcer therapy (identification, clinical evaluation and treatment) focusing attention on the different types of wound dressing available. The purpose is to propose a "Comparative Clinical Trial" of a new dressing based on the ENEA Patent 557 (Carnevali F., van der Esch S.A. Prova clinica su Ulcere da Pressione – Discussion Document).

Effectiveness of a wound healing remedy (ENEA Patent 48211BE/2008) for healing cutaneous wounds: an experimental study in sheep

ENEA patented a remedy (n° 48211BE/2008) of natural extracts in oil from plants (*Azadirachta indica*, named Neem and *Hypericum perforatum*, named St John's wort), seems to possess the capacity to properly regulate most of the complex events of the healing process. Aim of this study was to verify the effectiveness of the remedy on sheep in promoting the healing process after performing full thickness skin excision from the back of 5 adult sheep. The interested areas were treated with the remedy, the two single components and a disinfectant working as control group. The healing rate was calculated using the Gilman's equation and the histological and immunohistochemical parameters were evaluated. The results showed no significant differences in the healing rate among the four groups, while the healing quality differed significantly. The highest quality and the lowest fibrosis was obtained in the group treated with the remedy, whereas the poorest performance was observed in the control group with the highest fibrosis; the groups treated with the two single components showed intermediate performances. The remedy proved to be an "ALL IN ONE" remedy, which could be applied in any phase of the healing process.

SCIENTIFIC COLLABORATIONS

- Università di Camerino, Dipartimento di Chirurgia e Anatomia Patologica e Dipartimento di scienze Naturali
- Università di Bari, Dipartimento di Chirurgia
- Università di Perugia, Dipartimento di Chirurgia
- Università di Firenze Dipartimento di Chimica Farmaceutica
- Reggimento Carabinieri a Cavallo, infermeria quadrupedi
- Reggimento Corazzieri, Quirinale
- Ospedale Veterinario dell'Esercito di Montelibretti
- Policlinico Gemelli, Dermatologia e Chirurgia
- Clinica Villa Grazia, ricovero per malati di Alzheimer
- Clinica Della Divina Provvidenza e Del Buon Pastore (ricovero per anziani)
- Ospedale San Camillo, Chirurgia Vascolare

- Ospedale Forlanini, Reparto terapia intensiva
- Istituto Nazionale di Ricerca e Cura per Anziani (INRCA)
- Casa di Cura Madonna del Rosario, Civitavecchia
- Istituto IRCCS Don Gnocchi, Roma
- Veterinari Liberi Professionisti
- Canili di Roma

TECHNOLOGY TRANSFER ACTIVITY REGARDING ENEA PATENT 557

- Seminari di presentazione effetti Brevetto Enea (Università di Bari, Università di Padova, Università di Parma, Università di Perugia)
- Partecipazione con RIMOS (licenziatario brevetto Enea) a Congresso SCIVAC Piccoli Animali Rimini e Congresso SIVE
- Bergamo Circolo Veterinario Bergamasco. 3° Seminario Multisala 2010, Seminario Hypermix: meccanismo d'azione e presentazione di casi clinici
- Promozione Hypermix con Rimos SEMINARIO "MEDICINA D'URGENZA NEL CAVALLO ADULTO" (SIVE) FISE LOMBARDIA c/o Univ. Stat. di Milano - sede "grandi animali – Lodi
- Malpensa Cavalli, Milano
- Promozione Hypermix con Rimos Roma Cavalli, Fiera di Roma
- Promozione Hypermix con Rimos Verona Cavalli, Verona
- Formazione informatori farmaceutici promotori di Hypermix per Rimos, Nova Siri
- Formazione informatori farmaceutici promotori di Hypermix per Rimos, Roma
- Promozione Hypermix con Rimos F.I.S.E. COMITATO REGIONALE LOMBARDO
- Promozione Hypermix con Rimos HORSE EMERGENCY Sede Congressuale FISE, Roma
- Congresso Wound Care Veterinario, Padova nell'ambito del Congresso AIUC

GRANTS

Impiego della Medicina Integrata (fitoterapia) in modelli di produzione primaria/Use of integrated medicine in primary animal production models (phytotherapy)/Numero identificativo = IZS LT 04/09 RC.

Messa a punto di un protocollo innovativo per la prevenzione della moria degli alveari/Development of an innovative protocol for the prevention of the hive mortality – NEWPROBEE.

LABORATORY OF TOXICOLOGY

Francesca Pacchierotti, Lab Director

The Laboratory of Toxicology carries out research in the area of environmental toxicology, epidemiology, and occupational safety, conducting laboratory investigations, human biomonitoring and epidemiological studies. A special attention is given to the assessment of reproductive health hazards.

One of the main goals is the development or improvement, and standardisation of genetic and epigenetic biomarkers of semen alterations, for human health monitoring applications, livestock breeding programmes, and experimental studies under controlled exposure conditions. Comparative analyses of the performance of different testing methods and biomarkers are conducted to understand their range of applicability and functional significance.

Laboratory experiments investigate the mechanism of action and the effects of ionizing radiation in reproductive cells and, in collaboration with the National Institute of Health, evaluate the genotoxicity of food and environmental contaminants. To this aim, mouse models are used, including some that are genetically defective in DNA repair. The specificity of DNA Damage Response in male mammal germ cells is characterised by early molecular, cytological and histological biomarkers and untargeted effects of radiation are mechanistically investigated. In 2010, a collaboration with the Technical Unit Material Technologies has started to investigate biological interactions and potential toxicity of nano sized materials for biomedical and industrial applications.

In the frame of a long standing collaboration with Scandinavian research Institutes and European Commission funded projects, a large biomonitoring study of human semen is ongoing to evaluate global sperm DNA methylation levels. The final aim is to possibly relate variations of DNA methylation levels with chemical, biological and epidemiological parameters describing environmental exposure and reproductive health.

In collaboration with the Technical Unit on Renewable Energy Sources, a research and education programme that aims at reducing the environmental impact from cigarette butts has been initiated. In fact, this has been estimated to be a major overlooked source of widespread toxic chemical contamination with potential impact on living organisms. A networking activity has been undertaken to involve in a synergistic effort all the major stakeholders, including local and national administrations, industry, Environmental Protection and Health Agencies, no-profit associations, Universities and the High School Educational system.

Epidemiological studies, aimed at identifying environmental sources of hazardous exposures and describing geographical and temporal variations of mortality causes in Italy, are carried out by means of the ENEA mortality data-base, shared and run in collaboration with the Technical Unit Environmental Technologies. The searchable data-base contains general and cause specific mortality data recorded and codified by the National Institute of Statistics for all Italian municipalities. Recently, an updating programme with the most recent available mortality data has been undertaken.

The Laboratory collaborates with national and European University hospitals in designing and statistically evaluating clinical epidemiological studies in the area of molecular oncology aimed at assessing the diagnostic and prognostic significance of

specific cellular markers. Through its medical statistics expertise, it also participates to the ENEA Programme on Cardiovascular Disease Prevention.

In the field of occupational safety, the Laboratory has a long standing collaboration with Academia and Public Health Services addressing the implementation of good practices in various occupational settings, with special attention to gender issues. In addition, a recent study lead to the development of novel quantitative methodologies to assess occupational health risks in the woodworking industry.

REPRODUCTIVE HEALTH

Development and application of novel epigenetic biomarkers to assess the impact of environmental contaminants on reproductive health

Marcello Spanò, Giorgio Leter, Patrizia Eleuteri, Maria Giuseppa Grollino, Claudia Consales, Barbara Benassi, Cecilia Bartoleschi, Maria Chiara Pardini, Raffaella Uccelli, Michele Rescia[*], Antonia Di Caprio[^]
[*]Post-graduate fellow, [^]Urbino University graduate student

The Laboratory participation to the EU 7th FP Project "CLEAR – Climate change, Environmental Contaminants, and Reproductive Health", started in 2009. CLEAR is a research project aiming at investigating the possible impact of global climate change on reproductive health in Arctic (Greenland Inuits) and in two local European populations (Warsaw, Kharkiv). The key questions addressed are: (i) how may climate change impact on human exposure to widespread environmental contaminants; and, (ii) how may contaminants impact on occurrence of reproductive disorders as sensitive indicators of health. CLEAR, building upon 3 established cohorts in Greenland, Poland and Ukraine, will (i) identify and describe mechanisms by which a changing climate may affect the exposure of Arctic and other human populations to contaminants through change in chemical use and emissions, delivery to the Arctic ecosystem as well as processing within the Arctic physical environment and human food chain; (ii) expand the existing knowledge database on human exposure to POPs, metals, polybrominated diphenylethers, perfluorinated surfactants and phthalates by analyses of 1000 biobanked serum samples collected in the EU FP5 programme INUENDO (in which ENEA was also partner); (iii) increase the limited knowledge on links between human exposure to contaminants and reproductive health. The Laboratory of Toxicology, which has already measured sperm genetic integrity in these samples, shall measure the sperm DNA global methylation level (DGML) in some 200 samples representative of the 3 cohorts to investigate possible epigenetic effects of environmental exposures. The resulting values will be entered into the CLEAR general database and correlated with an array of chemical, biological, and epidemiologic parameters.

Due to the big number of samples to measure, an economically competitive method based on flow cytometric (FCM) immunocytochemical detection of methylated cytosines has been selected as the default option to estimate DGML. There is a limited record of publications on the application of this method to human sperm. Thus, a phase of optimization and standardization of the method has been undertaken. The critical issues identified and tackled were the indirect immunostaining procedure and the chromatin denaturation steps. A variety of denaturing conditions have been deployed aiming at making sperm chromatin structure more homogeneously accessible to the antibody binding. Particular care was also devoted to minimize washing and centrifugation steps to reduce unpredictable cell losses. Using ad hoc reference samples, efforts were focalized to test the overall performances in terms of optimal fluorescent signal-to-noise ratio associated with an acceptably low (<20%) level of measurement stability and repeatability. The introduction in the protocol of the Zenon technology to fluorescently label the IgG1 mouse anti 5-mC monoclonal antibody coupled to the use of high salt buffers containing reducing agents resulted in a net improvement of both intra-day and inter-day reproducibility of the DGML measurements: in the former case CVs <7% and in the latter case <14% were obtained. These figures represent an

essential requirement to provide sound and reliable measurements of the samples to be evaluated, presently stored in the INUENDO biobank.
The application of additional methods to estimate genome-wide DNA methylation levels in sperm is also being explored. This activity has been carried out in collaboration with Gunnar Toft (Aarhus University Hospital, Aarhus, Denmark).

Application of Sperm Chromatin Structure Assay to assess sperm quality in livestocks

Marcello Spanò, Patrizia Eleuteri, Claudia Consales, Barbara Benassi, Cecilia Bartoleschi, Maria Chiara Pardini, Eugenia Cordelli, Paola Villani, Michele Rescia,

Within a project funded by the Italian Ministry of Agriculture, Food and Forests (RiproSel), aiming at defining the semen fertility potential of bulls and boars, the FCM Sperm Chromatin Structure Assay (SCSA) was applied to sperm samples from selected animals coming from the Istituto Sperimentale Italiano 'Lazzaro Spallanzani", Rivolta d'Adda (Cremona). Assisted reproductive technologies (ART) are widely applied in the reproduction of economically relevant livestocks, cattles and boars included. Conventional semen quality analysis, essentially based on microscopic observations, is believed to be insufficient to adequately predict the fertilization chances. In analogy with what is happening for humans, the assessment of sperm genetic integrity can complement semen quality characterization and offer a new biomarker of the fertility potential. Among the tests recently developed to detect sperm DNA breaks (TUNEL assay, Comet assay, Sperm Dispersion Test), SCSA has certainly been the most applied in human and veterinary reproductive sciences. The assay indirectly detects DNA strand breaks and chromatin alterations by measuring increased susceptibility of sperm nuclei to acidic denaturation in situ. The activities of the year focused on the analysis of cryopreserved bull semen from 50 different animals. The results, expressed in terms of DFI (DNA Fragmentation Index, a parameter mirroring the sperm fraction with DNA breaks) and HDS (High DNA Stainability, a parameter reflecting the immature sperm fraction) showed strikingly low levels of chromatin structural damage suggesting that breeding selection produced animals with high semen quality also characterized by quite a negligible fraction of sperm with defective chromatin. The values obtained for each animal has entered a database where reproductive performances will be evaluated and associated to a variety of biomarkers, SCSA parameters included. Furthermore, a parallel, independent assessment of bull sperm chromatin integrity on a selected group of animals was carried out using the TUNEL assay, a more direct and specific assay for single- and double-strand breaks detection and the alkaline Comet assay. The preliminary results indicate a close correlation between the 3 techniques meaning that, even if the DNA integrity is approached by different points of view, the overall damage estimate is consistently similar.
Preliminary experiments have been carried out to evaluate the possibility to analyze non-coding RNAs in sperm as a novel epigenetic biomarker of livestock sperm quality.
This activity has been carried out in collaboration with Donatella Balduzzi and Andrea Galli (Spallanzani Institute, Cremona).

SCIENTIFIC COLLABORATIONS

- Centre for Arctic Environmental Medicine, Nuuk, Greenland
- Department of Environmental and Occupational Medicine, Aarhus University, Denmark
- Department of Environmental Toxicology, National Institute of Hygiene, Warsaw, Poland
- Department of Occupational and Environmental Medicine, Lund University Hospital, Sweden
- Department of Physical and Environmental Sciences, University of Toronto Scarborough, Canada
- Spallanzani Institute, Cremona
- Italian National Institute of Health, Department of Environment and Primary Prevention, Rome
- Italian National Institute of Health, Department of Veterinary Public Health and Food Safety, Food and Veterinary Toxicology Unit, Rome
- National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
- National Medical University, Kharkiv, Ukraine
- Reproductive Medicine Centre, Skåne University Hospital, Malmö, Sweden
- School of Health Systems & Public Health, University of Pretoria, South Africa
- TNO, Nutrition and Food Research, Zeist, The Netherlands

GRANTS

European Union, VII Framework Programme. Project "Climate Change, Environmental Contaminants and Reproductive Health – CLEAR" (www.inuendo.dk/clear)

Italian Ministry of Agriculture, Food and Forests, Research Project 2007-2010 "Reproduction and Selection" – RIPROSEL (www.riprosel.it)

RADIATION TOXICOLOGY

Genomic instability induced by ionizing radiation in male germ cells

Eugenia Cordelli, Cecilia Bartoleschi, Barbara Benassi, Patrizia Eleuteri, Maria Giuseppa Grollino, Maria Chiara Pardini, Paola Villani, Marcello Spanò, Orsio Allegrucci, Francesca Pacchierotti, Edoardo V. Di Caprio[*], Lorena Paris[^], Tullia Salvitti[^]
[*]Urbino University graduate student, [^]La Tuscia University graduate student

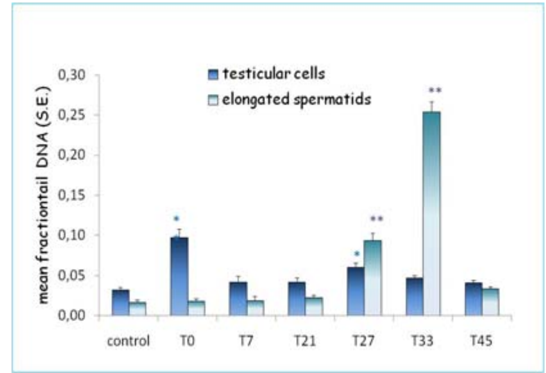
The specificity of male germ cells in their response to ionizing radiation is investigated. In particular, radiation induced double-strand breaks (DSB), their repair pathways and the molecular mechanisms underlying genetic instability are studied. Comet assay and immunodetection of phosphorylated H2AX (γ-H2AX) were applied to detect the production of DNA strand breaks in testicular cells and spermatozoa after in vivo X-ray irradiation. The level of DNA damage was measured throughout the whole spermatogenic process from spermatogonia to mature spermatozoa. Moreover, the gene expression profile by cDNA macroarray was analyzed in irradiated germ cells at different times after treatment. The expression of transcripts specific to different germ cell types was used to evaluate and follow the cellular changes induced by irradiation and subsequent post irradiation recovery; the expression profile of apoptotic genes was evaluated to verify the hypothesis that apoptotic processes could be involved with DNA fragmentation observed long times after irradiation.

Results obtained by comet assay in testis cells at different times after 4 Gy X-ray irradiation showed that irradiation did not enhance the fraction of tail DNA in elongated spermatids. On the contrary a clear induction of DNA damage immediately after irradiation was observed in the other testicular cells. Most of the damage was repaired shortly after irradiation but the presence of DNA damage was again observed at longer times. Elongated spermatids, in which no damage had been detected immediately after irradiation, showed a marked increment of DNA migration starting at 27 days, peaking at 33 days and decreasing back to control level at 45 days after irradiation (Fig. 1). It is noteworthy that, according to spermatogenic cycle, elongated spermatids analyzed at these timepoints derive from irradiated differentiating spermatogonia. Similarly to elongated spermatids, spermatozoa showed an increase of DNA damage starting from 27 days after irradiation. A further increase was observed afterwards with a peak at 45 days (Fig. 1).

H2AX phosphorylation in spermatozoa was analyzed by flow cytometry immediately, 1, 33 and 45 days after irradiation (Fig. 2). As for comet assay, immediately and short time after irradiation the level of phosphorylation and the percentage of positive spermatozoa were not increased over control values, while an increase of these parameters was observed at 33 and 45 days after irradiation.

Affymetrix macroarray analysis was performed in RNA samples extracted from testes of untreated mice, or 4 Gy irradiated mice 1 or 27 days after treatment (Fig. 3).

Direct comparison of the gene expression profiling of control mice versus 24 hours treated animals revealed that no significant changes occured by 24 hours following the end of the treatment. Only 4 transcripts (out of the about 30,000 probe sets spotted in the array) were down-regulated (P values ≤ 0.05, fold change ≥ 2) in the treated *vs* the untreated mice.



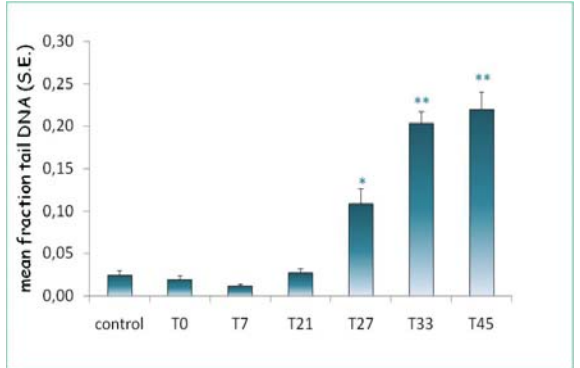


Fig. 1 - Comet assay results. Effects of 4 Gy X-irradiation on testicular cells (left) or epidydimal spermatozoa (right) assessed immediately (T0) or at different times after treatment. Asterisks indicate values significantly different from control (*$p \leq 0.05$; **$p \leq 0.0001$).

