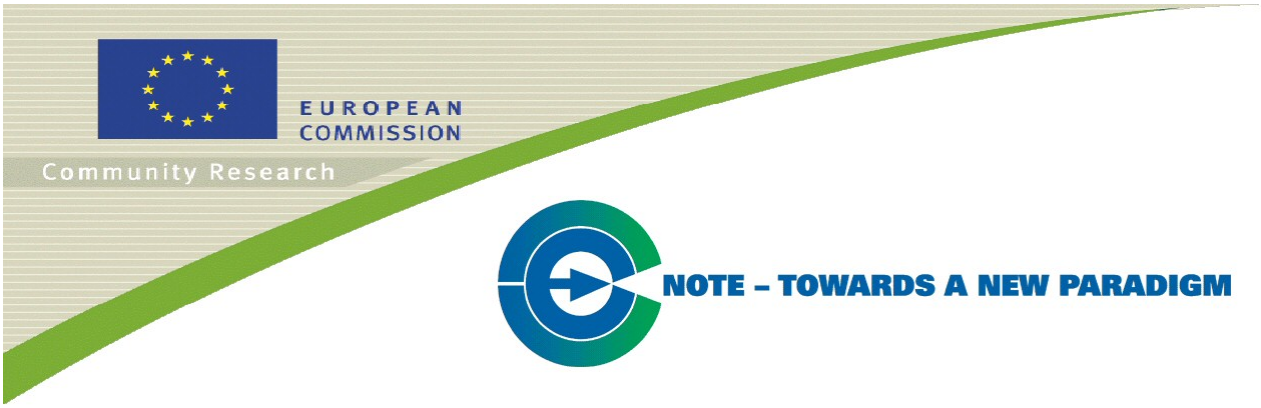

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NOTE

Non-targeted effects of ionising radiation

Integrated Project

Research and Training on Nuclear Energy (EURATOM)

Publishable Final Activity Report

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Authors: Sisko Salomaa (Coordinator), Eric Wright, Guido Hildebrandt, Munira Kadhim, Mark Little and Kevin Prise

Contents

Abbreviations used

ATM	Ataxia Telangiectasia Mutated
BE	bystander effect
GI	genomic instability
IIA	intercellular induction of apoptosis
LNT	linear no threshold
NO	nitric oxide
NOTE	Non-targeted effects of ionizing radiation (FP6 project)
ROS	reactive oxygen species
WP	Work package
IL6	interlenkine-6
IL8	interlenkine -8
MN	micronucleus, micronuclei
LET	linear energy transfer
DDREF	dose and dose rate effectiveness factor
Gy	gray, joule per kg
mGy	milligray
DNA	deoxyribonucleic acid

Partner acronyms

STUK	Radiation and Nuclear Safety Authority
UNIVDUN	University of Dundee
UL	Leipzig University
MRC	Medical Research Council
ICFM	Imperial College
GCI	Gray Cancer Institute
SCK-CEN	Belgian Nuclear Research Centre
RESC	Dublin Institute of Technology,
ISS	Istituto Superiore di Sanita
ULEICS	University of Leicester
MAC	McMaster University
AECL	Atomic Energy of Canada Limited
NRIRR	National Research Institute for Radiobiology and Radiohygiene,
HMGU	Helmholtz Zentrum München
UNIPV	University of Pavia
FAU	Friedrich-Alexander University of Erlangen-Nürnberg
UDE	University of Duisburg-Essen
OUS	Oslo University Hospital
OHIRC	University of Ottawa Heart Institute
GUF	Johann-Wolfgang Goethe University of Frankfurt
QUB	Queens University Belfast
UROS	University of Rostock
OBU	Oxford Brookes University

1 Overview of the NOTE's objectives and scientific program management

The universality of the target theory of radiation-induced effects is challenged by observations of non-targeted effects such as bystander effects and genomic instability. Essential features of non-targeted effects are that they do not require direct nuclear exposure by radiation and they are particularly significant at low doses. This new evidence suggests a need for a *new paradigm* in radiation biology. The new paradigm should cover both the classical (targeted) and the non-targeted effects. New aspects include the role of cellular communication and tissue-level responses. A better understanding of non-targeted effects may have important consequences for health risk assessment and, consequently, on radiation protection. Non-targeted effects may contribute to the estimation of cancer risk from occupational, medical and environmental exposures. In particular, they may have implications for the applicability of the Linear-No-Threshold (LNT) model in extrapolating radiation risk data into the low-dose region. This also means that the adequacy of the concept of dose to estimate risk is challenged by these findings. Moreover, these effects may provide new mechanistic explanations for the development of non-cancer diseases. Further research is required to determine if these effects, typically measured in cell cultures, are applicable in tissue level, whole animals, and ultimately in humans.

The general objectives of the NOTE project were:

- to investigate the mechanisms of non-targeted effects, in particular, bystander effects, genomic instability and adaptive response;
- to investigate if and how non-targeted effects modulate the cancer risk in the low dose region, and whether they relate to protective or harmful functions;
- to investigate if ionising radiation can cause non-cancer diseases or beneficial effects at low and intermediate doses;
- to investigate individual susceptibility and other factors modifying non-targeted responses;
- to assess the relevance of non-targeted effects for radiation protection and to set the scientific basis for a modern, more realistic, radiation safety system;
- to contribute to the conceptualisation of a new paradigm in radiation biology that would cover both the classical direct (DNA-targeted) and non-targeted (indirect) effects.

The overall organisational structure of NOTE is described in Figure 1. Work package leaders Eric Wright, Guido Hildebrandt, Munira Kadhim, Mark Little and Kevin Prise had a major role in the scientific coordination of the project. STUK was responsible on the administrative coordination of the project (Sisko Salomaa) as well as the dissemination and exploitation activities (Oleg Belyakov and Virpi Launonen). All WP leaders were members of the Management Board, steering the work program. External view was provided by Advisory Board (William F Morgan, Christian Streffer, Francis Cucinotta, Susanne Schultz-Hector and Barry Michael).

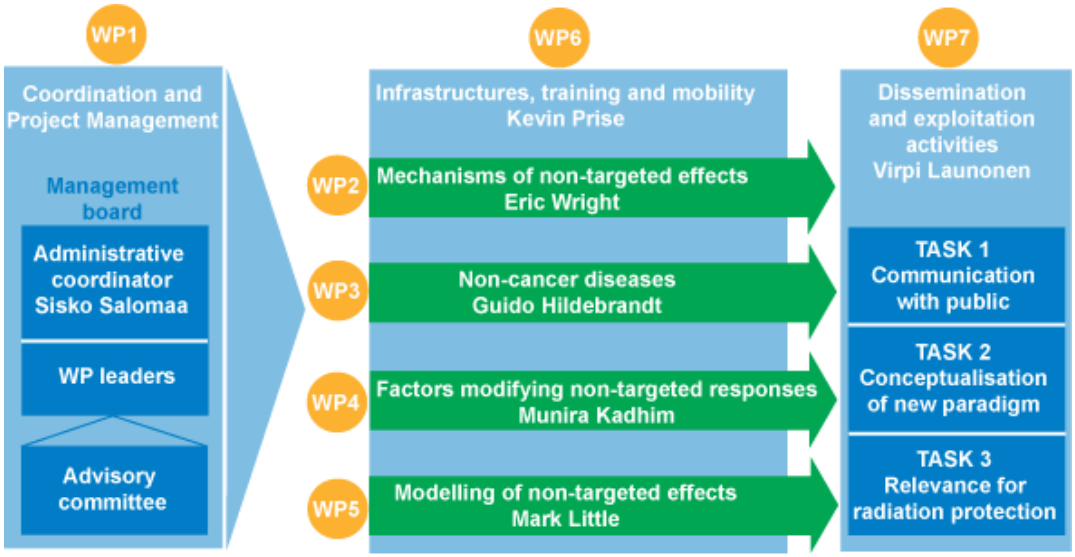

Figure 1: Organisational structure of NOTE

The work program was updated annually, phasing down activities that were considered less relevant and strengthening areas where promising advances in science could be expected. Resources for such shifts in work program, better integration of groups and training of young scientists were allocated from the flexibility budget. During the project lifetime, research was re-oriented from higher doses (used for the validation of model systems) to low doses. A low dose was defined as 0.1 Gy or less and moderate doses as 0.1 – 1 Gy for low-LET radiation. For high-LET radiations and experimental settings, one particle per cell was considered as low dose. Gradually, investigations on cell cultures were replaced by studies on tissue and animal models. Interaction between experimentalists and modellers was actively encouraged as assessment on spatial and temporal distribution of the non-targeted effects was considered an essential step towards judgements on the shape of dose responses and judgements on the concept of dose. Conceptualisation of the new paradigm and potential implications for risk assessment and radiation protection were discussed in several scientific and stakeholder workshops. Towards the end of the project, evidence for departure from linearity for human malignant and non-malignant disease was addressed by meta-analyses of epidemiological data and evaluation of potential mechanisms in disease induction and progression.