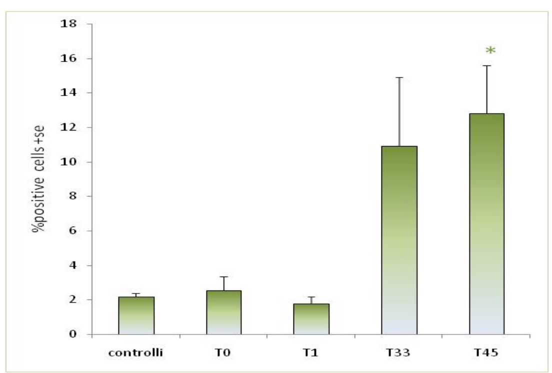


Fig. 2 - γ-H2AX in epidydimal spermatozoa. Flow cytometric detection of γ-H2AX immunostaining of epidydimal spermatozoa in controls or at different times after 4 Gy X-irradiation. Asterisk indicates a value significantly different from control ($p \leq 0.05$).

A complete different gene signature was triggered by irradiation at 27 days post-treatment. Heat map representation (Fig. 3) of the whole set of samples clearly showed that the testis transcriptoma fingerprint was significantly modified in the irradiated mice compared to control animals.

The involvement of genes positively driving programmed cell death was further investigated. In the list of apoptosis-related transcripts (85 probe sets out of 3437) we found many key regulators of both extrinsic (receptor-driven) and intrinsic (mainly triggered via mitochondria) cell death. After the validation of the macroarray cell death-related findings by qRT-PCR analysis of the same duplicates we assessed the kinetics of stimulation of key pro-apoptotic genes at different times after irradiation (0, 7, 21, 27, 33 and 45 days). We tested FADD, Caspase 8, p21, Bax and Caspase 7 mRNA expression. Fig. 4 shows that the whole set of genes analyzed follows a time-dependent stimulation after treatment. After an initial lag period, in which genes are kept at basal

level as in the controls, at 21 days a wave of transcriptional induction begins, although with a different trend.
Taken together, these studies showed that, in spite of the initial DNA repair, irradiated spermatogonia survivors originate spermatozoa bearing DNA strand breaks and that these breaks are detectable in testis since the stage of elongated spermatids.
Moreover, we also showed that pro-apoptotic genes are transcribed between 21 and 33 days post-irradiation in testis cells, suggesting that an active apoptotic process could be involved in the production of these DNA strand breaks.

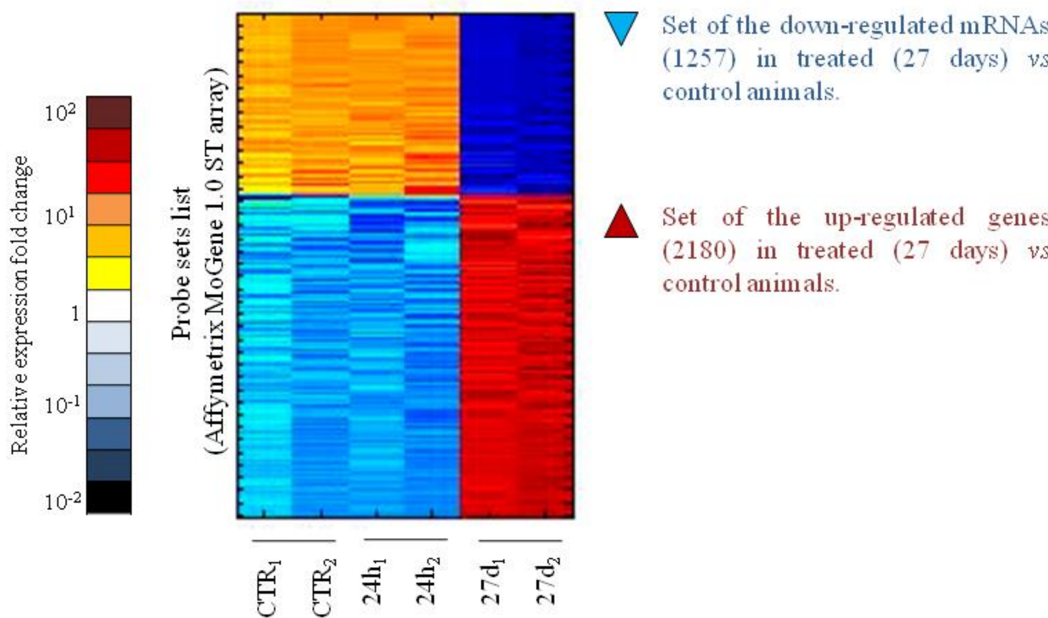


Fig. 3 – Gene expression profile. Heat map visualization of gene expression profiling carried out by Affymetrix platform (MoGene 1.0 ST chips) in testis total RNA sample extracted from two control untreated mice (CTR), two mice at 24 hours after treatment (24h) and two mice 27 days after irradiation (27d). The set of the 3437 transcripts, found differentially expressed at day 27 post-irradiation when compared to controls, are represented. The position of the down-regulated (1257) and up-regulated (2180) transcripts are indicated. Each individual horizontal line represents a probe set (transcript) with a high (red) or a low (blue) signal above the average.

In this period we also tried to better characterize the repair capability of male germ cells and to investigate, in these cells, the meaning of the long persistence after irradiation of a precocious marker of DNA damage like phosphorylated histone H2AX. With this aim, we measured the presence of Mdc1 foci. Mdc1 is a protein involved in the first phases of DNA strand break repair which focalizes into discrete foci at the sites of damage. The longer persistence of γ-H2AX with respect to Mdc1 foci (Fig. 5) supported the hypothesis that in germ cells the phosphorylated H2AX retained at long time after irradiation became disconnected from DNA repair pathways. This activity has been carried out in collaboration with Roberta Meschini (University of Tuscia, Viterbo) and Giovanni Blandino (National Cancer Institute Regina Elena, Rome).

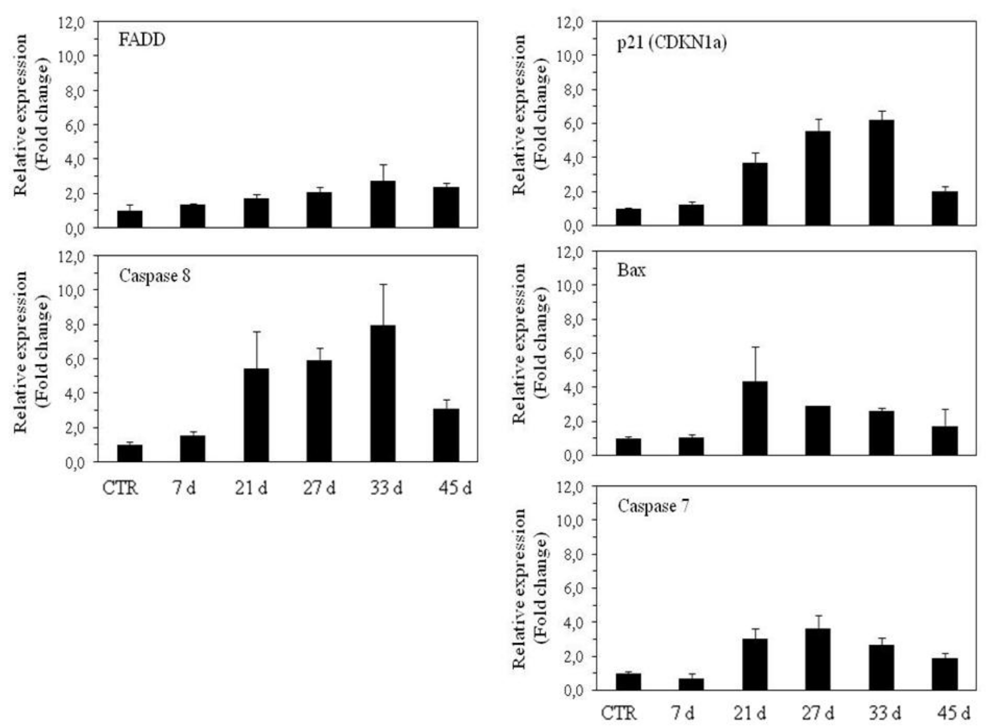


Fig. 4 - Expression kinetics of a set of pro-apoptotic genes in mouse testis samples at 0, 7, 21, 27, 33 and 45 days after irradiation. Each histogram shows the relative expression of each indicated transcript (represented as fold change values *vs* controls) performed by qRT-PCR assay. Bars indicate the mean values with standard errors.

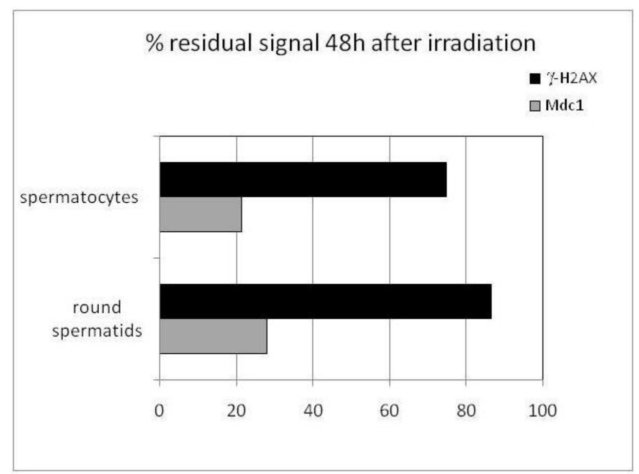


Fig. 5 - Percentage of spermatocytes and round spermatids with residual γ-H2AX or Mdc1 foci observed at 48 hours after 4 Gy irradiation.

SCIENTIFIC COLLABORATIONS

- National Cancer Institute “Regina Elena” of Rome, Translational Oncogenomic Laboratory
- Italian National Institute of Health, Department of Environment and Primary Prevention, Rome
- Department of Agrobiology and Agrochemistry, University of Tuscia, Viterbo

Reduction of environmental impact from cigarette butts

Raffaella Uccelli, Carmine Ciro Lombardi

Nowadays cigarette butts may be considered an environmental emergency because of their large amount and their ubiquitous presence (Fig. 1). Residual parts are collected with other wastes and send to incinerators. On the basis of ENEA's estimate, about 13.000 tons per year of butts are produced in Italy (Fig. 2). Their presence in natural or urban environments, and the consequent release of the dangerous chemicals they entrapped, has negative impact on the environment matrices, on living organisms and on urban decorum (Fig. 3).
Two projects aimed at reducing the impact of cigarette butts were elaborated by ENEA's researchers of two Technical Units (Radiation Biology and Human Health, and Renewable Sources, in particular with Eng. Vincenzo Colaci) and shared with some potential external partners such as the Municipality of Rome, British American Tabacco (BAT) Italia, Eko Technology (Industrial plant projecting and constructing Society), GEA Progetto Salute (no-profit organization mainly involved at developing territorial services to help tabagists) and the Italian Society of Tobaccology (SITAB).

Fig. 1 – Cigarette butts emergency. Some reports evidencing cigarette butt impact on the environment.

Estimated amount of cigarette butts in Italy

- The number of **smokers** In Italy is about **13 million** (ISS - Doxa/2009 Investigation*)
- Assuming that each of the above smokes 15 cigarettes a day, the total amount of **butts** produced will be:

195 million per day

>72 billion per year (≅ 13,000 tons)

* **ISS:** Italian National Institute of Health; **Doxa:** Italian National Institute for Statistical Research and Analysis of Public Opinion

Fig. 2 – Estimated amount of cigarette butts in Italy. Data estimated from ENEA on the basis of the number of smokers given in the ISS-Doxa investigation of 2009.

Estimated yearly input of pollutants into the Environment through cigarette butts in Italy

- **Nicotine:** **324 tons**
- **Polonium-210:** **1,872 millions Bq**
- **VOC:** **1,800 tons**
- **Toxic Gases:** **22 tons**
- **Tar:** **1,440 tons**
- **Cellulose Acetate:** **12,240 tons**

Assuming that 50% of the substances present in the cigarette remains in the butts

Fig. 3 – Estimated yearly input of some pollutants into the environment through cigarette butts in Italy. These quantities were estimated from ENEA on the basis of the previously calculated number of cigarette butts in Italy, assuming that 50% of the substances present in the cigarette remains in the butt (VOC = volatile organic compounds).

Project 1: Reduction of environmental impact from cigarette butts and production of energy through a pyro-gasificator plant

The first project included 3 different phases: separated collection of butts, their elimination by a pyro-gasification treatment with reduction of pollutant emission and production of energy and heath.
The separate collection of butts could be carried out in Rome or in other municipalities in collaboration with the local Authorities. Information and training of citizens to avoid dispersion of cigarette butts in the environment, the distribution of specific butt collectors and transportation of butts to a selected site will also be necessary.
The pyro-gasificator plant will be a pilot one properly designed for using butts as biomass fuel (due to their high calorific power) and it will allow to investigate and characterize all the parameters involved in the combustion process.

Project 2: Educational project on reduction of environmental impact from cigarette

The second project is an educational one submitted by GEA Progetto Salute, SITAB and ENEA to the Ministry of the Environment and Territory for grants. It has been proposed in the framework of a Ministry ban aimed at Environmental Education and Sustainable Development. The proposed project focuses at spreading information about the damage of cigarette butts on the environment and on urban decorum and at changing smokers' behaviour in terms of enhanced attention to and respect for the environment. These goals will be achieved through the activation of the first national network of stakeholders (Universities, Scientific and Education Institutions, Environmental Protections Agencies, Health Agency, etc) sensitive to the cigarette butt problems and capable of transferring information and building up education activities through the national territory. The target population will include personnel of schools, public administrations, prevention services, medical centres, tourist centres, environmental organizations, physicians, general population.

SCIENTIFIC COLLABORATIONS

- British American Tobacco (BAT) Italy
- GEA Progetto Salute
- Italian Society of Tobaccology (SITAB)
- Municipality of Rome, Rome Mayor's Delegate for Local Health District and for relations with Institutional Health Organizations.
- International Society of Doctors for the Environment (ISDE).
- Eko Technology S.r.l., Lucrezia di Cartoceto (PU), Italy
- Ekosmoke, Guspini (VS), Italy

ENVIRONMENTAL EPIDEMIOLOGY

ENEA mortality database

Raffaella Uccelli, Pierluigi Altavista

The current Italian health data, but mortality, are usually collected through different sanitary structures so that an expensive and time consuming work is necessary to find, acquire and elaborate them.
Mortality data, both general and cause specific, codified and recorded since more than 100 years by the National Institute of Statistics (ISTAT), are immediately available for all Italian municipalities. Moreover they are stored and organized in the ENEA mortality data-base shared and run in collaboration between two Technical Units: the UT Radiation Biology and Human Health and the UT Environmental Technologies (in particular with Dr. Marina Mastrantonio).
Through this data-base, using the decennial census populations as denominators, it is possible to calculate many epidemiological indexes, at municipal level since 1980 and at provincial level since 1969 (Fig. 1).
Even though mortality is influenced by several confounding factors including individual susceptibility, lifestyle, socio-economic characteristics and population mobility, some causes of death are good indicators of hazardous exposures, according to previous epidemiological and toxicological studies.
Mortality distributions of causes of death may be used in geographical epidemiological investigations for several purposes: characterize the health state of populations living in particular areas, formulate hypotheses on the existence of some risk factors, point out areas with the presence of rare diseases and monitor the temporal trends of total and cause specific mortality.
Mortality trends may also be used as indicators of response in order to evaluate the efficacy of environmental, sanitary or political actions.

At present ENEA mortality database needs some up dates with the most recent available municipal mortality data, namely those related to the years 2002, 2003 and 2006. They have already been acquired from ISTAT but must still be loaded. In addition, starting from the year 2003, a change in the International Classification of Diseases (ICD) has occurred.
Therefore it is necessary to add, besides the previous VIII and IX ICD Classification codes, the X ICD ones.
Some modification of the database software, developed in Oracle environment, are also required to link the new data to the already existing ones and all of them to the elaborating procedures for the selected epidemiological indices. The Software House who previously developed, up-dated and serviced the database has already been charged to perform such task under the supervision of and in collaboration with the ENEA's researchers of the two UT involved in the topic.

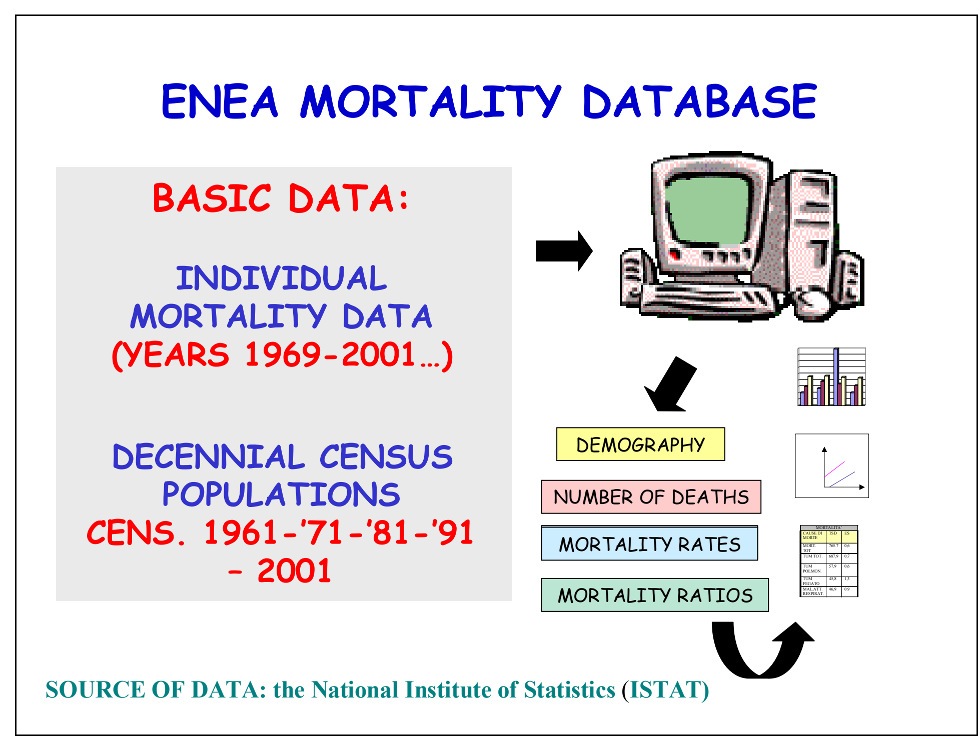


Fig. 1 – Schematic representation of ENEA epidemiological database.