2 Contractors involved

The NOTE project started in 2006 and lasted till the end of 2010. The consortium brought together 21 partner organisations from seven EU countries, Canada and Norway.
The organisations involved were:

- Radiation and Nuclear Safety Authority, STUK (Sisko Salomaa)
- University of Dundee, UNIVDUN (Eric Wright)
- Leipzig University, UL (Guido Hildebrandt, Annegret Glasow, Tilman Butz)
- Oxford Brookes University, OBU (Munira Kadhim), Medical Research Council, MRC until 2008
- Imperial College, ICFM (Mark Little)
- Queens University Belfast, QUB (Kevin Prise), Gray Cancer Institute, GCI until 2008
- Belgian Nuclear Research Centre, SCK-CEN (Paul Jacquet)

- Dublin Institute of Technology, RESC (Fiona Lyng)
- Istituto Superiore di Sanita, ISS (Maria Antonella Tabocchini)
- University of Leicester, ULEICS (Yuri Dubrova)
- McMaster University, MAC (Carmel Mothersill)
- Atomic Energy of Canada Limited, AECL (Ron Mitchel/Nick Priest)
- National Research Institute for Radiobiology and Radiohygiene, NRIRR (Géza Sáfrány)
- Helmholtz Zentrum München, HMGU (Werner Friedland)
- University of Pavia, UNIPV (Andrea Ottolenghi)
- Friedrich-Alexander University of Erlangen-Nürnberg, FAU (Franz Rödel, Udo Gaipl)
- University of Duisburg-Essen, UDE (George Iliakis)
- Oslo University Hospital, OUS (Jostein Dahle)
- University of Ottawa Heart Institute, OHIRC (Stewart Whitman)
- Johann-Wolfgang Goethe University of Frankfurt, GUF (Franz Rödel)
- University of Rostock, UROS (Guido Hildebrandt)

3 Work performed and end results

The dogma that genetic alterations are restricted to directly irradiated cells has been challenged by observations in which effects of ionizing radiation, characteristically associated with the consequences of energy deposition in the cell nucleus, arise in non-irradiated cells. These, so called, non-targeted effects are demonstrated in cells that have received damaging signals produced by irradiated cells (radiation-induced bystander effects) or that are the descendants of irradiated cells (radiation-induced genomic instability). Radiation-induced genomic instability is characterized by a number of delayed adverse responses including chromosomal abnormalities, gene mutations and cell death. Similar effects, as well as responses that may be regarded as protective or adaptive, have been attributed to bystander mechanisms.

3.1 What are the mechanisms of non-targeted effects and what factors modify their response?

In order to formulate the new radiobiological paradigm that would cover non-targeted effects in addition to the classical DNA targeted effects, it is necessary to shed light on the mechanisms of non-targeted effects and to evaluate the universality of these responses in cellular, tissue, organ and at species level.

The studies of mechanisms were structured around a number of specific questions relating to mechanisms underlying non-targeted effects; in this context, taken to mean bystander effects, genomic instability and some examples of adaptive response:

- What cells produce signals?
- What is the trigger for the production of signals?
- What cells respond to signals?
- What intracellular processes are involved in responding to signals?
- What are the messengers of bystander responses?
- What are the mechanisms of non-targeted effects in tissues?
- What are the mechanisms of genomic instability perpetuation?

- What are the mechanisms of transgenerational instability?

At the start of this project, the mechanism of non-targeted effects demonstrated in some, but not all, cells and in some, but not all, genotypes. It was not understood nor was it known whether all endpoints reflect any commonality of an underlying mechanism. A number of *in vitro* studies had pointed to an association with free radicals/oxidative processes, inter-cellular signaling and the production of cytokines. *In vivo*, major causes of "spontaneous" DNA damage are associated with oxidative stress and such processes have also been implicated in the tissue responses to irradiation. Mechanistically these are also features of inflammatory responses that may be protective or damaging, depending on context.

Whilst many published studies have concentrated on damaging responses of non-targeted effects a limited number of studies have drawn attention to the possibility of protective responses. The determinants of whether a non-targeted effect will occur and whether it would be damaging or protective were not known. In addition, one of the major questions was whether effects demonstrated by *in vitro* systems can be extrapolated to the *in vivo* situation where additional protective mechanisms that are not operating *in vitro*, are important. Thus, major objectives included the characterization of signaling mechanisms involved in non-targeted effects.

At the start of the project, the mechanistic studies addressed six main tasks and the research has utilized both *in vitro* and *in vivo* model systems with significant effort directed towards characterizing the mechanisms underlying the various bystander effects demonstrable in the models. The six main tasks were:

- to establish and validate robust model systems of non-targeted effects
- to investigate gene expression changes in bystander cells using primary human fibroblasts of known and differing radiosensitivities irradiated with high and low doses of X-rays
- to investigate signalling molecules involved in non-targeted effects
- to investigate non-targeted effects in tissue culture systems: the artificial human tracheal/bronchial tissue and explants cultures established from mice exposed to low dose, low-LET radiation
- to investigate non-targeted genetic instability effects in irradiated mice and their first generation offspring. Complementing these various investigations, adaptive response and bystander effects were also investigated using fish and amphibian models to determine evolutionary linkages and to explore relationships between these non-targeted effects.
- to test the hypothesis that radiation-induced and transgenerational instability in mice can be attributed to epigenetic changes in gene expression and non-targeted effects in the bone marrow of irradiated mice involve activation of stress and damage response signalling pathways.