SCIENTIFIC COLLABORATIONS

- Italian National Institute of Health (ISS), Department of Environment and Primary Prevention and National Centre for Epidemiology, Surveillance and Health Promotion, Rome
- Italian Association Doctors for the Environment (ISDE)
- Italian National Institute for Occupational Safety and Prevention (ISPESL), Department of Occupational Medicine, Epidemiology Unit.
- University of Modena and Reggio Emilia, Department of Public Health Sciences

CLINICAL EPIDEMIOLOGY

Pierluigi Altavista

Galectine 3 expression in neuroblastoma tumours

A collaborative study on Galectine-3 (Gal-3) expression in Neuroblastoma tumours is being carried out with the Department of Pediatrics of La Sapienza University of Rome (Italy), the Department of Oncology of Alder Hey Children's NHS Foundation Trust of Liverpool (UK) and Bambino Gesù Pediatric Hospital of Rome (Italy). The study, including 101 pediatric patients enrolled from the Alder Hey Children's NHS Foundation Trust of Liverpool and the Bambino Gesù Pediatric Hospital of Rome, is aimed at the evaluation of the diagnostic and prognostic significance of nuclear and cytoplasmatic expression of Gal-3 in neoplastic cells as evidenced by immunohistochemical methods. Gal-3 is a member of the lectin family involved in many biological processes but his role in neuroblastoma tumours has not yet been well established.

Gal-3 has resulted expressed in the nucleus of 14.9% and in the 7% of cytoplasm of tumour samples. A preliminary analysis showed a possible association between the absence of Gal-3 nuclear expression and a shorter survival in the subgroup of patients with age less than 15 months. Absence of Gal-3 nuclear expression is also significantly associated with unfavourable Shimada prognostic category and less differentiated tumours.

Cardiovascular diseases prevention program in ENEA

Pierluigi Altavista collaborates with Dr. Giuliana Di Cicco in the ENEA Cardiovascular Diseases Prevention Program. This program has been run for many years mainly in the Casaccia Research Center and has interested more than 500 workers, on voluntary participation. The aim of the program is the identification of risk factors, the assessing of cardiovascular risk and the creation of a customized plan to correct risk factors and protect cardiovascular health through Diet Therapy, Health Education and specific pharmacologic treatment, when needed.

Pierluigi Altavista has elaborated individual specific diets and has created a computer program (Visual Basic and Access) for the management of patients data and analysis of the efficacy of the prevention program.

SCIENTIFIC COLLABORATIONS

- University of Rome "La Sapienza", Department of Pediatrics
- Bambino Gesù Pediatric Hospital of Rome
- Alder Hey Children's NHS Foundation Trust, Department of Oncology, Liverpool, United Kingdom.

OCCUPATIONAL SAFETY

Good practices and health

Silvana Salerno, Valentina Kolmann*
*Tor Vergata university bachelor student

Good practice in medical care

Gender oriented good practices (PRAT.O) have been studied in a Project shared with the University La Sapienza (Prof. I. Figà Talamanca), the University of Tor Vergata (Prof. A. Magrini, Dr. L. Livigni), the University of Chieti (Prof. P. Boscolo) and the Health Local Unit H, Rome (Dr. L. Dimitri). A total of 802 technical actions have been studied in three main hospital units, showing that 26% of the actions of direct patient's care were communicative actions (mainly giving psychological support) while providing physical care (double actions). The non formal work of nurses was mainly represented by double actions. These results have been recently submitted to a peer-reviewed journal, highlighting the importance of the dual task paradigm in gender oriented research to achieve prevention. From a methodological standpoint, the Organization Congruence Method was shown to be a useful approach to reveal and measure the "non formal" work of women.

Good practice in the cleaning job sector

A new project (P.R.E.S.A) has been started in collaboration with the University La Sapienza (Prof. I. Figà Talamanca), the University of Tor Vergata (Prof. A. Magrini, Dr. L. Livigni) and the Health Local Unit B (Dr. M.G. Bosco), in order to evaluate the occupational risks in the cleaning sector where women are mostly occupied. The preliminary results (based on 52 hours of observation, 731 technical actions and 11 interviews) show how the cleaning work in hospital is a highly constrained job with repetition of simple tasks (< 10 minutes), long standing, need of using protective gloves in almost all the working schedule, slipping and falling risk conditions, contact with harmful wastes (needles, cutting objects, biological wastes), long walks for supplying, handling chemical products, using not ergonomic trolleys, artificial lights, hot microclimate.

Gender issues

In Italy, a recent legislation introduced norms concerning gender based risk assessment. In this frame, a collection of recent European and international publications and Congress Proceedings on gender issues in occupational health has been produced. A monograph has been published on the outstanding contribution of Ersilia Majno Bronzini to the field of occupational health.

Internet-based information on electromagnetic fields

In collaboration with National Institute for Occupational Safety and Prevention (ex-ISPESL, Dr. C. Giliberti and A. Bedini), 300 websites containing information on the relationship between exposure to electromagnetic fields and health, from public Institutes and private sources, have been analyzed to evaluate their content from a good practice viewpoint. The results show that the few "official" websites (mostly mastered by public Institutes) provide *good practice information* and can represent a model for

websites design, even though their capability to interact with end users should be improved. A follow-up of this study has recently started.

Feasibility analysis of epidemiological surveys in war zones

A feasibility analysis for an epidemiological survey of military and voluntary personnel in warring zones has been presented in collaboration with the Department of Epidemiology of the National Institute for Cancer Research in Genoa (Dr. V Gennaro). The study showed ten main limitations and pitfalls in such epidemiological surveys, namely: 1) the lack of both environmental and health information is regarded as evidence of no risk, 2) the population exposed under the law limits is considered unexposed; 3) the synergistic effects of multiple exposures are not evaluated; 4) the exposed population is not distinguished from the unexposed one; 5) only one group of rare diseases is considered (i.e. Hodgkin lymphoma); 6) the detection of all cases is not systematic (but casual); 7) the follow-up period is shorter than the latency time; 8) the military staff are mistakenly compared with the general population; 9) total attributable cases are not quantified; 10) the statistical interpretation is privileged with respect to the epidemiological one.

SCIENTIFIC COLLABORATIONS

- University of Rome “La Sapienza”, Department of Public Health and Infectious Diseases University of Rome “Tor Vergata”, Faculty of Medicine
- University of Chieti, Department of Biomedical Sciences
- Italian National Institute for Occupational Safety and Prevention (ISPESL)
- B and H Health Local Units of Rome.

Development of methodologies and algorithms for the evaluation of occupational risk in research laboratories and woodworking.

Carmine Ciro Lombardi

The aim of this study was the development of an alternative method to facilitate the evaluation of risk assessment for woodworkers. To this purpose, we have validated a mathematical algorithm model combining eight factors indicative of exposure levels. Some parameters refer to the type and the state of wood conservation, others are related to the presence/absence of natural or forced/local aspiration system, the type and speed of production of dust, the frequency of use of machineries, the exposure time, the hygienic condition in wood factories. Arbitrary values (ADV) were assigned to each parameter and combined by algorithm trough random additive and multiplicative factor in order to obtain a parameter (IRL) that quantifies the work exposure.
On the ground of different ADV, taken as representative of the exposure risk, we have established three level of limit value corresponding to three different level of risk.

IRL < 90	*Low Risk*
IRL > 90 > 224	Alert level
IRL > 224	**High Risk**

Risk assessment was performed in 7 wood factories in the Lazio region. To validate algorithm we have performed field experiments for the monitoring of actual dust exposure using IOM sampler.
Experimental monitoring result for airborne wood dust (Tab. 1) were compared either with the Wood Risk Index (IRL) or with the limit set by regulation = 5 mg/m^3.

Tab. 1 - Comparison between airborne wood dust measurements and Wood Risk Index (IRL).

Factories	Function	Dust monitoring (mg/m^3)	IRL algorithm
1	Planning	3,04	227
2	Dissection	3,98	176
3	Planning	4,36	155
4	Dissection	*1,29*	*61*
5	Planning	**6,14**	**269**
6	Planning	*1,55*	*64*
7	Planning	*1,25*	*89*

Preliminary data suggest that the selected parameters are good indices of exposure and the algorithm could be used to predict wood exposure risk.

SCIENTIFIC COLLABORATIONS

- Technical High School "Gian Lorenzo Bernini", Rome
- Italian National Institute for Occupational Safety and Prevention (ISPESL), Department of Occupational Hygiene, Monte Porzio Catone Rome

LABORATORY OF BIOTECHNOLOGY

Eugenio Benvenuto, Lab Director

The Biotechnology laboratory was founded in April 2010, following the new ENEA Agency directives. It consists of a group of twelve permanent staff researchers with an extensive set of expertise ranging from molecular biology to "omic" sciences and from virology to immunology in a synergistic approach to exploit the whole potential of plants for human and animal health. The knowledge of the connections between plants and health is a high-priority task for a new generation of botanical therapeutics that includes plant-derived recombinant proteins, besides the well-known natural drugs, dietary supplements and functional foods. Interest in the "plant-based pharming" started in the nineties when monoclonal antibodies were demonstrated to be functionally expressed in tobacco plants. For more than twenty years, despite technological, economic and social issues, public and private enterprises went ahead to produce antibodies, vaccines and therapeutic proteins of medical and veterinary significance. The first plant-derived produced human therapeutic protein (taliglucerase alfa), approved just recently by the US Food and Drug Administration for human use, definitely validates and promotes the plant cell-based platforms for the production of "biologics".

Plant resources available for "herbal recombinant medicine" are often poorly understood by non-plant scientists and research funding agencies. Consequently, the strengths and needs of the "plant-based pharming" research community are also often underestimated. It is the mission of our group to increase the knowledge on the plant cells as a genetic and physiological system for the production of high-added value molecules, countering the concept of growing crops only for food, fiber or fuel.

In order to achieve this mission, we will actively pursue the goal of widening the breadth and scope of existing plant molecular biology research towards health applications.

In October 2010, a workshop on: "*Modern Vaccines and Delivery Technology*" was organized at ENEA Casaccia Research Center with the aim to stimulate a coordinated synergistic approach in this important research area. More than seventy attendants participated in the workshop, intersecting knowledge acquired by both academia and industry in basic immunology, "omic" sciences, bioinformatics, drug delivery, animal models.

Lab topics include, but are not limited to: antibody engineering and pharming, mAb targets in the therapeutic areas of cancer, infectious diseases and in diagnostics, recombinant vaccines derived from plants and plant viruses, proteomics and biochemistry, plant cell biology.

As a whole, the Laboratory has produced more than ten peer-reviewed papers in year 2010 with an average journal impact factor of 4.1, basically in the area of plant-derived vaccines, antibody engineering and pharming, proteomics.

Potato virus X as a candidate carrier for a universal flu vaccine

Chiara Lico, Riccardo Siligato*, Eugenio Benvenuto, Selene Baschieri
*Graduate student at ENEA

We have developed a peptide vaccine-delivery strategy based on the plant *Potexvirus* Potato virus X (PVX). This plant virus is an ideal, highly ordered, multivalent scaffold to be used to this purpose. In fact, it is characterized by a simple filamentous structure made of a ss(+)RNA molecule embedded in a capsid of approximately 1300 units of the same coat protein (CP). Genetically modified PVX particles can display approximately 1300 copies of the peptide as fusion with the exposed N-terminus of CP units. We isolated and characterized a PVX mutant (PVX*Sma*), that lacks the first 21 amino acids of the CP (Lico et al., J. Gen. Virol. 87:3103, 2006) and by using this mutant we have demonstrated for the first time that chimeric plant virus particles (CVPs) produced in plants, and carrying a peptide derived from the Influenza A Nucleoprotein (PVX*Sma*-NP), enter antigen presenting cells compartments resulting in MHC class I presentation and in the activation of CD8+ T lymphocytes. Remarkably, the best response was obtained in the absence of adjuvant co-delivery (Lico et al., Vaccine 27:5069, 2009).
With the aim of developing a universal plant virus-based influenza vaccine able to stimulate different compartments of the immune system, we have recently fused to the CP of PVX*Sma* a peptide derived from the M2 protein ($M2_{6-13}$ with amino acid sequence: EVETPIRN; PVX*Sma*-EVE). This peptide is highly conserved among different influenza virus subtypes and known to stimulate antibody-dependent cell-mediated cytotoxicity by Natural Killer cells. PVX*Sma*-EVE CVPs were constructed on the basis of previously defined "rules" for peptide-display on PVX surface (Lico et al., J. Gen. Virol. 87: 3103, 2006) and successfully produced in plants (Fig. 1). The production on large scale of these CVPs is under development to then proceed with immunization experiments aimed at verifying if their co-delivery in association with PVXSma-NP CVPs induces a synergistic increment of the immune response and protection in challenge experiments.

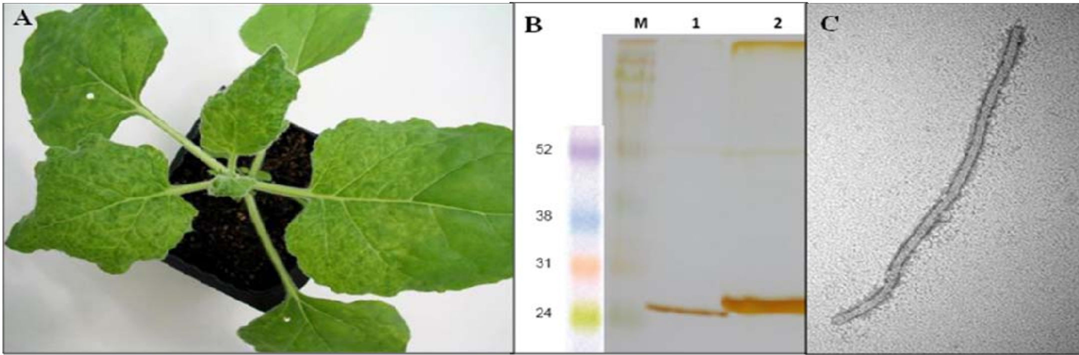


Fig. 1 - *In planta* production and purification of PVX*Sma*-EVE (A) Typical mosaic symptoms produced in *Nicotiana benthamiana* plants 10 days after the inoculation with the viral vector encoding the chimeric CP, bearing the M2 derived peptide (pPVX*Sma*-EVE). **(B)** Silver stained SDS-PAGE of the purified wild-type PVX (lane 1) and of the chimeric PVX*Sma*-EVE (lane 2). The shift of the bands, corresponding to the monomeric CP, is due to peptide fusion (M: molecular mass marker). **(C)** Transmission electron microscopy of purified PVX*Sma*-EVE CVPs, showing at high resolution the filamentous aspect of PVX.

Oil bodies-based vaccine delivery platform

Floriana Capuano*, Camillo Mancini, Eugenio Benvenuto, Selene Baschieri
*Postdoc at ENEA

Oil bodies are special plant cell organelles, mainly found in seeds, where lipids are stored until germination. These organelles are made of a triacylglycerol (TAG) *core* surrounded by a half-unit phospholipid membrane and an outer shell of specialized proteins. Oleosins represent 80% of oil body proteins. One of the strategies adopted to produce in plants heterologous proteins by inducing their accumulation in the seeds, consists in expressing the heterologous protein as fusion to an oleosin. Because oleosins accumulate at high levels and almost exclusively on oil bodies, this strategy allows the localization of the heterologous protein on oil bodies resulting in the formation of chimeric organelles. The advantage of this approach is that the lipid *core* confers to oil bodies special physicochemical properties that allows to easily separating these organelles from other cellular components. The properties of oil bodies as carrier for the delivery of peptides of immunological interest have been tested. To this purpose *Arabidopsis thaliana* plants were engineered to stably express a peptide (ASNENMETM) from the nucleoprotein of influenza A virus (A/PR/8/34) as fusion to the 19KDa sunflower oleosin. Both wild type and chimeric oil bodies were analysed by mass spectrometry both to characterize the carrier and to confirm the presence of the influenza epitope in the preparations. The mass spectrometry-based technique AQUA was further used for peptide quantification. The immunological properties of the chimeric oil bodies were assessed by subcutaneously injecting C57Bl/6J mice and evaluating the frequency of peptide-specific T lymphocytes by IFN-γ ELISPOT assay (Fig. 1). The ability to activate CD8+ T cells having a crucial role in protection against viral pathogens was demonstrated. This study highlights the potentials of oil bodies as valuable carriers of antigens in vaccine formulations.

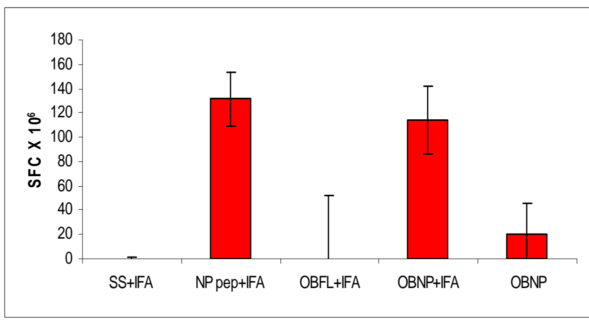


Fig. 1 - Evaluation by IFNγ ELISPOT assay of the *in vivo* induction of ASNENMETM specific T cell responses by the s.c. Immunization with Incomplete Freund's Adjuvant (IFA) emulsions of apyrogen saline (SS), ASNENMETM synthetic peptide (NP pep), non-chimeric (OBFL) or chimeric (OBNP) oil body preparations. An extra-group of mice was immunized with the chimeric oil bodies without IFA. Results are expressed in terms of spot forming cells (SFC)/10^6 of splenocytes in culture on the y-axis. Each value represents the mean of triplicate wells after subtraction of the SFC counted in the respective negative control (unstimulated cells) ± SD. Cells were considered responsive only when the number of SFC in the wells stimulated with the peptide was at least two times that counted in the corresponding unstimulated control. The *in vitro* response to the H-2d-restricted peptide TYQRTRALV is not shown because for all the groups the number of SFC counted in the stimulated wells was equal to that counted in negative controls. Mice immunized with saline, with non-chimeric oil bodies or with chimeric oil bodies without IFA do not show a peptide-specific response. *t-student $p<0.01$.

Plant heat shock protein-based vaccine delivery platform

Giampaolo Buriani*, Camillo Mancini, Eugenio Benvenuto, Selene Baschieri
*Postdoc at ENEA

Mammalian Heat Shock Proteins (HSP), have potent immune-stimulatory properties due to the natural capability to associate with polypeptides and bind receptors on antigen presenting cells.
We have recently published the results of a study aimed at exploring whether plant HSP, and in particular HSP70, share similar properties. In particular we have evaluated if HSP70 extracted in association to naturally bound polypeptides from plant tissues (pHSP70) transiently expressing a recombinant "reporter" antigen, carry antigen-derived polypeptides and can be used to activate antigen-specific immune responses. This application of HSP70 has been very poorly investigated so far. The analysis started by structurally modelling the plant protein and defining the conditions that ensure maximal expression levels and optimal recovery from plant tissues (Fig. 1a and b). Afterwards, HSP70 was purified from *Nicotiana benthamiana* leaves transiently expressing as "reporter" protein the coat protein (CP) of plant virus. The purification was carried out taking care to avoid the release from pHSP70 of the polypeptides chaperoned within plant cells. The evaluation of antibody titers in mice sera subsequent to the subcutaneous delivery of the purified HSP70 demonstrated that it is highly effective in priming humoral immune responses specific to the CP, also after endotoxin removal (Fig. 1c). Overall results indicated that pHSP70 shares structural and functional properties with the mammalian homologue. This study paves the way to further investigations targeted at determining the properties of HSP70 extracted from plants expressing foreign recombinant antigens as a readily available immunological carrier for the efficient delivery of polypeptides derived from these antigen

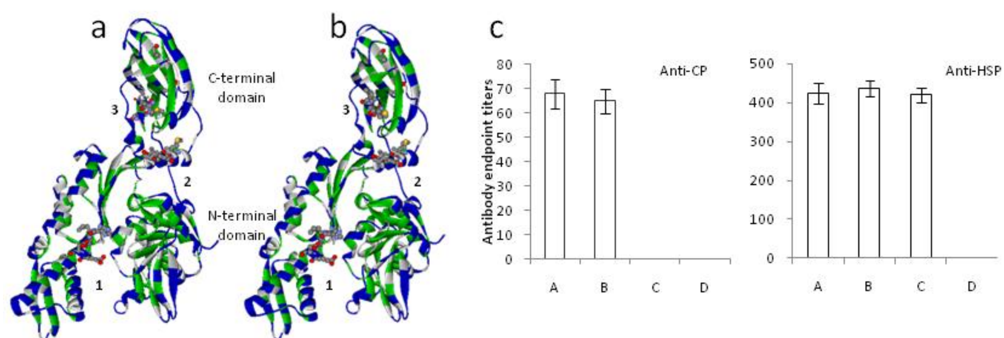


Fig. 1 - Comparison between mammalian HSP70 crystal structure and pHSP70 model. (a) *B. taurus* HSP70. **(b)** *N. tabacum* HSP70. Ribbons with arrowheads: b-strands. Coiled ribbons: α-helices. Tubes: connecting loops. Structures are colored on the basis of solvent accessibility (% maximum Solvent Accessibility Surface, SAS) Blue: exposed residues (SAS>30%); white: partially exposed residues (10%<SAS<30%); green: buried residues (SAS<10%). N-terminal and C-terminal domains are indicated. Conserved residues critical for function are indicated in "ball and stick" mode and colored in conventional CPK mode. Figures are obtained with Accelrys DS Visualizer 2.0. **(c)** Effect of endotoxin removal from pHSP70 preparation on anti-CP and anti-pHSP70 antibody titers. Left Panel: CP-specific IgG titers. Right panel: pHSP70-specific IgG titers. A: mice immunized two times with 25 μg of HSP70 extracted from plant tissues in association to naturally chaperoned peptides and not endotoxin-depleted. B: mice immunized two times with 25 μg of HSP70 extracted from plant tissues in association to naturally chaperoned peptides and endotoxin-depleted. C: mice immunized two times with 25 μg of HSP70 extracted from plant tissues in association to their naturally chaperoned peptides, but *in vitro* unloaded of their binders. D: mice immunized with apyrogen saline. Titers are expressed as arithmetic mean of individual mice titers obtained in independent experiments ± standard deviation. The differences among each group and controls are significant for P<0.05.

Plant virus-like particles as carriers of HIV-1 neutralizing epitopes: computational and experimental virology

Patrizia Circelli*, Raffaele Lombardi*, Eugenio Benvenuto and Carla Marusic
*PhD Student at ENEA

Virus-like particles (VLPs) consist of one or several self-assembling viral proteins expressed through recombinant technologies. For both animal and plant viruses examples have been found where the structural proteins retain their ability to self-assemble without the presence of the encoding viral genome. These features make VLPs a safe and effective vaccine platform for inducing strong B and T-cell immune responses. Moreover VLPs could be used not only as vaccine against the corresponding virus but also as carriers for the presentation of foreign epitopes to the immune system in an ordered and repetitive way. The genes encoding viral protein able to form VLPs have been found in animal and plant viruses. An attractive approach for producing epitope-based plant-derived vaccines is the construction of chimeric VLPs based on a plant virus, which display peptides of interest for vaccine formulations. Several plant viruses (e.g. cowpea mosaic virus, papaya mosaic virus, potato virus X, tobacco mosaic virus) are currently being utilized for the expression of heterologous peptides. The "epitope-display" strategy using plant virus coat proteins (CPs) vaccine carriers for both viral and bacterial antigens has been successfully tested in animal models. Currently we are investigating the Tombusvirus Artichoke Mosaic Crinkle virus (AMCV) as epitope presentation system. We have transiently expressed the sequence encoding the viral CP in *Nicotiana benthamiana* and the assembled recombinant CPs have been detected in plant extracts by ELISA (data not shown). Moreover, VLPs were purified by sucrose gradient and shown by Electron Microscopy (EM) to be similar in structure to genuine AMCV particles. Furthermore, we are developed an approach that combines *in silico* tools and experimental virology for a rational design of immunologically active chimeric VLPs, in an attempt to select suitable sites on the AMCV CP for the insertion of different HIV-1 neutralizing epitopes without affecting VLPs assembly (Fig. 1). Based on bioinformatics analysis, we have fused the HIV-1 2F5 neutralizing epitope (ELDKWA) to the CP C-terminus. ELISA assays of plant extracts from agro infiltrated plants using the human 2F5 mAb showed that the chimeric CP, presenting 2F5 epitope, and EM analysis that it retains the ability to self-assemble. Project carried out in collaboration with Caterina Arcangeli, ENEA UTMEA-CAL and George Lomonossoff, JII, UK.

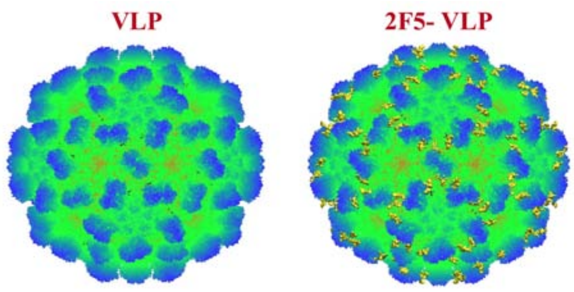


Fig. 1 - VLPs *in silico* models. The models were generated using the bionformatics tool http://viperdb.scripps.edu. VLP: homology model of AMCV derived VLPs; 2F5-VLP: AMCV derived chimeric VLPs exposing on its surface the HIV-1 2F5 neutralizing epitope. All the 2F5-VLPs (30 models) homology models obtained suggest that the 2F5 epitope inserted at the CP C-terminus does not significantly affect the VLPs assembly and stability.

Plants as biofactories and plant proteins as immuno-modulating molecules in the development of new anti-cancer (dna/protein) vaccines

Silvia Massa, Olivia Costantina Demurtas*, Elena Illiano°, Orsola Bitti, Rosella Franconi
*PhD Student at ENEA, °Postdoc at ENEA

Our main field of interest is the development of new therapeutic/prophylactic vaccines by exploiting plants both as biofactories (for safe and low-cost formulations) as well as a source of innovative immuno-stimulating sequences/molecules with less clinical use constraints (i.e. auto-immune reactions/pre-existing T-cell immunity to the carrier) of some immune response modifiers currently tested in experimental DNA/protein-based immunization. We are particularly focused on the Human Papilloma Virus (HPV)-derived cancers. A therapeutic vaccine against cervical cancer should trigger effector T cell trafficking, overcome local immuno-suppression and generate acute inflammation at the tumour site. These requirements can be fulfilled improving the poor immunogenicity of the target antigen, in particular of the HPV E7 oncoprotein (responsible for the malignant state onset and maintenance).

Plants as biofactories

We demonstrated previously that a plant extract of *Nicotiana benthamiana* obtained after the ectopic expression of HPV16 E7 tumor-associated antigen induced, a cell-mediated immune response in vaccinated mice, able to protect them after tumor challenge, even in absence of adjuvant (Franconi R. *et al.*, *Cancer Research* 2002, *Int. J. Immunopathol. Pharmacol.* 2006). The plant extract with intrinsic adjuvanticity was patented and, thereafter, it was demonstrated that it induced maturation of human dendritic cells and primed a HPV16 E7-specific cytotoxic activity (Di Bonito P. *et al.*, *Int. J. Immunopathol. Pharmacol.* 2009). Subsequently, a purified plant-derived E7 fusion protein, produced by agro-infiltration through an advanced plant viral expression vector, inhibited tumor development in vaccinated mice (Massa S. *et al., Vaccine* 2007) and exhibited a dramatic therapeutic effect in presence of fully established tumors (Venuti A. *et al., Vaccine* 2009). These formulations are being tested in prime-boost heterologous experiments performed in a newly developed tumor model of HPV-related Head/Neck tumors in mice. Preliminary experiments indicated that the combination of DNA and fusion proteins vaccines, induced size reduction of mouth implanted experimental tumours. The intrinsic adjuvant activity of the plant extract as well as the characterization of the purified E7 fusion protein are under study with the aim to start a Phase 1 clinical trial. Activity carried out in collaboration with: A. Venuti, IRE, Rome; P. Di Bonito, L. Accardi and C. Giorgi, ISS, Rome; V. Yusibov, Fraunhofer, USA.

Plant proteins as immuno-stimulants

After having demonstrated the improved anti-cancer activity of a DNA vaccine obtained by fusing E7GGG (a mutagenized E7 gene from the high risk HPV type 16) to a plant virus coat protein (Massa S. *et al.*, *Hum. Gene Ther.* 2008), we focused our attention on other possible plant-derived carriers like the 'Ribosome inactivating proteins' (RIPs), so far used to develop immuno-toxins for targeted cancer therapy. Beside toxicity, RIPs have other features (i.e. antigenicity, ability to modulate immune functions, apoptosis induction) that could be useful tools to use in tumor immunotherapy. A non-toxic

mutant of saporin (SAP-KQ) was used as a carrier for the E7GGG gene in the context of a DNA-based vaccine. We demonstrated that fusion constructs of SAP-KQ with E7GGG induce E7-specific immunoglobulins, CTLs and ‘Delayed-Type Hypersensitivity’ affecting the growth of E7-expressing tumors in mice (Fig. 1). These data demonstrate that mutant plant genes hold promise to improve the poor immunogenicity of tumor-associated cancer antigens and could contribute to the evolution of new cancer immunotherapy (Franconi R. *et al.,* PCT/IT2010/000324, 21 Jul 2010). Virus vector systems are also being exploited to formulate plant-derived E7GGG/SAP-KQ fusion protein-based vaccines. Project carried out in collaboration with: A. Venuti, IRE, Rome; L. Spanò, University of L’Aquila.

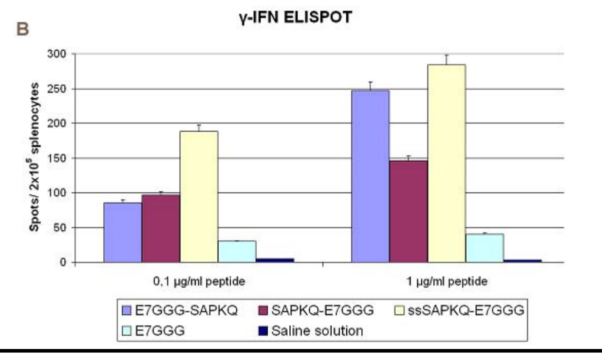


Fig. 1 - ELISPOT analysis of splenocytes from vaccinated mice stimulated with the specific CTL E7 peptide (aminoacids 49–57, RAHYNIVTF). Data are presented as mean number of spots ± S.D. per 2×10^5 splenocytes.

Plant-derived vaccines for veterinary uses

Silvia Massa, Olivia Costantina Demurtas*, Elena Illiano°, Rosella Franconi
*Postdoc at Ylichron, °Postdoc at ENEA

DNA vaccines against bovine papillomavirus (BPV)

Cattles infected by bovine papillomavirus (BPV) with associated bladder carcinoma have been identified in an area of Southern Italy and utilised as a model to study virus-mediated cancerogenesis. In the same area a large number of animals are also affected by chronic enzootic haematuria that may predispose to cancer development. In all of these pathologies the oncogene E5 of BPV Type 2 has been identified and its presence is linked to the maintenance of the transformed status. Moreover, the presence of the E5 sequence has been recently revealed in the bloodstream of these infected animals demonstrating the key role of this oncogene in all the steps of the BPV pathogenesis. Therefore, the E5 represents a realistic target for immunotherapy as it can be considered not only a tumour-associated (specific?) antigen but also an infection-associated antigen. We built a recombinant DNA vaccine expressing the BPV2 E5 protein fused to the coat protein of PVX plant virus. This construct is able to express the proper E5 oncogene upon the transfection of bovine palatal fibroblasts (PalF) demonstrating the possibility to utilise this DNA vaccine. This fusion enhanced the “visibility” of the tumour antigen rendering more efficacious the immunological response, at least in a mouse model. Starting clinical trials in cattle suffering of BPV induced pathologies (i.e. enzootic haematuria/bladder carcinoma) will ascertain the effectiveness of anti-E5 immunotherapy in modifying the natural history of BPV-induced tumours as well as the

efficacy of a new pharmaceutical product ready for the utilization in veterinary pathologies. Project carried out in collaboration with: F. Roperto, G. Borzacchiello, S. Roperto, N. Corteggio, University FEDERICO II Naples; M.S. Campo, University of Glasgow, F. Paolini, A. Venuti, IRE, Rome.

Plant extracts as adjuvants in fish vaccination

We are currently exploring the possibility to use plant extracts as adjuvants to improve the efficacy of commercially available veterinary vaccines. We reported the immunomodulatory activity on human Monocyte Derived Dendritic Cells (MDDCs) of a crude extract-based vaccine preparation shown to be effective against an HPV16-related tumour in an animal model (Di Bonito P. *et al*., *Int. J. Immunopathol. Pharmacol*. 2009). The vaccine was composed of extract from *Nicotiana benthamiana* leaves containing HPV16 E7 protein expressed by a potato virus X-derived vector (NbPVX-E7) *(Franconi R. et al., 'Subunit Vaccines and Processes for the Production Thereof'. EUROPEAN PATENT n.1401493, 2007).*

The chemical nature of the compounds responsible for the immunomodulatory properties of the *N. benthamiana* extracts has to be determined for a possible, useful application in humans. In any case, this study, giving a clue about the immunomodulatory activity of the plant extract, opened the way to their possible use in veterinary immunotherapy. Ongoing experiments on fish vaccination are expected to confirm this hypothesis. Project carried out in collaboration with N. Romano, University 'Tuscia', Viterbo; A. Venuti, IRE, Rome; P. Di Bonito, L. Accardi and C. Giorgi, ISS, Rome.

Production of biopharmaceuticals in contained systems (i.e. roots and microalgae)

Olivia Costantina Demurtas*, Silvia Massa, Rosella Franconi

*PhD Student at ENEA

The use of contained plant systems (i.e. cell suspensions, roots, including microalgae) represents a powerful alternative combining the merits of whole-plant systems with microbial and cell cultures benefits. *In vitro* contained production systems show intrinsic benefits like control over growth conditions, batch-to-batch consistency, production in compliance with good manufacturing practice (GMP), avoidance of political resistance to release of genetically modified field crops. Several products from contained systems are expected to reach the clinical trial stage and commercial development in the next future.

Hairy roots are being exploited to produce functional and pharmaceutical proteins in view of combining the advantages of recombinant protein-based vaccines with the potential benefits provided by contained plant-production. Our previous data showed the anti-cancer activity of a HPV16 E7 based experimental vaccine expressed in *N. benthamiana*, by engineering HPV16 E7 coding sequence (wild type or mutagenised sequence, E7GGG) as a fusion to β-1,3-1,4-glucanase (LicKM) of *Clostridium thermocellum* (Massa S. *et al*., *Vaccine* 2007). The expression of LicKM-E7 hybrid proteins is currently being tested in clonal roots obtained from stable transformation of leaf explants from *Petunia hybrida* and of *N. benthamiana* with recombinant *Agrobacterium rhizogenes*.

The selected clonal roots expressed the E7 antigen at concentrations that were comparable with leaf production. Organ cultures have been obtained in order to produce extracts, lyophilized material or purified fusion proteins to be tested in a pre-clinical vaccination model. Project carried out in collaboration with G. Giuliano, P. Ferrante, ENEA UTAGRI-GEN, V. Yusibov, Fraunhofer, USA, A. Venuti, IRE, Rome.

Efficient *agrobacterium*-based transient expression system for the production of biopharmaceuticals in plants

Patrizia Circelli*, Marcello Donini, Maria Elena Villani, Eugenio Benvenuto and Carla Marusic
*PhD Student at ENEA

We have recently described an efficient transient expression system mediated by *Agrobacterium tumefaciens* for the production of HIV-1 Nef protein in *Nicotiana benthamiana* plants.
In order to enhance the yield of recombinant protein we assayed the effect of three gene-silencing viral suppressor proteins [P25 of Potato Virus X, P19 of Artichoke Mottled Crinckle Virus (AMCV) and Tomato Bushy Stunt Virus] on Nef expression levels. Results demonstrated that AMCV-P19 gave the highest Nef yield (1.3% of total soluble protein) and that this effect was correlated to a remarkable decrease of Nef-specific small interfering RNAs indicating an effective modulation of RNA silencing mechanisms.
To demonstrate that this transient expression system, based on the use of AMCV-P19 gene-silencing suppressor (isolated in our lab), can be successfully employed for the high-yield production of different proteins, three constructs encoding a human immunoglobulin heavy (HC) or light chain (LC) and the AMCV coat protein were tested.
Six weeks old *N. benthamiana* plants were infiltrated with *A. tumefaciens* strain LBA 4404 containing the binary vectors carrying the expression cassettes for *hc, lc* or *cp* genes. Plants were also co-infiltrated with either two *A. tumefaciens* clones harbouring HC and the AMCV-P19 or LC and AMCV-P19 or CP and AMCV-P19.
Leaves were collected at 3, 5, 7, 9 days post infiltration (dpi) and the protein expression was assayed by quantitative ELISA (Fig. 1). Maximum expression levels in plants infiltrated with just the LC or HC constructs were obtained at 5 dpi, while in the plants co-infiltrated with both LC or HC and AMCV-P19 the expression peak was observed at 7 dpi (Fig. 1A).
Plants infiltrated with the HC construct, revealed a similar expression behaviour to those infiltrated with LC (Fig. 1B).
Comparable results were obtained in *N. benthamiana* leaves co-agro-infiltrated with mixed Agrobacterium cultures carrying AMCV-P19 and AMCV-CP expression cassettes (Fig. 1C). The results showed that the *Agrobacterium*-based transient expression system boosted by AMCV-P19 strongly enhances the production of different types of proteins including HIV-1 Nef antigen, human immunoglobulin heavy and light chains as well as a plant virus coat protein, offering several advantages over the generation of transgenic plants represented by higher protein yields, rapidity of production and cost-effectiveness.