The establishment and validating of robust model systems of non-targeted effects was completed by mid-term of the project. An appropriate range of robust *in vitro* systems to investigate non-targeted effects had been established and there was no compelling argument for further collection of phenomenological findings. The systems fell into 4 categories (i) cells irradiated with microbeam ions or low fluence alpha-particles where not all cells are irradiated (ii) co-cultures, where irradiated cells share the medium with bystander cells (iii) transfer of medium in which cells were irradiated onto unirradiated cells (iv) obtaining tissue explants or cell suspensions from tissues irradiated *in vivo* at various times post-irradiation and by centrifugation obtaining medium that is transferred onto non-irradiated cells. Much of the early research in establishing model systems and obtaining preliminary data on mechanisms operating after

low-LET exposures had used relatively high (>1 Gy) radiation doses. Later on, there was a significant emphasis on lower doses.

In early studies the National Research Institute for Radiobiology and Radiohygiene had demonstrated that about 200 genes in human fibroblasts were shown to respond to radiation and the expression profile depended on individual genetic backgrounds of the cells (Kis et al, 2006). Several genes were induced by low dose radiation both in directly hit and bystander cells and cell-type differences and differences in transcriptional kinetics were also detected. Based on the gene expression data, two genes (TP53INP1 and GDF15) were selected for further study. *TP53INP1* (tumor protein p53 inducible nuclear protein 1) promotes p53 phosphorylation and subsequent apoptosis and *GDF15* (growth differentiation factor 15 precursor) is a secreted cytokine induced in a dose dependent manner in directly hit cells. Gene silencing technology was used to reduce their expression in cells and silencing the GDF-15 gene increased the radiation sensitivity of the irradiated cells and inhibited the response of bystander cells (Hegyesi et al, 2011). The bystander response was unaffected in TP53INP1 silenced cells.

Investigations on signalling molecules were performed by several laboratories. *In vitro* studies were carried out by Istituto Superiore di Sanita, University of Pavia, Gray Cancer Institute, Queens University of Belfast, Dublin Institute of Technology and MacMaster University. Istituto Superiore di Sanita demonstrated that alpha particle-induced signaling factors trigger a rapid production of NO in bystander AG01522 cells and an increase in micronuclei (MN) production. Comparison with results obtained after gamma irradiation doesn't show significant difference as a function of radiation quality for DNA damage induction. Experiments on the timing and dose-dependence of bystander induced cell killing after gamma-rays or alpha-particle irradiation, using the medium transfer approach have shown a reduction in clonogenic survival after incubation with medium from alpha-irradiated cells, independent of dose and time, and a slight dose and time dependence for gamma-irradiation. Magnetic Resonance Spectroscopy (MRS) has shown the presence of an hyper metabolic state in bystander cells with a modest radiation quality dependence. No evidence of bystander-mediated adaptation, using medium from both gamma- or alpha-irradiated cells, was obtained in these studies. The adaptive response model implemented at ISS has given suggestion for the design of new experiments to be performed (Esposito et al., 2011). University of Pavia carried out parallel studies with medium collected from gamma- and alpha-irradiated cells and sham irradiated samples for the presence of a broad spectrum of cytokines. Medium collected 20 hours after irradiation displayed different amounts of IL6, IL8 and IL-12 compared to controls. But amounts and timing of release depended on radiation quality. These results have been applied to a mathematical model developed during the project to understand the single cell mechanisms of release. Gray Cancer Institute, Queens University of Belfast have conducted microbeam-based studies and demonstrated that targeting only the cytoplasm of a cell is capable of eliciting damage in both hit and bystander HeLa cells, independently of the dose or the number of cells targeted. The mechanism involves reactive oxygen and nitrogen species and active mitochondrial function is required for these responses. When a fraction of cells within a T98G glioblastoma population was individually irradiated micronuclei (MN) in the nontargeted cells was increased by a mechanism involving NO and TGF-beta1. TGF-beta1 induces free radicals and DNA damage in bystander cells and involvement of ATM in bystander cells has demonstrated the involvement of the Fanconi's Anemia proteins (known to play a role in response to oxidative damage). Dublin Institute of Technology have used a Cell Observer System to simultaneously measure levels of reactive oxygen species (ROS) and nitric oxide (NO) and membrane signalling in real time and demonstrated that calcium and/or ROS induce irradiated cells to release long-lived signalling factors