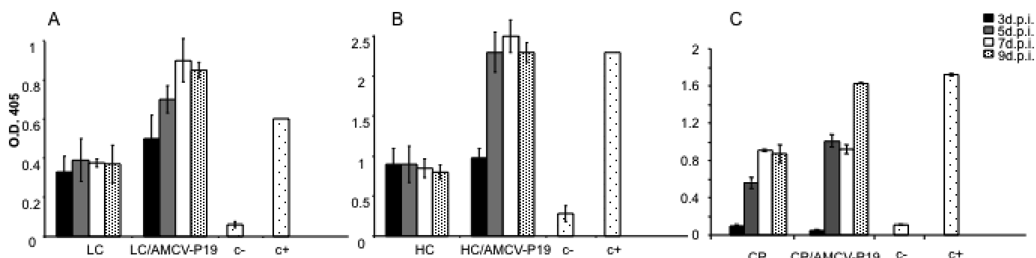


Fig. 1 - Transient expression analysis of human immunoglobulin heavy (HC) and light (LC) chains and AMCV coat protein. Plant extracts, expressing the LC and HC, collected at different time points were analysed by DAS-ELISA (A, B). LC: plants agro-infiltrated with LC only; LC/AMCV-P19: plants co-agro-infiltrated with LC and AMCV-P19; HC: plants agro-infiltrated with HC only; HC/AMCV-P19: plants co-agro-infiltrated with HC and AMCV-P19; C+: purified IgG_1 human antibody used as a positive control; C-: mock infiltrated plants. (C) ELISA of plant extracts expressing the AMCV-CP. CP: plants agro-infiltrated with AMCV-CP only; CP/AMCVP19: plants co-agro-infiltrated with AMCV-CP and P19; C-: mock infiltrated plants used as a control; C+: purified AMCV virus particles used as positive control. Plant extracts were normalized for total soluble proteins.

A chimeric potato virus x encoding a heterologous peptide affects *nicotiana benthamiana* chloroplast structure

Chiara Lico, Camilla Betti*, Eugenio Benvenuto, Selene Baschieri
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The cytopathology of a Potato virus X (PVX) recombinant variant (PVX*Sma*-P18DD, encoding as fusion with the N-terminus of the coat protein, CP, a peptide of immunological interest) has been compared with that induced by the wild-type virus (PVX wt.) in *Nicotiana benthamiana* plants. Both wt. and chimeric PVX caused in mesophyll cells of the infected leaves the typical PVX-induced ultra structural alterations, in particular the formation of the so-called laminated inclusion components (LICs) and bulk virus accumulations. However, PVX*Sma*-P18DD infection showed some relevant differences in cytopathology, at least for what it concerns the aspect of the viral aggregates and chloroplast alterations. In both inoculated and systemically infected leaves the typical banded inclusions of virus particles were never observed, as virions usually were accumulated in looser and scarcely organized bundles of different sizes (Fig. 1A) as compared to PVX wt. (Fig. 1B). The most striking difference was the presence in chloroplasts of an unusual vesiculation involving the thylakoids membranes (Fig. 1C-E) and resulting, in the end, in organelle collapse by disruption of the envelope membrane.
These findings indicate that the peptide display technology, aimed towards the high-mass production of pharmaceutically interesting heterologous sequences in plants, could be also a useful tool to unravel the complexity of plant–virus interactions during the infection cycle. The knowledge of these basal mechanisms is, in turn, fundamental for the successful development of the peptide display technology, as the large-scale production of the chimeric virus particles in plants requires optimal viral fitness.

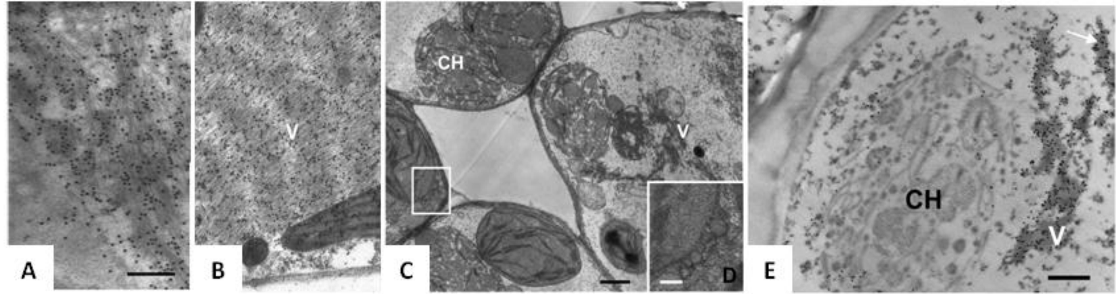

Fig. 1 - Ultrathin sections of *N. benthamiana* leaves infected with PVX wt or PVX*Sma*-P18DD, immunogold labeled with an anti-CP primary antibody. (A) Disordered PVX*Sma*-P18DD accumulations. **(B)** PVX wt accumulations in the form of typical banded aggregates. **(C)** Different stages of chloroplasts vesiculation; an early stage of vesiculation is framed in the white box and enlarged in **(D)**. **(E)** A cell in an advanced stage of infection, containing aggregates of filamentous virus particles and CP (arrow) and completely collapsed chloroplasts. (V), virus particles; (CH), chloroplast.

ANTIBODY ENGINEERING AND PHARMING

Production of a tumor human targeting antibody

Marcello Donini, Maria Elena Villani, Raffaele Lombardi*, Mariasole Di Carli°, Patrizia Brunetti *, Eugenio Benvenuto
*PhD Student at ENEA; °Postdoc at ENEA

Antibodies have been expressed in plants using different formats; among these, monoclonal antibodies (mAbs) result efficiently folded and assembled within the ER of plant cells, retaining the full binding activity (Ma *et al*., Science 268:716-9, 1995). Antibody production has been achieved with different expression strategies including stable transformation of the nuclear/chloroplast genome, or transient transformation using viral or *Agrobacterium* vectors (Sainsbury *et al*. Plant Biotechnol J 6:82-92, 2008). Generation of transgenic plants is generally labour-intensive and time-consuming with yields not extremely high thus representing the major drawback for the success of this technology. In contrast, transient expression systems have the advantage of a rapid production of assembled antibodies in a very short time, although only few transient expression technologies have proved to be ready for the large-scale production (Giritch *et al*. Proc Natl Acad Sci 103:14701-14706, 2006). We have previously demonstrated that the tumour-targeting antibody mAb H10 can be transiently expressed and purified at high levels in *Nicotiana benthamiana* by using a vacuum-infiltration system (Fig. 1) with an *Agrobacterium* expression vector boosted by the artichoke mottled crinkle virus (AMCV) P19 virus silencing suppressor protein. In this work we analysed the different steps of protein extraction from Agro-infiltrated leaves to optimise the purification process of the secretory mAb H10 providing new insights in the field of large-scale plant production.

Fig. 1 - The H10 tumour-targeting antibody was produced using an optimised transient expression system based on vacuum Agro-infiltration.
The vacuum chamber used for the Agro-infiltration of *N. benthamiana* plants in an environmentally contained greenhouse is shown here.

Two different extraction procedures (mechanical shearing/homogenisation and recovery of intercellular fluids -IFs-) were evaluated and compared in terms of purified antibody yields, antibody degradation and total phenolic compounds content. Mechanical grinding from fresh leaf tissues gave the highest purification yield (75 mg / Kg Fresh Weight -75% intact tetramer IgG-) and total phenolic concentration in the range of 420 μg / g FW. The second extraction procedure, based on the recovery of IFs, gave purification yields of 15-20 mg / Kg FW (corresponding to 27% of total

soluble protein) in which about 40% of purified protein is constituted by fully assembled IgG with a total phenolic compounds content reduced by one order of magnitude (21 μg / g FW) (Fig. 2). Based on these results, we optimised a pilot-scale purification protocol using a two-step purification procedure from batches of fresh agro infiltrated leaves (250 g) allowing purification of milligram quantities (average yield 40 mg/Kg FW) of fully assembled and functional IgG with a 99.4% purity, free of phenolic and alkaloid compounds with low endotoxin levels (< 1 EU / ml).

Fig. 2 - Visual comparison between clarified total extract from fresh Agro-infiltrated leaves obtained by mechanical homogenization (1) or intercellular fluids (IFs) (2). In the bottom part, protein A chromatography columns after three purification cycles from the corresponding extracts are shown. Extraction procedure, based on the recovery of IFs, gave purification yields of 15-20 mg / Kg FW (corresponding to 27% of total soluble protein) with a total phenolic compounds content reduced by one order of magnitude (21 μg / g FW) compared to total extract.

Overall data highlight that different extraction procedures can dramatically affect the proteolytic degradation and quality of antibody purified from Agro-infiltrated *N. benthamiana* leaves. Despite a higher antibody degradation, purification from intercellular fluids demonstrated to be very promising since extraction procedures resulted extremely fast and amenable to scaling-up. The reported results open the way to over-express human anticancer monoclonal antibodies through a rapid, low-cost molecular farming approach.

Production of anti-β-glucan antibodies for immunotherapy of fungal infections in humans

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*PhD Student at ENEA

Human diseases caused by opportunistic fungal agents (in particular *Candida albicans*, *Aspergillus* spp and *Cryptococcus neoformans*) are dramatically increased in the recent

years; these infections unfortunately caused high morbidity and mortality mainly in immunocompromised and hospitalized hosts. There is an increasing interest in the development of therapeutic antibodies (Ab) to improve the control of these invasive and mucosal infections, but none of these reagents is yet available for clinical use. A murine monoclonal antibody (mAb 2G8) targeting βeta-glucan, a fungal cell wall polysaccharide, conferring significant protection against *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans* in animal models was chosen as an ideal candidate to test a wide-spectrum antifungal therapy. Herein, the antigen binding domains of this mAb were fused to human constant IgG1 domains to obtain a chimeric complete IgG and a IgG like format (scFv-Fc), in addition these engineered molecules were expressed in a safe and economical plant expression system. Both recombinant Abs purified from Agro-infiltrated *Nicotiana benthamiana* leaves with high yields (40 and 50 mg/kg of fresh plant tissue for the IgG and the scFv-Fc, respectively) were found to fully retain the βeta-glucan binding specificity and the antifungal activities of the cognate mAb against *C. albicans*. In fact, they recognized preferentially β-(1,3)-linked glucan molecules present on the cell surface (Fig. 1) and were able to inhibit directly the growth of *C. albicans* and its adhesion to human epithelial cells *in vitro*. In addition, the recombinant IgG and scFv-Fc promoted the killing of fungal cells by isolated, human polymorphonuclear neutrophils in *ex-vivo* assays (Fig. 2) and conferred significant antifungal protection in animal models mimicking a systemic or vulvovaginal *C. albicans* infection (Fig. 3). This study highlights that these recombinant Abs represent valuable molecules that could be rapidly scaled up for developing novel immunotherapeutics against candidiasis and, possibly, other fungal diseases. Project carried out in collaboration with Paola Chiani, Carla Bromuro, Flavia De Bernardis, Antonio Cassone, and Antonella Torosantucci, ISS, Roma and Angelina S. Palma, Yan Liu, Ten Feizi, Imperial College (UK).

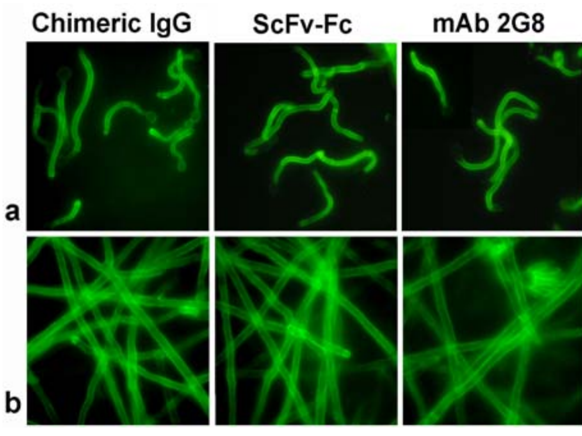

Fig. 1 - Immunofluorescence staining of major pathogenic fungi by the humanized anti-β-glucan Abs. *Candida albicans* germ-tubes (a) and *Aspergillus fumigatus* hyphae (b) were stained with the indicated monoclonal reagents at 50 μg/ml.

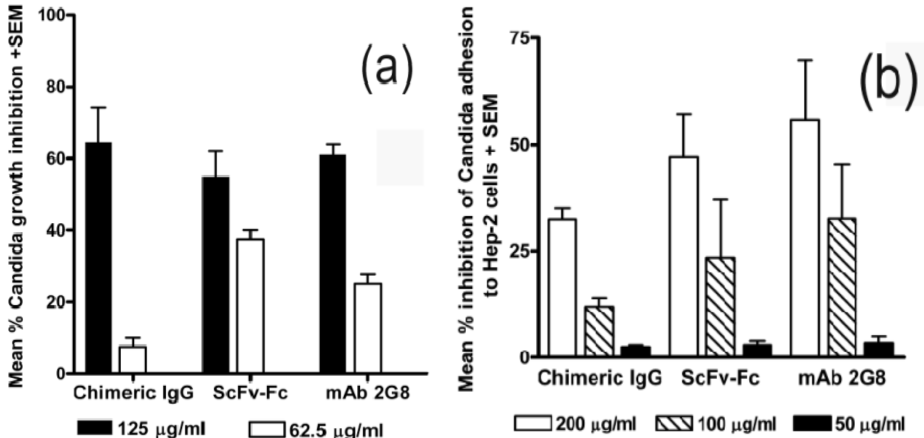

Fig. 2 - *In vitro* anti-*Candida* activities of the recombinant Abs. (a) Inhibition of *Candida albicans* growth. Percentage of inhibition was calculated by comparing fungal CFU in cultures supplemented with the recombinant Abs to those with equal doses of an irrelevant murine IgG2b mAb. (b) Ability by the Abs to prevent the adherence of *Candida* to human epithelial cells. Values in the graph are mean percent reduction (recombinant anti-β-glucan Abs *versus* irrelevant Ab of the correspondent format) of the number of adherent fungal cells measured in three independent experiments, each performed in triplicate. Differences in activity between the different Abs are not statistically significant.

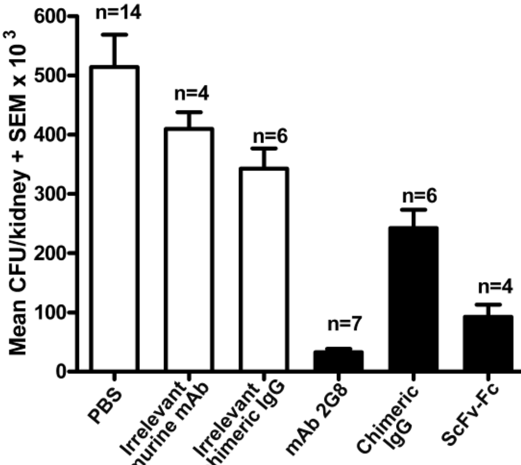

Fig. 3 - Anti-*Candida* protective activity of the recombinant Abs in animal models. Kidney invasion following a systemic infection with *C. albicans* in Ab-treated mice. Data presented in Figure are cumulative, mean CFU values from five independent experiments; n= number of animal examined for each experimental condition. Untreated or control animals *vs.* mice treated with any mAb, P<0.001; mAb 2G8 *vs.* chimeric IgG or ScFv-Fc, P<0.0001; chimeric IgG *vs.* scFv-Fc, P<0.05; any difference among negative controls is statistically not significant.

Anti aflatoxin B1 recombinant antibodies for uses in agri-food diagnostics

Marcello Catellani*, Cristina Capodicasa, Camillo Mancini, Eugenio Benvenuto
*PhD Student at ENEA

Mycotoxins are secondary metabolites that are produced by several fungi mainly belonging to the genera: *Aspergillus*, *Penicillium* and *Fusarium*. In particular, aflatoxin B1 is a common contaminant and can occur in a wide range of important raw food commodities. Consumption of mycotoxin-contaminated food or feed does, however,

lead to the induction of teratogenic, cancerogenic, oestrogenic, neurotoxic, and immunosuppresive effect in humans and/or animals. Mycotoxin contamination may occur in the field before harvest, during harvesting, or during storage and processing and, although the use of pesticides and good agronomic practices may reduce mycotoxin accumulation, eradication is still a major challenge. Therefore, diagnostics remains a fundamental tool to reduce risks associated to the assumption of contaminated food. To this aim, we concentrated our efforts on the isolation and characterization of new diagnostic antibodies to be expressed in cost-effective systems as an alternative to classical mammalian cell culture system. Therefore monoclonal antibodies (mAbs) with high specificity for aflatoxin B1 have been selected using hybridoma technology. Since aflatoxin is a low MW(~300 Da) and poorly immunogenic molecule, this toxin has been chemically conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH) and this conjugate has been used to immunize mice. The monoclonal antibodies of three different hybridomas isolated were able to recognize free aflatoxin B1 in competitive ELISA. Two of these mAbs (2D2 and 9E11) revealed a different cross-reactivity towards the different aflatoxins (B2, G1, G2) (Tab. 1) and a similar high affinity to the toxin, in the picomolar range.

Tab. 1 - Cross-reactivity of 2D2 and 9E11 mAbs towards different aflatoxins.

	Cross-reaction (%)			
mAb	Afl B1	Afl B2	Afl G1	Afl G2
2D2	100	20,6	20,7	2
9E11	100	77,2	248,6	18,2

The genes encoding heavy and light chains of this mAbs were cloned from the hybridomas cDNA and exploited to device a panel of recombinant formats, namely scFv, scFv-Fc and full IgG to be expressed in low-cost heterologous systems. The scFv format was expressed in *E. coli* and retained a very high specificity and affinity to aflatoxin B1, as evaluated by ELISA and SPR (Surface Plasmon Resonance) analysis. Whereas, the scFv-Fc and the light and heavy chain genes were separately cloned in a plant expression vector (pBI) and expressed in *N. benthamiana* plants by Agro-infiltration. Western blot analysis of plant extracts revealed high expression levels of both scFv-Fc and IgG, while the correct assembly and functionality of all antibodies was confirmed by ELISA. The IgGs, purified from infiltrated leaves, bind aflatoxin B1 to the same extent as the cognate antibodies purified from murine hybridomas in ELISA (Fig. 1). Finally, the plant-produced IgG was successfully used to determine aflatoxin B1 in multi-contaminated food matrices (Fig. 2), suggesting the idea that plants are convenient bioreactors for antibodies production, alternative to classical mammalian cell culture systems, for agri-food diagnostic applications.

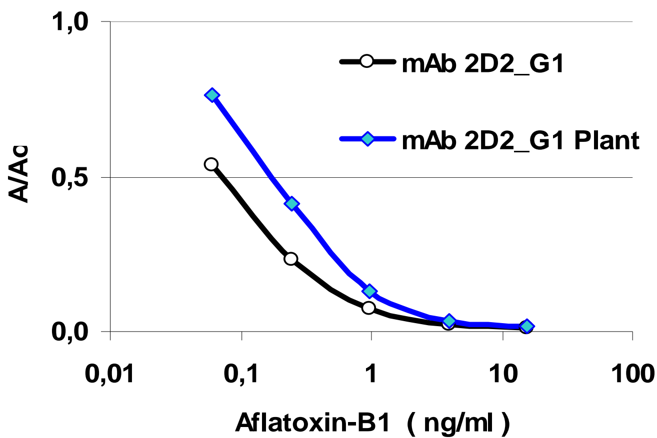

Fig. 1 - Comparison of hybridoma and plant-produced 2D2 mAbs binding to aflatoxin B1 in competitive ELISA. Binding is expressed as mean O.D.$_{405nm}$ readings from triplicate wells after negative controls value subtraction.

Flour mix A:
AB1 0,31 ppb
OTA nr
DON 144
FB1 1000
FB2 320
ZEA nr
T2 nr
HT2 nr

Flour mix B:
AB1 0,58 ppb
OTA 0,40
DON 955
FB1 2000
FB2 550
ZEA 72
T2 25
HT2 nr

Flour mix C:
AB1 1,17 ppb
OTA 10,52
DON 1211
FB1 2000
FB2 480
ZEA 312
T2 100
HT2 100

	AFB1 content
Flour A	**0,249** ppb
Flour B	**0,704** ppb
Flour C	**1,331** ppb

Fig. 2 - Analysis and determination of aflatoxin B1 content in three flours naturally contaminated with different mycotoxins. The AFB1 content of each flour mix, determined by competitive ELISA, are reported in the table as ppb (parts per billions) and matched with the real AFB1content, determined by chemical analysis (HPLC). The data are the mean of three technical replicates and have been elaborated with a commercial software.

Development of new diagnostics for health and food quality monitoring

Elena Illiano°, Silvia Massa, Rosella Franconi
°Postdoc at ENEA

Recombinant antibodies for mycotoxin detection.

Mycotoxin contamination (aflatoxins, ocratoxins, fumonisins and trichothecenes from the type B group) occurs in raw and transformed foodstuffs and is brought by different several fungi species (among which *Aspergillus*, *Penicillium, Alternaria* e *Fusarium)*. The production of mycotoxins is strictly connected to fungal growth. Nevertheless, neither the presence of toxigenic fungi is a necessary condition for mycotoxin presence, nor the absence of toxigenic fungi is an indicator of mycotoxin presence.

In the frame of the project 'Me.Di.T.A. 'Metodologie diagnostiche e tecnologie avanzate per la qualità e la sicurezza dei prodotti alimentari del mezzogiorno d'Italia' financed by MUR (end November 2010) our goal was to develop analytical systems based on recombinant antibodies isolated from 'phage display' libraries in order to detect the presence of mycotoxins produced by different species of fungi on durum wheat (grains or derived products) and other foodstuffs. We produced antibody phage display libraries from immunized animals and, in parallel, selected recombinant antibodies (scFvs) specific to T-2 and Deoxynivalenol (DON) mycotoxins from the 'F8 library' (Desiderio A., Franconi R. *et al., J. Mol. Biol.* 2001 and EUROPEAN PATENT EP1120464). From this repertoire, we had already isolated scFvs specific for different protein targets such as plant pathogens (i.e. CMV, AMCV, TSWV, PVX), lysozyme and glutathione S-transferase (GST) (Villani ME *et al*., *Plant Mol Biol.* 2005, Villani ME *et al*., *J. Imm. Meth.* 2007; Di Carli M *et al., J. Proteome Res.* 2008) that were also used for the development of new diagnostics and sensing devices (Maly J. *et al., Mat. Sci. Eng.* 2002). The scFvs specific to the mycotoxins have been purified, biochemically characterized (SPR-Biacore X and competitive ELISA) and will be used to develop new immunodiagnostic tools for mycotoxin contaminants (i.e. lateral flow) also by genetic modifications to obtain oriented immobilization or direct detection by fusion with enzymatic activities. The new immunological detection devices will be compared with other detection systems. Project carried out in collaboration with F. Vitali, C. Nobili, ENEA UTAGRI- INN; C. Fanelli, Sapienza University of Rome.

Production of recombinant proteins (i.e. HPV-related)

We are planning to realize two new diagnostic kits in order to:

1) detect pre-existing HPV antibodies in women who receive VLP-based HPV vaccination and to perform serological surveillance within HPV prophylactic vaccination follow-up;
2) early diagnosis of HPV-related disease and cancer, in parallel with currently performed HPV test and Pap test.

To these purposes, low-density protein microarrays will be realized with recombinant antigens produced by ENEA, IRE and ISS, based on a technology already existing at LLL ('Dr Chip').

We are able to produce L1 and the E6 and E7 oncoproteins from several HPV types in native conditions and in a biologically active form (Franconi R. & Illiano E., 2007*, ITALIAN PATENT-RM2007A000220*) useful to monitor HPV infection/vaccination as well as progression toward disease/cancer by detecting serum antibodies. Project carried out in collaboration with C. Padula, LLL S.r.l., Pomezia, Rome; A.Venuti IRE, Rome; P. Di Bonito, L. Accardi, ISS, Rome.

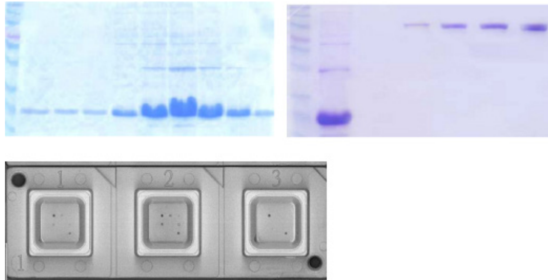

Fig. 4 - Purification (SDS-PAGE) of His_6-E6 and His_6-E7 proteins from *E. coli* and spotting for immunodetection of serum antibodies onto 'Dr Chip' platform.

Introduction of new genetic variability into Orchidaceae via somatic hybridization and polyploidization

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*PhD Student at ENEA

Orchidaceae is one of the most highly developed monocotyledonous families; their pot plant and cut flower production have high economical value in international flower markets. In this family, *in vitro* culture protocols have been developed for propagation via protocorm-like body formation and for shoot multiplication and callus culture. One of the aim of the genetic improvement in orchids is to obtain new varieties and hybrid combinations commercially attractive. The breeding programs conducted by traditional methods have so far led to good results, but some desirable combinations have been excluded from these programs due to problems of incompatibility between the species.
The present work its focused on the introduction of new variability in *Dendrobium* and *Phalaenopsis spp* by means of a biotechnological approach combining cellular and molecular manipulations (Novaorchid Project – http://novaorchid.casaccia.enea.it).

Protoplast Isolation
Protoplast isolation was performed on young leaf explant. Approximately 100-150mg of fresh tissue were incubated at 24 °C for 18h in the dark, in an appropriate enzymatic solution (1% Cellulase Onozuka R-10, 0.01% Pectolyase Y23, 0.2% Macerozyme R-10) (Kanchanapoom *et al*. 2001). Following incubation the digestion mixture was filtered trough Partec Celltrics 50µm nylon filter units and pelleted at 100×g for 5 min. The supernatant was discarded and the pellet resuspended in CPW 25 % sucrose solution layering on it a CPW 13% mannitol solution and centrifuged at 100×g for 10 min. The protoplasts were recovered at the interface of these two solutions and resuspended in 1ml of BH_3 solution (Frearson *et al*. 1973). The viability of the cells was estimated with FDA (*fluorescein diacetate*) at the final concentration of 1µg/ml under a Nikon TE2000-S Eclipse inverted microscope equipped with a DXM1200F camera and the NIS AR 3.1 software.

Protoplast fusion
Somatic hybridization with PEG. Protoplasts were fused with a PEG (Polyethylene glycol) 6000 40% solution and diluted according to the procedure reported in Tusa et al. (1990) with some minor modifications integrated in our laboratory (Fig. 1).

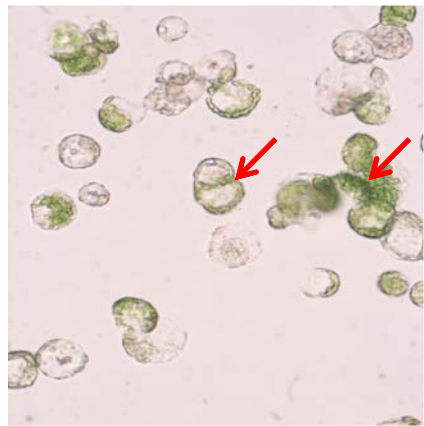
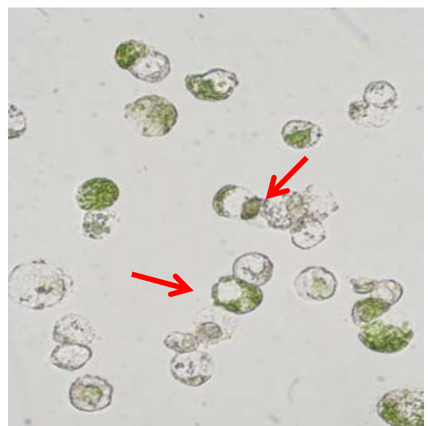

Fig. 1 - Protoplasts fusion performed with PEG. Red arrows pointing fusion products.

Somatic hybridization with electrofusion. Electrofusion was induced by using "ZellfusionCFA-400" (Kruss, Germany). Protoplasts were suspended into an acqueos solution made of 12% Mannitol and 3% sucrose. Settings for the electrofusion were: 1000-1100 KHz frequency, AC7 V/cm primary voltage for 50s (pearl chain formation) and DC 1.5 KV/cm three pulses for 30 µs to fuse protoplasts (Lucretti et al. 1992).
Characterization: We have characterized with FCM (Flow Cytometry Measurement, Fig. 2) and molecular marker RAPD (Random Amplified Polymorphic DNA) techniques the species and the hybrids used in the somatic hybridization trials for the early screening of the future new combinations obtained through somatic hybridization via PEG and electrofusion (Fig. 3).
The protocol for protoplast isolation from leaf tissues of *Dendrobium* and *Phalaenopsis* has been optimized, while callus and PLB (*protocorm like bodies*) regeneration protocol is under evaluation. We have developed and optimised the somatic hybridization protocols via PEG and electrofusion. The molecular characterization by polymorphic RAPD primers for all the orchids accessions used in the somatic hybridization program has been achieved. A simple procedure for ploidy evaluation has been developed for FCM analysis on leaf nuclei suspensions for all the accessions.
Project carried out in collaboration with Debora Giorgi, Anna Farina and Sergio Lucretti, ENEA UTAGRI - GEN, Benedetto Aracri and Dimitri Pashkoulov, Floramiata SpA.

Fig. 2 - Flow cytometric ploidy histogram of *Phalaenopsis amabilis*. *Raphanus sativus* was used as an internal standard.

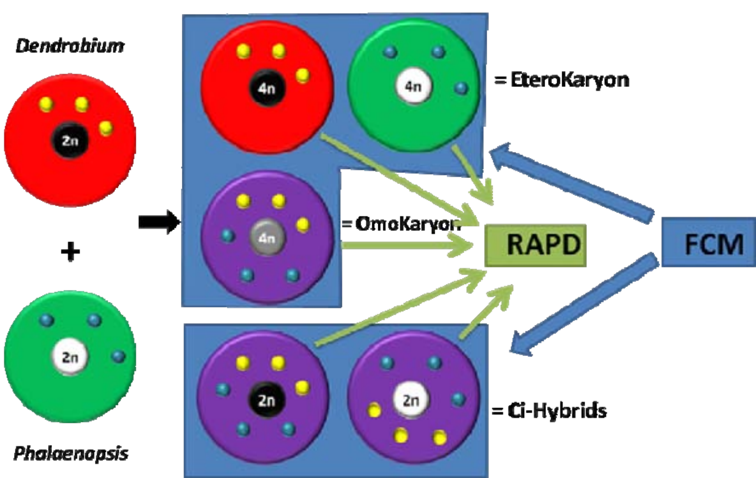

Fig. 3 - The synergic integration of FCM and RAPD analysis allow us to detect and discriminate heterokaryons, homokaryons and ci-hybrids. The first step is the discrimination via FCM ploidy analysis of tetraploid (homo- or heterokaryons) and diploid (non fused and ci-hybrids) hybrids. The second step is the RAPD characterization of tetraploid hybrids to discriminate between heterokaryon and homokaryon.

PROTEOMICS

Proteomic analysis of grape berry during postharvest withering

Mariasole Di Carli*, Angiola Desiderio, Eugenio Benvenuto
*Postdoc at ENEA

Grape berry proteomic analysis

The practice of postharvest withering is commonly used to correct quality traits and sugar concentration of high quality wines. To date, changes in the metabolome during the berry maturation process have been well documented. However, the network of events occurring in the proteome has yet to be fully investigated. To gain insight into the postharvest withering process, we studied the protein expression profiles of grape (*Corvina* variety) berry development at this stage, exploiting the two-dimensional differential in gel electrophoresis (2D-DIGE) proteomic platform. Comparative analysis revealed changes in the abundance of numerous soluble proteins during the maturation and withering processes (Fig. 1). On a total of 870 detected spots, 90 proteins were differentially expressed during berry ripening/withering and 72 were identified by MS/MS analysis. The majority of these proteins were related to stress and defence activity (30%), energy and primary metabolism (25%), cytoskeleton remodelling (7%) and secondary metabolism (5%). Moreover, this study demonstrates an active modulation of metabolic pathways throughout the slow dehydration process, including *de novo* protein synthesis in response to the stress condition and further evolution of physiological processes originated during ripening. These data represent an important insight into the withering process of *Vitis* berry*,* which can assist quality improvement of vinification process.

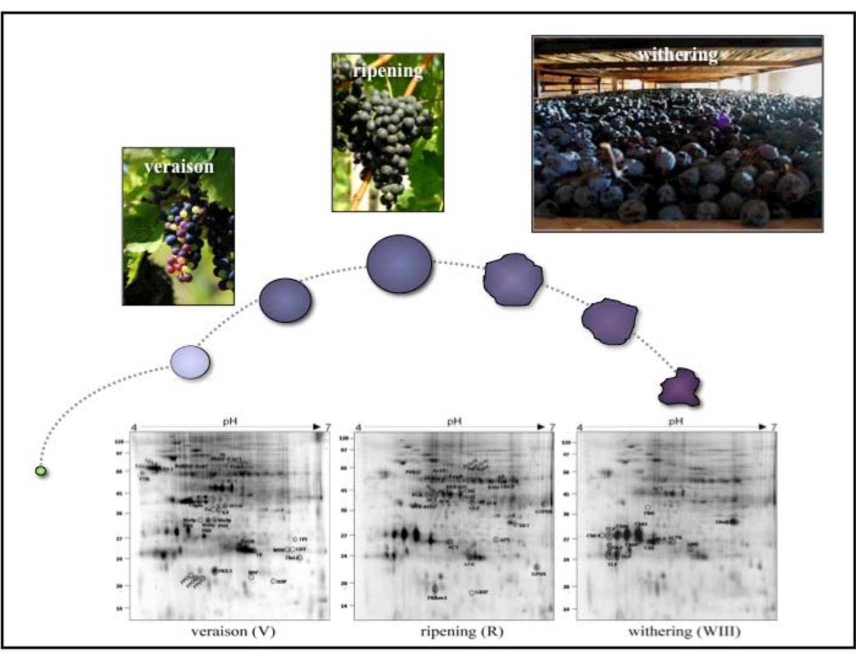

Fig. 1 - Distribution of differential protein spots identified on 2D-DIGE gels of *Corvina* berry samples at veraison, ripening, and withering. Cy-dye labeled proteins (50 μg) were separated by

IEF at pH 4-7, followed by 12.5% SDS-PAGE (Di Carli M., Zamboni A., Pè M.E., Pezzotti M., Lilley K.S., Benvenuto E., Desiderio A. *J. Proteome Res.*, 10: 429-446 (2011). Copyright © American Chemical Society)

Identification of Putative Stage-Specific Grapevine Berry Biomarkers and Omics Data Integration into Networks

Proteomic data were integrated with the results of transcriptomic and metabolomic analyses of the same grapevine berry samples, using two different strategies, one hypothesis-free and the other hypothesis-driven. A multistep hypothesis-free approach was applied to data from four developmental stages and three withering intervals, with integration achieved using a hierarchical clustering strategy based on the multivariate bidirectional orthogonal projections to latent structures technique. The hypothesis-driven approach was used to integrate data from three withering intervals, starting with subdata sets of transcripts, proteins, and metabolites. We can confirm that berry formation involves active cell wall metabolism and photosynthesis, whereas ripening and withering are characterized by the induction of stress responses (Fig. 2). Novel findings include the discovery that carbonic acid acts as a putative supplier of CO_2 to RUBISCO and that sphingolipid fatty acids act as signals during the first berry growth phase. This identification of stage-specific functional networks of linked transcripts, proteins and metabolites provided important insights into the key molecular processes that determine the quality characteristics of wine. Project carried out in collaboration with Anita Zamboni, Massimo Delledonne, Alberto Ferrarini, Sara Zenoni, Flavia Guzzo Ketti Toffali, Paola Tononi, Mario Pezzotti at Dept. of Biotechnology, University of Verona; Kathryn S. Lilley at Cambridge Centre for Proteomics, Dept. of Biochemistry, University of Cambridge; Matteo Stocchero at S-IN Soluzioni Informatiche, Vicenza and Mario Enrico Pè at Scuola Superiore Sant'Anna, Pisa.