which can trigger membrane signalling and an influx of calcium further inducing ROS in unirradiated cells (Lyng et al, 2011). Differences in cell death pathways were observed for directly irradiated cells and cells exposed to medium from irradiated cells (Jella et al, 2011). Gene expression studies to investigate apoptotic and stress activated signalling pathways, including Bax, Bcl-2, JNK, ERK, initiator caspases (caspase 2, 8, 9) and effector caspases (caspase 3, 6, 7), showed different responses for directly irradiated cells and for cells exposed to medium from irradiated cells. MacMaster University demonstrated a dose threshold of 2-3 mGy gamma rays for calcium signal induction in irradiated cells correlating with bystander-mediated clonogenic reduction. A threshold for neutron bystander effect induction is in the region of 100-300mGy. A role for bcl-2/cmyc ratio was found and mitochondria are suspected of being the target for bystander effect induction. The mutually exclusive expression of adaptive response with hyper-radiosensitivity / induced radioresistance effect (HRS/IRR)or bystander effects in various cell types was also studied. It appears that IRR, which is the phenomenon of a sudden increase in resistance as the dose increases in a dose response curve, mirrors the adaptive response seen in some cell systems. If a break point occurs in the dose response curve then a bystander effect is not seen above that point. HRS *per se* really means that the break point does not occur hence really sensitive cells which do not show IRR can show bystander signaling and response. Building on previous work showing that serotonin binds to cells following irradiation leading to the calcium flux, several serum samples were tested and serum serotonin level with radiation-induced bystander activity correlated.

In vivo or tissues model studies on signalling molecules were carried out by University of Dundee, STUK, and University of Leipzig. University of Dundee demonstrated that signals generated *in vivo* in the bone marrow of CBA/Ca mice irradiated with 4 Gy gamma-rays 24 hours previously, but not immediately post-irradiation, are able to induce DNA damage and apoptosis in non-irradiated bone marrow cells. The signaling mechanism involves FasL, TNF-alpha, nitric oxide and superoxide and macrophages are implicated as a source of damaging signals (Burr et al, 2010). However, dose-response studies indicate that the signals detected at high doses are not detected at low doses (below~50-100mGy) (Zyuzikov et al, 2011). A pilot study for the characterization of clastogenic factors (Lindholm et al, 2009) from plasma of irradiated subjects was carried out by STUK and University of Leipzig. Blood cells from an unexposed person were cultured with patient plasma taken before or after radiotherapy and the presence of clastogenic factors in the irradiated plasma was investigated by studying DNA damage by γH2AX, chromosome aberration and micronucleus production assays. Subsequent studies measured proteomic changes via protein separation on two-dimensional gel electrophoresis (2DE) and identification of proteins by mass spectrometry. The results obtained imply that the plasma from the particular patient groups investigated in the current study did not contain clastogenic factors induced by radiotherapy. STUK also conducted investigations of non-targeted effects using a commercial artificial human tracheal/bronchial tissue. Accurate assessment of apoptotic cells in this model after microbeam irradiation has proven to be difficult. The overall background frequency of apoptosis was very low and variable between experiments as well as within an experiment. The location of the irradiated portion of the tissue cannot be defined with sufficient accuracy resulting in uncertainty in distance-dependent analysis. The tissue handling after fixation also produces uncertainties to the distance management. Overall this model system is relatively laborious and influenced by multiple and often uncontrollable variables. Bystander effects are detected in some experiments but further studies are needed to confirm the reliability of any data. MacMaster University developed techniques for explant cultures from tissues of irradiated mice and fish and for using the explants as a source of bystander signals tested in HPVG and other cell line reporter cells. Tissue differences in response have been demonstrated and the toxicity of the signals produced is correlated with the ratio of bcl2/cmyc in the tissue. No

bystander signal production was detected in mice fed an antioxidant diet for 2 months prior to radiation exposure. In several cell line bystander reporters, signal production appears to depend on monoamine binding to surface membrane receptors and can be inhibited using serotonin inhibitors.

Somatic and transgenerational instability effects were addressed by University of Leicester and University of Dundee. *In utero* irradiation (1 Gy X-rays) experiments by the University of Leicester induced ESTR mutations in both male and female germline. These data for the first time show that ESTR mutation induction occurs in replication-proficient tissues. *In utero* exposure of male (but not female) mice resulted in ESTR mutation frequencies being significantly elevated in the offspring and the magnitude of instability was similar to that in the offspring of males irradiated during adulthood. In contrast to the offspring of adult male mice exposed to high-dose acute gamma-rays (1 Gy), the offspring of male mice exposed to10 cGy irradiation on transgenerational instability are genetically stable. Taken together with the results of the effects of low-dose-rate paternal irradiation on transgenerational instability where 1 Gy delivered acutely (0.5 Gy/min) produces instability but delivered at 0.001 Gy/min does not, these data imply the presence of a threshold dose below which an instability signal cannot be induced in the germline of irradiated male mice. The University of Dundee demonstrated that following whole body irradiation of CBA/Ca mice with doses above 1 Gy there was evidence for chromosomal instability in the bone marrow. However, at doses below ~1 Gy there was no evidence for such an instability phenotype (Zyuzikov et al, 2011). These data are consistent with a threshold for the induction of chromosomal instability in the bone marrow. When instability is demonstrated *in vivo* the bone marrow contains signals able to induce an instability phenotype *in vitro*. The instability is expressed in bone marrow cells of the radiation-induced acute myeloid leukaemia (r-AML) susceptible strain (CBA/Ca) but not in mice resistant to r-AML (C57BL/6). Macrophages are a source of the signals and the signaling mechanism involves TNF-alpha nitric oxide and superoxide (Lorimore et al, 2008).