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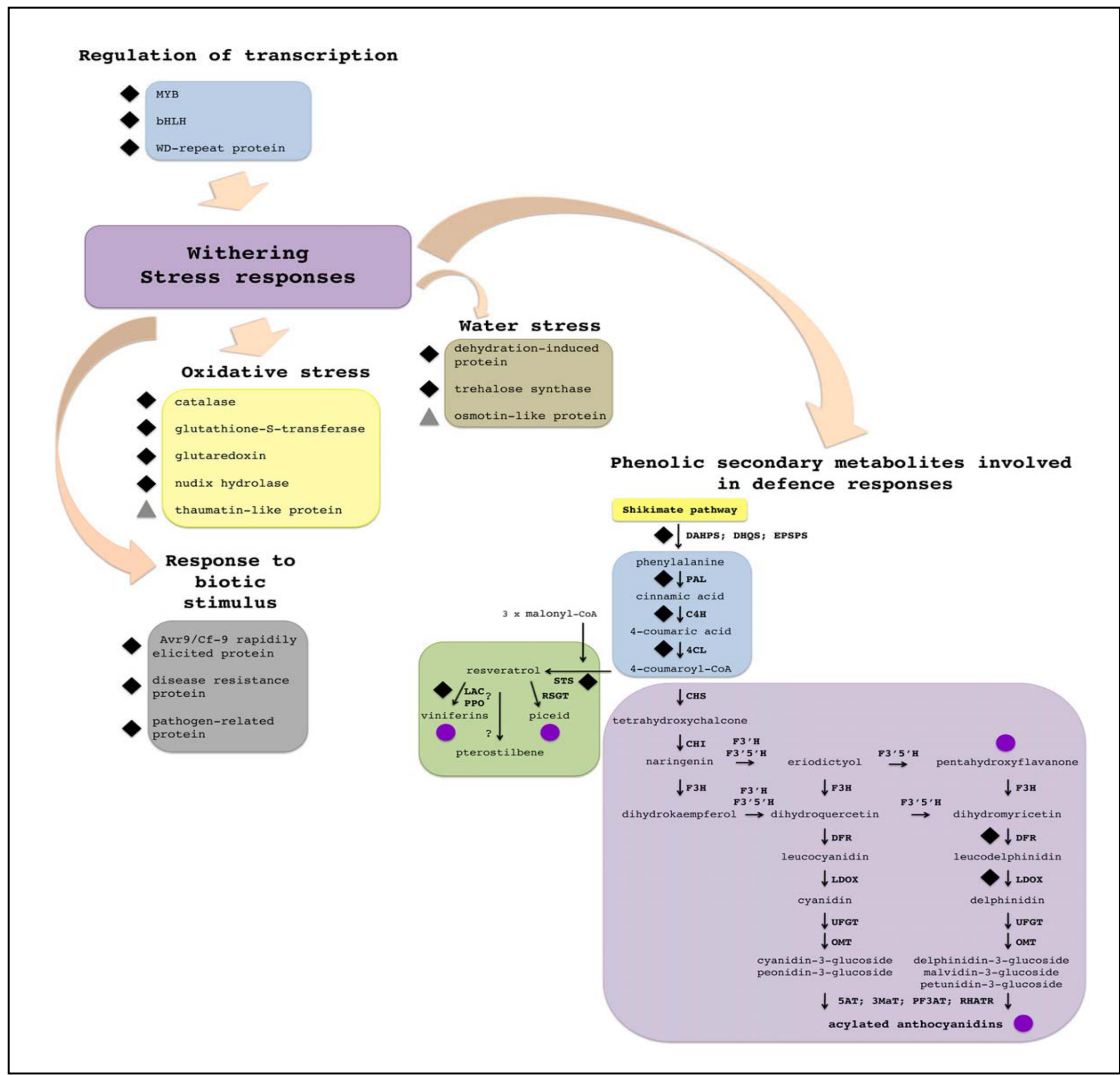


Fig. 2 - Schematic representation of the molecular events characterizing grapevine berry withering determined by hypothesis driven data integration. The well-correlated variables involved in these molecular events, resulting from two O2PLS models performed with the new transcript, protein, and metabolite data sets, were tagged with different symbols (black diamonds, transcript; grey triangles, protein; violet circles, metabolite) and identifier numbers are shown in parentheses.

Proteomic analysis of cucumber mosaic virus resistant transgenic tomato

Mariasole Di Carli*, Maria Elena Villani, Raffaele Lombardi°, Marcello Donini, Eugenio Benvenuto
*Postdoc at ENEA, °PhD Student at ENEA

Cucumber mosaic virus (CMV), member of the *Cucumovirus* genus, is the causal agent of several plant diseases in a wide range of host species, causing important economic losses in agriculture (Palukaitis et al. 2003). Because of the lack of natural resistance genes in most crops, different genetic engineering strategies have been adopted to

obtain virus-resistant plants. The expression of exogenous antibodies in plant is an effective strategy to confer protection against viral infection. In a previous study we described the production of transgenic tomato plants expressing a single-chain variable fragment antibody that are specifically protected from CMV infection (Villani *et al*., 2005). A comparative proteome analysis of transgenic CMV immune-modulated tomato plants with their wild type counterpart did not evidence any significant alteration in protein profiles, demonstrating that even very low antibody expression levels were sufficient to prevent virus infection (Di Carli *et al*, 2008). Thus, these transgenic plants represent an interesting biological system to study the plant-pathogen interaction and identify molecular mechanisms underlying virus resistance.
The aim of the present study was to identify proteins involved in the response to CMV infections in susceptible wild type (WT) and transgenic (T) immune-modulated tomato plants (Fig. 1). Leaf proteome profiles of inoculated and apical leaves of both genotypes were analysed by two Dimensional Differential in Gel Electrophoresis (2D-DIGE) (Fig. 2). The number of detected protein spots, showing a molecular weight between 10 and 200 kDa and a pI between 3 and 11, corresponded to an average of 2084 (±3.6%) (Fig. 3). Among all T and WT CMV inoculated and apical leaf samples a total of 80 proteins with a fold change threshold of 1.4 were selected as differentially expressed and among them 50 proteins were identified by nLC-ESI-IT-MS/MS analysis. Statistical analysis revealed that apical leaves of transgenic CMV inoculated plants had no protein profile alteration compared to control WT uninfected (mock) (Fig. 4) plants although trace levels of virus coat protein (CP) were found. This result strengthens the hypothesis that virus infection is limited to the inoculated leaves and systemic viral spread is hindered by the CP-specific scFv. These results emphasized the strong effect of the scFv antibody on virus infection, and the ability to confer a strong resistance to engineered tomato plants. Wild type CMV infected plants showed protein profile alterations in different functional categories such as photorespiration, photosynthesis, pathogen resistance and primary metabolism, substantially matching literature data on plant-virus interactions. Moreover, in both wild type and transgenic symptomatic CMV inoculated leaves we observed a virus-specific down-regulation of typical plant defence mechanisms leading to high viral accumulation. The proposed mechanism for virus resistance in transgenic plants is the interference of CP specific scFv during virus assembly in the cell cytoplasm. Evidence of similar symptoms and protein profiles in the inoculated leaves of transgenic and wild type plants indicates that the low scFv intracellular levels are not sufficient to hinder virus replication and cell to cell movement. Overall, presented results give a highlight on proteins expressed during plant-virus interaction in both wild type and transgenic resistant tomato. Project carried out in collaboration with Linda Bianco, Gaetano Perrotta, ENEA UTTRI- BIOTEC.

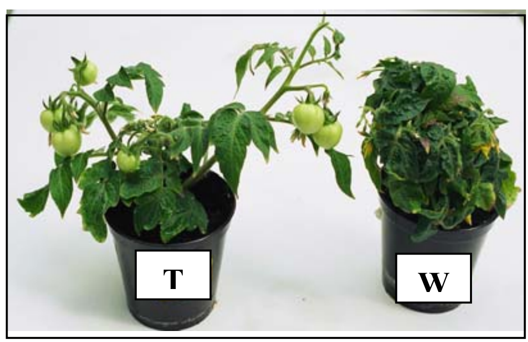
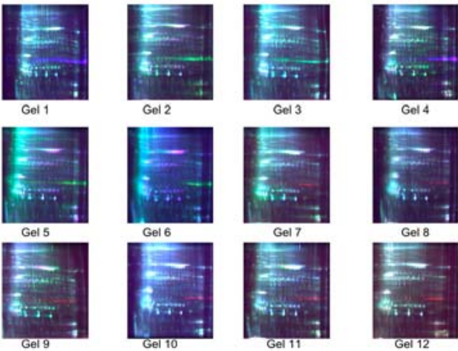


Fig. 1 - CMV resistance analysis on T4 transgenic tomato plants. Phenotypes observed at 18 days post inoculation (d.p.i.) in transgenic (T) and untransformed (WT) plants. At this stage, inoculated and apical leaves have been collected from three different plants as biological replicas for proteome analysis. Apical leaves of transgenic plants show no visible symptoms, while WT plants show typical CMV symptoms (chlorosis, stunting and leaf deformation).

Fig. 2 - Multicolour images of the 12 gels from 2D-DIGE analyses. At 18 d.p.i. Both inoculated (i) and apical (a) leaves of CMV infected transgenic (T_{CMV}) and wild type plants (WT_{CMV}) together with mock-inoculated uninfected control plants (T_M and WT_M) were collected and analyzed according to the DIGE labelling design. 50 μg of total proteins were loaded for each sample and for the standard (mixture of all samples). Prior to 2-DE, protein samples were labeled using the CyDyes^{TM} DIGE Fluors (Cy2, Cy5 and Cy3). Three biological replicates were prepared for each sample, to ensure statistical significance of analyzed data. Each sample was dye-swap labeled with Cy3 and Cy5, and Cy2 was used for labeling the internal standard created by pooling an aliquot of all biological samples.



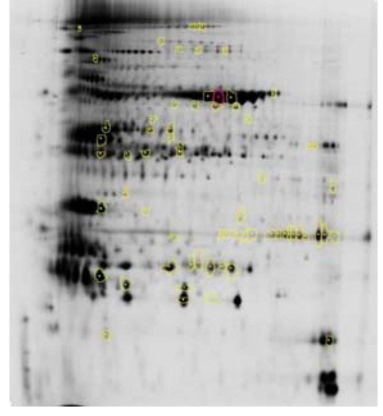
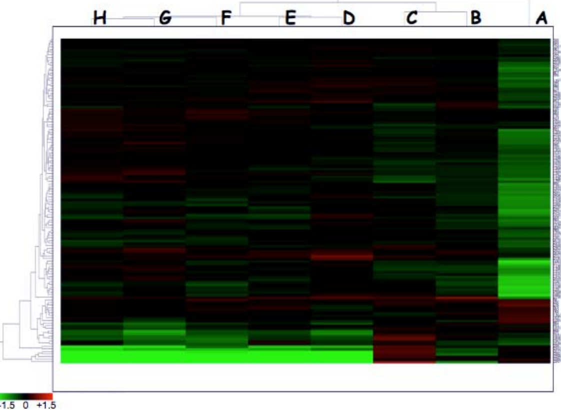


Fig. 3 - 2-DE proteomic map of identified proteins analysed by DIGE. Proteins were separated by IEF at pH 3-11NL, followed by 12.5% SDS PAGE and visualized by scanning using Typhoon 9410. Spots marked with circles refer to protein identified by nLC-ESI-IT-MS/MS analysis.

Fig. 4 - Heat map of differentially expressed proteins (ANOVA, $p \leq 0.05$) that showed at least 1.4 fold change in level compared to internal standard. Red indicates increase of protein levels while green represents repression. **A,** WT apical leaves; **B,** T inoculated leaves; **C,** WT inoculated leaves; **D,** T mock-inoculated leaves; **E,** WT mock-inoculated leaves; **F,** T apical leaves; **G,** T mock apical leaves; **H,** WT mock apical leaves.



BIOCHEMISTRY

Inhibitors of protein aggregation

Alessandra Pasquo

Some neurodegenerative disorders, as for example the Alzheimer‘s disease, are characterized by an extracellular deposition of protein aggregates (insoluble fibrils) that are deposited as plaques. Fibril formation is preceded by a modification of protein folding (Amyloid βeta fragments 1-42 of Amyloid precursor protein) followed by formation of higher molecular mass intermediates deriving from the regular stacking of beta–sheet structures**)**. A therapeutic approach may be realized by the synthesis of molecules capable to interact with A βeta peptides without becoming part of beta-sheet structure and preventing its formation. This approach can be carried out either with specific antibody or with synthetic peptides, named beta-sheet breaker peptides (Fig. 1), in order to destabilize the amyloidogenic A βeta conformers hence precluding amyloid formation. Project carried out in collaboration with Valerio Consalvi, Roberta Chiaraluce, Dipartimento di Scienze Biochimiche Università di Roma Sapienza, and Cesare Giordano, Istituto di Chimica Biomolecolare, CNR Roma.

Fig. 1 –Schematic representation of peptides 1-4.

Kinases and phosphatases

Alessandra Pasquo

Protein phosphatases and kinases play a key role in the regulation of different cellular functions, cell development, proliferation, differentiation, migration and cell cycle progression. They are also involved in several signalling pathways and in the regulation of apoptosis. Moreover, kinases and phosphatases families, identified in humans, have been reported as signalling protein important in tumor biology. High expression levels of kinases have been found in many cancer types like leukaemia, lymphoma, prostate cancer and multiple myeloma. Pim kinases family are considered to be involved in the initiation and progression and invasion of cancer malignant phenotype. Several variants of protein tyrosine kinase and phosphatases have been detected in neoplastic tissues. Many of these natural variants are non-synonymous single nucleotide polymorphisms (nsSNPs) occurring in the coding region and leading to the same polypeptide sequence with a change in the amino acid sequence. A large number of amino acid substitutions

originate from nsSNPs and an increasingly large number of diseases and defects reported in HGMD and (OMIM) are referred to nsSNPs (Fig. 2).

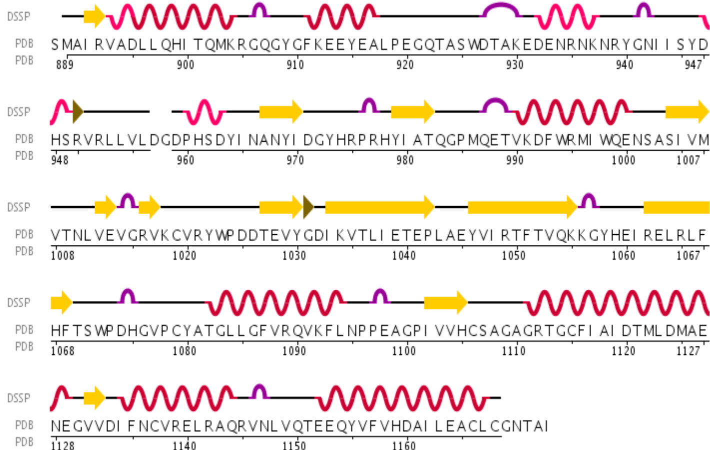


Fig. 2 - PTPRT phosphatase pdb: 2ooQ no° 359 di SGC; fast SNP http://fastsnp.ibms.sinica.edu.tw www.uniprot.org/uniprot/O14522#O14522-1, OMIM 608712 Snps: D927G, R952Q, Q987K, A1118P, V1020A, N1128I; red arrows in the sequence.

Although most nsSNPs are considered not to affect protein function, computational analysis predicts that around 30% of protein variants resulting from nsSNPs have altered stability than the most common variant. In this study, we selected tumor-related nsSNPs kinases and phosphatases variants, expressed. The variants have been expressed and purified in large amount to investigate the effect of single amino acid substitution on thermodynamic stability, function, structure and related to the wild-type protein (Fig. 3). A detailed study of these variants is particularly important because these mutated proteins involved in tumours are important target for direct inhibition by small molecules. Project carried out in collaboration with Valerio Consalvi, Roberta Chiaraluce, Dipartimento di Scienze Biochimiche Università di Roma, Sapienza and Stefan Knapp, SGC University of Oxford.

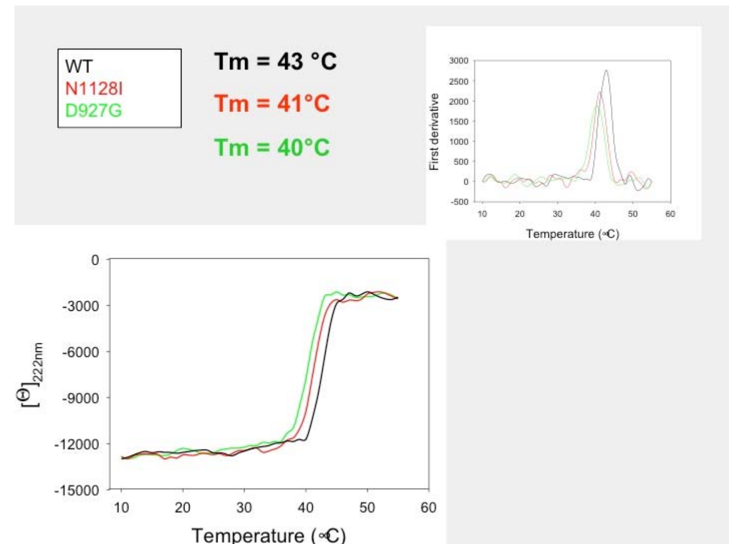


Fig. 3 - CD and Fluorescence Spectra of mutant proteins 7G(SNPS N1128I and D92) in comparison with the wild-type PTPRTAρ protein.

Crystallization of a highly stable ScFv antibody

Alessandra Pasquo, Elena Illiano°, Rosella Franconi
°Postdoc at ENEA

Single chain antibody fragments (scFv) are engineered antibodies composed of the variable regions of both heavy and light chains, encompassing the antigen-binding site, joined together by flexible linkers allowing functional expression as a single polypeptide sequence. All scFv antibodies selected from an ENEA proprietary phage-display library (named "F8 library") designed on a single antibody scaffold, are binders endowed with high thermodynamic stability that can be expressed as soluble molecules in bacterial and eukaryotic cell cytoplasm. These features make them ideal candidates for extra- and intra-cellular applications in human therapy and *in vivo* diagnosis. Site-directed mutagenesis and three-dimensional structure determination represent a rational approach to identify the structural features conferring stability to this peculiar antibody scaffold. Activity carried out in collaboration with Veronica Morea and Andrea Ilari CNR IBMP, Roma

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GRANTS

2010 Italian Ministry of Foreign Affairs funded Project: 'Challenge for global health. Plant-derived biopharmaceuticals'. Italy-Japan Bilateral Project. Project Coordinator, E. Benvenuto; Scientific Responsible, M. Donini.