Evolutionary conservation and linkages of various non-targeted effects across different species and phyla were addressed by Atomic Energy of Canada Limited and MacMaster University. Atomic Energy of Canada Limited had previously demonstrated that a single whole body low dose of gamma-radiation increased the latency period for spontaneous and radiation-induced tumours and decreased their frequency in C57BL6/J mice. However, short-term *in vitro* adaptive response studies using assays of cytogenetic damage and apoptosis in spleen lymphocytes and lung fibroblasts of C57BL6/J adult female mice showed no statistically significant adaptive response. No radio-adaptive responses in foetal lung fibroblasts or liver mononuclear cells irradiated were demonstrable. Experiments in which both adaptive and challenge irradation were given as whole body dose *in vivo* were conducted (spleen weight and cellularity) and bone marrow lymphocyte cytogenetic damage) and these also indicated the lack of a short-term adaptive response. However, frogs living in radiologically contaminated natural environments (compared with non-contaminated natural environments) exhibited adaptive responses to *in vivo* irradiation. Fatty acid composition of muscle tissue measured in these amphibians is a useful marker of environmental effects and may reflect factors influencing cellular responses to low doses of ionizing radiation. MacMaster University conducted collaborative experiments with AECL on effects of exposure to very low doses (10-50 mGy) on female mice and their offspring. However results were very variable. Similarities between cell to cell bystander communication and fish to fish communication have been demonstrated and proteomic studies indicate the importance of protective responses. Additional studies examined transgenerational effects in the fish-fish model where the offspring of irradiated fish show bystander signal production.

Studies testing the hypothesis that radiation-induced and transgenerational instability in mice can be attributed to epigenetic changes in gene expression were carried out by University of Leicester. To establish the pattern of gene expression, total RNA was extracted from liver, spleen, kidney and brain of 8-week-old non-exposed male offspring of control and irradiated males. RNA samples were studied using NimbleGen expression arrays. The data showed that the pattern of expression of a number of circadian genes is signficantly compromised in the offspring of irradiated parents. Overall, the results show a substantial dysregulation of a number of essential signal pathways in the offspring of irradiated male mice, consistent with the hypothesis of paternal preconceptional irradiation producing an altered epigenetic landscape in the offspring assessed as changes in DNA methylation.

Studies testing the hypothesis that non-targeted effects in the bone marrow of irradiated mice involve activation of stress and damage response signalling pathways were carried out by University of Dundee. The *in vivo* responses of haemopoietic cells to low doses of X-rays by immunohistochemical analysis of the p53 damage response pathway. After the lowest doses, where not all cells may have been irradiated (1-2 mGy) there was only a small (statistically non-significant) increase in p53 expression and for p53 and p21 protein expression and apoptosis, no dose-dependence was observed between 2 and 25-50 mGy in bone marrow (depending on mouse strain) Linear increases in the endpoints were observed at higher doses. Doses below 100 mGy produced no significant increase in apoptosis and at 30 days after whole-body irradiation with doses up to 500 mGy there was no evidence for the expression of a chromosomal instability phenotype. However, after 1 Gy the expression of instability was similar to that after higher doses (Zyuzikov et al, 2011). Additional investigations also demonstrated that 24hours or longer after *in vivo* doses greater than 100 mGy, macrophages produced bystander-type damage in non-irradiated bone marrow cells. The mechanism involves FasL, TNF-alpha and reactive nitrogen/oxygen species (Burr et al, 2010).

Although exact details vary with the model system studied in the mechanistic studies, some general concepts concerning mechanisms non-targeted effects have emerged from this project:

- Production of signals involved in non-targeted effects is cell-type and genotype-dependent
- Production of signals can be influenced by radiation quality. Often, high-LET is a more effective inducer
- Responses to signals are cell-type and genotype-dependent
- The trigger for the production of signals may involve targets other than nuclear DNA;
- Both damaging and protective responses may be elicited in a context-dependent manner;
- Non-targeted effects are mediated by stress response pathways that have evolved to deal with cellular injury
- Signalling pathways involving reactive oxygen/nitrogen species and cytokines are commonly implicated in non-targeted mechanisms.
- *In vivo*, a somatic instability phenotype may not necessarily be a reflection of genomically unstable cells but responses to the ongoing production of pro-inflammatory and pro-apoptotic signals acting in a bystander manner.
- *In vivo,* non-targeted somatic effects are difficult to detect / may not be significant after doses below ~100mGy of low-LET radiation (at least for murine bone marrow)
- Generally, *in vivo* responses have properties in common with inflammatory responses.
- Induction of genomic instability in murine germ cells is dose-rate -dependent (1 Gy delivered acutely but not chronically induces instability) and there may be threshold dose (~100mGy) below which such instability is not induced by acute exposure
- Germ-line instability may be perpetuated by intrinsic epigenetic changes such as methylation

An illustration of the cellular pathways involved in the signalling of non-targeted responses is given in Figure 2 below.

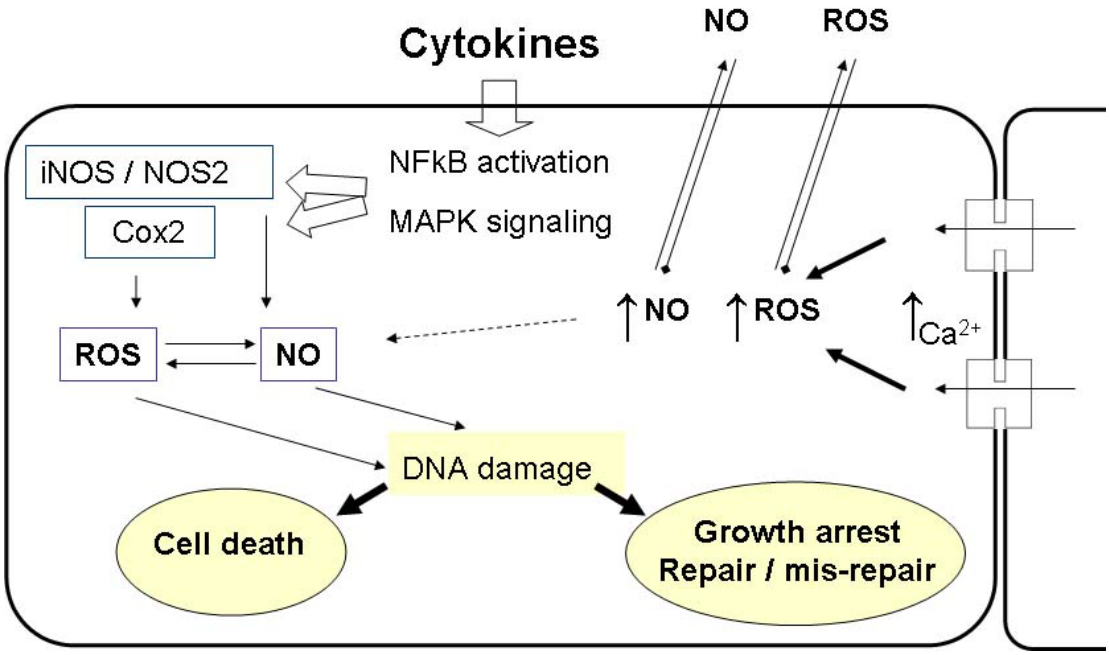

Figure 2. Cellular pathways involved in the signaling of non-targeted responses.

3.2 Do non-targeted effects modulate the cancer risk in the low dose region?

For the purposes of radiation protection, a linear-non-threshold (LNT) assumption is used to describe the shape of dose response for cancer down to low doses. Furthermore, a dose and dose rate effectiveness factor (DDREF) of 2 is used at low doses. Large scale epidemiological studies have reached the limits of their statistical power to detect an excess cancer risk in the low dose region (below about 100 mSv). Therefore, fundamental mechanistic understanding of the processes that drive radiation carcinogenesis and those that modify the risk is important.

Non-targeted effects have been shown to exhibit highly non-linear responses, the overall biological response at low doses may be influenced by them. Therefore, NOTE considered it is important to:

- study the shape of dose responses for the various non-targeted effects across a relevant dose range, down to low (0.1 Gy of low-LET) and to very low (10 mGy) doses;
- consider if the non-targeted responses increase or decrease the risk;
- study the effect of various LET on non-targeted effects;
- study the spatial and temporal distribution of non-targeted effects;
- consider the concept of dose, especially in situations where low fluences of radiation are encountered

A number of modeling approaches have been applied by NOTE partners to address these issues. Much of the experimental data was obtained from the mechanistic studies previously described.