2007-2010 Me.Di.T.A. project (Metodologie Diagnostiche e Tecnologie Avanzate per la qualità e la sicurezza di prodotti alimentari del Mezzogiorno d'Italia).

PATENT

Franconi R, Spanò L, Venuti A, Massa S. (2009). 'Vaccines based on genetic chimera of viral and/or tumoral antigens and plant proteins'. ITALIAN PATENT RM2009A000383- INTERNATIONAL PATENT PENDING (PCT/IT2010/00324, 21 July 2010).

MAIN FACILITIES

The Technical Unit of Radiation Biology and Human Health includes some peculiar research facilities: the Animal House, a 250 keV X-Ray generator, the Contained Greenhouse

Even though tissue culture are used wherever possible for investigating biological mechanisms, the Unit operates a strictly controlled Animal House to allow studies based on in vivo models, which are essential for patho-physiological research and preclinical testing. The Animal House at C.R. Casaccia (Responsible Dr. Marta Piscitelli) is able to host over than 5 thousand animals (mainly mice). Mice are wild type but also knockout out strains; some are supplied by external laboratories and facilities, other lines are selected in our laboratories. The Animal House is actually managed by Charles River Laboratories Italia Srl, whose personnel is qualified for taking care of the animals and for treating them on the bases of the appropriate experimental protocols. The Animal House allows us to collaborate and to create network with different institutions at national and international level, supporting our participation to the European research programs.

Animal House

Biomedical researchers at ENEA Casaccia are supported by an Ethical Committee composed by external and internal members.

The Ethical Committee has a mandatory item for animal care improvement suggestions and the application of the “3 R’s principles” which are the ethical keys of the national law. Ethical Committee plays an important role in approving projects involving animals, and represents also an important reference settlement that influences the culture of the ENEA research community. By promoting educational activities and debates, the Ethical Committee can contribute to develop a culture of responsible use of animals in lab, including the update of the best experimental practices.

Dosimetry for in vitro and in vivo experiments with the 250 keV X-Ray generator is settled in the framework of fruitful collaboration with colleagues of the ENEA Metrology Institute.

250 keV X-Ray Machine

The contained greenhouse was designed to be a complete and compact solution for growing genetically modified plants that may exhibit new characteristics or may be capable of interbreeding with related species present in the vicinity (Biosafety Level 2 Containment). The structure provides every convenience to make agricultural practices easy. In fact, it is organized in eight isolated and independent modules (14 sq. m.); all with differential programme setting of temperature and artificial illumination and two large working and deposit areas. The glasshouse is equipped with a heating/cooling system of 160 kW/h. At the present, primary physical containment is provided by both the facility and the equipment within the facility: personal badge access control system, air-wash devices, interlock doors, video surveillance, computer-controlled events monitoring, HEPA air filters, air and water UV-sterilizers. Containment in the greenhouse is also maintained through the negative air pressure, inward airflow, within the facility. Good Agricultural Practices (GAP) are required to manage these activities and appropriate measures are required to staff members.
Greenhouse structural components are in zinc-coated steel and coverings in alveolar polycarbonate (16 mm thick) clear enough to provide optimum light transmission and at

the same time be durable as well as economical. All shade casting members are removed from the eave line, so that the glazed portion extends in one unbroken span from the sill to the roof ventilators, allowing the greatest amount of light to enter the greenhouse from sunrise until sunset. The greenhouse measures 210 square meters and its width balances perfectly with its length.

ENEA Greenhouse

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del DNA in tumori cerebrali. XV Convegno Nazionale della Società Italiana per le Ricerche sulle Radiazioni. Roma 27-29 Ottobre 2010 (Premio poster S.I.R.R. 2010).

68. Testa A, Sterpone S, Cornetta T, Poggioli T, Patrono C, Donato V, GiammarinoD, Cozzi R. DNA repair capacity and acute radiotherapy adverse effects in breast cancer patients: evaluation of single-nucleotide polymorphisms in repair genes and biomarkers of in vitro DNA damage. SIMA National Symposium (Italian Society of Environmental Mutagenesis), 15-16 Nov 2010, Rome, Italy.

69. Testa A, Sterpone S, Cornetta T, Poggioli T, Patrono C, Donato V, Giammarino D, Cozzi R. DNA repair capacity and acute radiotherapy adverse effects in breast cancer patients: evaluation of single-nucleotide polymorphisms in repair genes and biomarkers of in vitro DNA damage. Simposio della Società Italiana di Mutagenesi Ambientale (S.I.M.A.) "Instabilità genetica e riparazione del DNA: nuovi paradigmi per la ricerca transazionale". 15-16 Novembre 2010, Roma.

70. Testa A. "Le applicazioni metodologiche e lo sviluppo di biomarcatori in tossicologia" XXVIII Conferenza Nazionale di Citometria – Scuola Nazionale di Citometria - Corsi Residenziali di Aggiornamento a Formazione. 29 Settembre - 2 ottobre 2010, Urbino.

71. Uccelli R. Reduction of environmental impact from cigarette butts and production of energy through a pyro-gasification plant. ENEA's Project Presentation to British American Tobacco (BAT). BAT Italy-ENEA Workshop; Rome, April 27, 2010.

72. van der Esch A, Carnevali F. Medicamento avanzato per la cicatrizzazione a base di Neem ed Iperico: dall'idea al mercato; dagli effetti ai possibili meccanismi d'azione. Workshop Terapie Innovative e Sostanze Naturali (TISNa), ISS, 12/04/2010.

SCIENTIFIC AND ORGANIZING CONGRESS COMMITTEES

1. Baschieri S., Benvenuto E. "Modern Vaccines and Delivery Technology", Casaccia Research Center, Rome, Italy, 14-15 October 2010 (Local Organizers).

2. Giovanetti A. Session: Ionizing radiation and antioxidants. 8th Indo-Italian Workshop on Chemistry and Biology of Antioxidants, Rome 29 October-1th November 2010.

3. Giovanetti A. Member of the Scientific Committee for the organization of the Symposium "From dosimetry to biological effect: radiobiology as guide to clinical practice in nuclear medicine", 29 October - 1 November 2011, Ischia.

4. Giovanetti A. Member of the Scientific Committee of the Scuola Superiore di Radioprotezione "C. Polvani".

5. Lombardi CC. Member of the Scientific and Organizing Committee and chairperson of National Workshop "The Environmental Impact of Tobacco Smoke", ENEA, Rome, January 21, 2010.

6. Pacchierotti F. Member of the Scientific and Organizing Committee of the XV National Congress of the Italian Society for Radiation Research, Rome, October 27-29, 2010.
7. Pacchierotti F. Member of the Scientific and Organizing Committee of the Workshop “Genetic instability and DNA repair: new paradigms for translational research” organized by the Italian Environmental Mutagen Society, Rome, 15-16 November, 2010.
8. Spanò M. Member of the Scientific Committee and chairperson of the session “Epigenetics”, 2nd Serono Symposia International Foundation Conference on “Gene, Environment, Lifestyle Interactions and Human Reproduction”, Malmö (Sweden), August 27-28, 2010.
9. Testa A, Patrono C. XV Convegno Nazionale della Società Italiana per le Ricerche sulle Radiazioni (S.I.R.R.). Roma, 27-29 October 2010.
10. Testa A. XV Convegno Nazionale della Società Italiana per le Ricerche sulle Radiazioni (SIRR), Sessione III - Effetti biologici delle radiazioni: approcci modellistici e sperimentali. Roma, 27-29 Ottobre 2010 (Chairperson).

PARTECIPATION TO SCIENTIFIC COMMITTEES

1. Amendola R. Member of the Editorial Board of “The International Journal of Low Radiation”. WONUC - World Council of Nuclear Workers.
2. Amendola R. Member of the Expert Group in the VII FP European Support and Coordination project “THESEUS” (Towards Human Exploration of Space: a EUropean Strategy) Space Radiation Biology WorkPackage. (http://www.esf.org/research-areas/space-sciences/activities/theseus.html).
3. Amendola R. Member of the reviewers panel of European Space Agency, European Science Foundation.
4. Donini M. and Lico C. Working group on food contaminants (antimicrobial resistance) part of the Joint FAO/WHO food standard programme, ‘Codex Alimentarius’ Commission 2010.
5. Franconi R. Expert Panel Meeting for the ex-post evaluation of 44 Belgian interuniversity networks in the framework of IAP-VI programme’, November 8-10 2010, ‘Belgian Science Policy Office’ (BELSPO), Brussels, Belgium.
6. Giovanetti A. FIRR: Representative of AIRP in the Secretary and Member of the Scientific Committee.
7. Lovisolo GA, Marino C. Scientific Committee of Italian Interuniversitary Centre on “Interaction between electromagnetic fields and biosystems” (ICEmB).
8. Lovisolo GA. Referees list for the Evaluation of research projects on behalf of Italian Ministry of Education, University and Research.

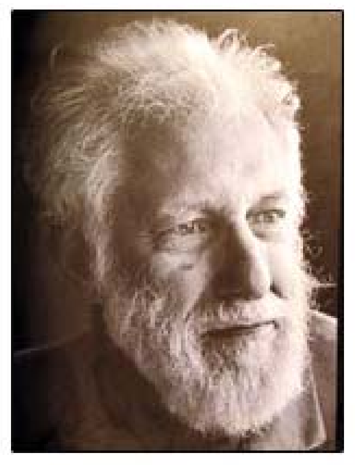
9. Marino C. COST ACTION BM704: Emerging EMF Technologies Health Risk Management (Italian delegate).
10. Marino C. Council of European BioElectromagnetic Association (President).
11. Marino C. Editorial Board of "Bioelectromagnetics".
12. Nardi L. Global Citrus Germplasm Network (FAO) workgroup "Global Computerised Citrus Germplasm Information System" workgroup; 1st European Triticeae Genomics Iniziative (ETGI); "International Wheat Genome Sequencing Consortium".
13. Pacchierotti F. OECD Expert Group on the Development of a Test Guideline on a Transgenic Rodent in vivo Gene Mutation Assay.
14. Pacchierotti F. OECD Working Party on Manufactured Nanomaterials, Steering Group Safety Testing of a Representative Set of Manufactured Nanomaterials.
15. Pioli C. International Oversight Committee for the Russian-French replication studies of the Soviet results on RF exposure promoted by WHO EMF project.
16. Testa A. Società Italiana per le Ricerche sulle Radiazioni. Roma (S.I.R.R.).
17. Uccelli R, Lombardi CC. Convention between ENEA and the Italian Association of Doctors for the Environment (ISDE) concerning coordination and collaboration in epidemiological and toxicological research and training activities.
18. Uccelli R. ENEA-ISS Joint Agreement for definition of priorities in epidemiological and toxicological research activities and for conduction of collaborative studies on risk factors for populations.
19. Uccelli R. NATO Committee Science for Peace and Security **(**SPS), Panel Chemistry/Biology/Physics (CBP).

TEACHING AND DISSEMINATION ACTIVITIES

1. Altavista P. Environmental Epidemiology. Contract professor at the University of Rome "Tor Vergata", bachelor course "Techniques for the Prevention in the Environment and in the Workplace".
2. Cordelli E. Comparison among different methods to evaluate sperm chromatin integrity. Lecture at the Professional upgrading and updating course of the National School of Cytometry. Urbino University, September 29-October 1, 2010.
3. Cordelli E. Environmental Mutagenesis. Contract professor at the University of Rome "Tor Vergata", bachelor course "Techniques for the Prevention in the Environment and in the Workplace".
4. Donini M. Biotechnology IFTS Course (Corso Tecnico Superiore per l'industrializzazione del prodotto e del processo: Biotecnologie per l'individuazione e lo sviluppo di molecole e prodotti per il settore chimico-farmaceutico e cosmetico'). Extraction of biopharmaceuticals from Plants, April 2010.

5. Donini M. Contract Professor at Faculty of Agriculture Tuscia University (Viterbo, Italy). MSc Degree course on: ‘Plant secondary metabolites’, March-June 2010.
6. Franconi R. Lecturer within the ENEA ‘Biotecnoform’ Project ‘Training of experts in the production of biomolecules from yeast, bacteria by biofermenters or extracted from plants’.
7. Franconi R. Contract Professor of ‘Plant Biotechnologies for the Environment and Human Health’, Faculty of Medicine, University of Rome, Tor Vergata, Italy, 2006-2010.
8. Franconi R. Contract Professor of ‘Pharmaceutical Biotechnologies’, Faculty of Sciences, Tuscia University,Viterbo, Italy, 2009-2010.
9. Giovanetti A. Diagnostic Imaging and Radiation Therapy. Triennal Course of Degree in Prevention Techniques in the Environment and the Work Place, Tor Vergata University of Rome, Italy.
10. Giovanetti A. International Summer School. Criteria and Approaches for Radioactive Waste Management and Nuclear Decommissioning, Milano 6-9 July 2010, lesson: Scientific bases for low dose effects.
11. Lombardi CC. Chemical and carcinogenic risk assessment. Contract professor at the University of Rome “Tor Vergata”, bachelor course “Techniques for the Prevention in the Environment and in the Workplace”.
12. Lombardi CC. Cigarette butts, a toxic waste forgotten. MUSIS initiative “Why is still a lot of dependence of youth from tobacco despite the information, health and environment”. 9th week of the Italian Scientific and Technological Culture. Lecture at the Technical High School Gian Lorenzo Bernini, Rome, March 22, 2010.
13. Lombardi CC. Environmental Hygiene. Contract professor at the University of Rome “Tor Vergata”, graduate program in Health Physics.
14. Lombardi CC. Environmental Impact of Cigarette butts. “The angle of science” broadcast, IES-TV (Health Information Television), 12 November 2010.
15. Lombardi CC. Environmental Impact of Cigarette butts. A BBC English Channel Interview, 29 January, 2010.
16. Lombardi CC. Environmental Impact of Cigarette butts. An Isoradio Interview, 4 December, 2010.
17. Lombardi CC. Environmental impact of cigarette butts. Television program GEO & GEO, 17 February, 2010.
18. Mancuso M. Applied Biology, Triennal Course of Degree in Prevention Techniques in the Environment and the Work Place, Tor Vergata University of Rome, Italy.
19. Marino C. Electromagnetic Fields II (Biological Effects). Course of Triennal Degree in Prevention Techniques in the Environment and in Work Place, Tor Vergata University of Rome, Italy.

20. Massa S. Board of examiners member ("honorary fellow") of ‘Pharmaceutical Biotechnologies’ Course - Faculty of Science, Tuscia University,Viterbo, Italy.
21. Massa S. Lecturer within the UNESCO-ENEA Desire-Net e-learning Project ‘Genetically modified plants’.
22. Massa S. Tutor for practical laboratory lessons within the ENEA ‘Biotecnoform’ Project ‘Training of experts in the production of biomolecules from yeast, bacteria by bio-fermenters or extracted from plants’.
23. Nardi L. Biotechnology IFTS Courses: Bioinformatics and Database analysis. Corso Tecnico Superiore per l’industrializzazione del prodotto e del processo: Biotecnologie per l’individuazione e lo sviluppo di molecole e prodotti per il settore chimico-farmaceutico e cosmetico, e Corso Tecnico Superiore in Biotecnologie e Tecnologie Alimentari. Extraction of biopharmaceuticals from Plants, April 2010.
24. Nardi L. Course on Plant Cytogenomic held by the National School of Cytometry GIC (Italian Group of Cytometry), Urbino, 30 September, 2010.
25. Pasquo A. Biotechnology IFTS Courses: Bioinformatics. (Corso Tecnico Superiore per l’industrializzazione del prodotto e del processo: Biotecnologie per l’individuazione e lo sviluppo di molecole e prodotti per il settore chimico-farmaceutico e cosmetico).
26. Pinto R. Electromagnetic Fields I (Non–ionizing electromagnetics fields: Dosimetry and Protection). Triennal Course of Degree in Prevention Techniques in the Environment and in the Workplace, Tor Vergata University of Rome.
27. Pioli C. Faculty member of the PhD program “Immunology and Applied Biotechnologies”, Tor Vergata University of Rome.
28. Pioli C. Contract professor of “Molecular Immunology” for the bachelor in Biological Sciences, Tor Vergata University of Rome.
29. Salerno S. Psychosocial risk evaluation in the workplace. Contract professor at the University of Rome “Tor Vergata”, bachelor course “Techniques for the Prevention in the Environment and in the Workplace”.
30. Testa A. Applied Biology, Triennal Course of Degree in Prevention Techniques in the Environment and the Work Place, Tor Vergata University of Rome.
31. Villani M.E. Biotechnology IFTS Course: Bioinformatics and Database analysis. Corso Tecnico Superiore per l’industrializzazione del prodotto e del processo: Biotecnologie per l’individuazione e lo sviluppo di molecole e prodotti per il settore chimico-farmaceutico e cosmetico. Extraction of biopharmaceuticals from Plants, April 2010.



Jiulian Otto Trevelyan, (20 February 1910 – 12 July 1988) was a British artist and poet.

Trevelyan was the only child of Robert Calverley Trevelyan and his wife Elizabeth van der Hoeven. His grandfather was the liberal politician Sir George Otto Trevelyan and his uncle the historian George Macaulay Trevelyan. Julian Trevelyan was educated at Bedales School and Trinity College, Cambridge, where he read English Literature. He moved to Paris to become an artist and enrolled at Atelier Dix-Sept, Stanley William Hayter's engraving school, where he learned about etching. He worked alongside famous artists including Max Ernst, Oskar Kokoschka, Joan Miró and Pablo Picasso. In 1935, Trevelyan bought Durham Wharf, beside the River Thames in Hammersmith, London. This became his home and studio for the rest of his life and was a source of artistic inspiration to him. He became a confirmed Surrealist and exhibited at the *International Surrealist Exhibition*, held at the New Burlington Galleries in London. He married Ursula Darwin, daughter of Bernard Darwin and great-granddaughter of Charles Darwin, but their marriage was dissolved in 1950. Their son is the film-maker Philip Trevelyan. Julian Trevelyan married the painter Mary Fedden in 1951. From 1950 to 1955, Trevelyan taught history of art and etching at the Chelsea School of Art. During 1955–63, he was Tutor of Engraving at the Royal College of Art, rising to Head of the Etching Department where he was influential to many younger printmakers, including David Hockney and Norman Ackroyd. In July 1986, Trevelyan was awarded a senior fellowship at the Royal College of Art and in September 1987 he was appointed a Royal Academician. Trevelyan died on 12 July 1988 in Hammersmith, London.

(From Wikipedia, the free encyclopedia)

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