Whilst many published studies have concentrated on damaging responses of non-DNA targeted effects a limited number of studies have drawn attention to the possibility of protective responses, the determinants of whether a non-DNA targeted effect will occur and if it would be damaging or protective are not known. One of the major questions is whether effects demonstrated *in vitro* can be extrapolated to the *in vivo* situation where additional protective mechanisms are important. However, the observations so far highlight the complexity of non-DNA targeted effects and there appears to be no single mechanism for non-DNA targeted effects.

Genomic instability occurs in the progeny of irradiated cells at a frequency that is several orders of magnitude greater than would be expected for mutation in a single gene, implying that a more complex, multigenic phenomenon may underlie the phenotype. The expression of genomic instability can depend on the genotype of the irradiated cell/animal with considerable inter-individual variation even in those genotypes that may generally express high levels of instability. Similarly there is evidence to suggest that the relationship between genomic instability and radiation dose is non-linear, but is maximally induced at the lowest doses investigated, including for instance a single alpha-particle traversal to a single cell nucleus in a population.

Mathematical modelling of non-DNA targeted effects, in collaboration with experimental investigation, has highlighted novel mechanisms that can be experimentally tested. Mathematical modelling also facilitates integration with models of DNA-targeted effects, and will allow determination of the role they may play in low-dose risk. The general objectives of modelling in NOTE were to devise mathematical models for non-targeted effects, to better understand low dose risks. By mathematical modelling the potential role of non-targeted effects in the development of different pathologies can be determined.

Initially, a fundamental part of the activity was reviewing the modelling and experimental literature, as performed by Imperial College, Istituto Superiore di Sanita, Helmholtz Zentrum München and University of Pavia. A number of major review articles were published by Imperial College in particular (in *Radiat. Res.*, *Radiat. Environ. Biophys.*). These review activities lead on to models of the systems and endpoints under consideration, described in more detail below.

A number of successful workshops contributed to the success of modelling activities (Challenges for Modelling of Radiation Damage in Biological Systems, Bad Honnef, Germany, October 2006; First International Workshop on Systems Radiation Biology, GSF, Neuherberg, February 2007; Second International Workshop on Systems Radiation Biology, Washington DC, January 2008; Third International Workshop on Systems Radiation Biology, Rovaniemi, Finland, January 2009; Fourth International Workshop on Systems Radiation Biology, New York, May 2010). These provided opportunities for interaction between experimentalists and modellers, many from outside the contract, indeed outside the EU. A project meeting was arranged in advance of the LH Gray workshop in Edinburgh in June 2008 to facilitate modeller-experimentalist interactions, and a training workshop on modelling and statistical analysis was organised as an adjunct to the NOTE Annual Contractors meeting in Rome in July 2009.

Helmholtz Zentrum München developed a mechanistic model describing selective induction of apoptosis in oncogenic transformed cells via signalling by surrounding normal cells. Earlier experiments indicated that this phenomenon may serve as a crucial anti-carcinogenic process occurring naturally, and that low dose radiation may enhance its rate and extent. Earlier experiments also provided detailed biological and biochemical knowledge on the major interaction mechanisms. Thus, intercellular induction of apoptosis (IIA) under the influence of low dose radiation represented a prime example of a non-targeted radiation effect mediated by in-

tra- and local intercellular signalling processes. The separation of the interaction system into three distinct steps allowed a modular development of the corresponding simulation code. Modules were developed for triggering of effector function in neighbouring cells by signalling emitted by transformed cells, for intracellular signalling through the two major pathways identified experimentally as well as their complex interplay via consumption reactions, and for triggering and execution of apoptosis. Using literature-based data on reaction kinetics, the model of intercellular signalling between normal and precancerous cells was calibrated against available *in vitro* data. The model predicts that in the long term the intercellular induction of apoptosis limits the growth of the transformed cell population and yields stable limits for transformed cell density. To the extent to which the results can be translated to the physiological situation, the modelling studies indicate that intercellular induction of apoptosis serves as a crucial anti-carcinogenic mechanism *in vivo*, limiting the growth of pre-neoplastic lesions, corresponding to a kind of dormancy in early stages of carcinogenesis. In addition, enhanced release rates of signalling species by irradiated cells, reported experimentally, are predicted by the model to lead to enhanced rates of intercellular induction of apoptosis. These results indicate that the model is capable of representing the experimentally observed enhancement of intercellular induction of apoptosis by ionizing radiation.

Furthermore, Helmholtz Zentrum München developed refined models of radiation-induced bystander effects in medium transfer experiments. Models were developed that account for non-linear response to bystander signals observed experimentally. The capabilities were investigated of the PARTRAC code to test quantitatively alternative hypotheses on size and multiplicity of targets for the initiation of bystander signal emission, based on experimentally determined dose-response relationship. Alternative signal emission scenarios were found in agreement with the data, assuming a rather broad distribution of target sensitivities to radiation, secondary signal amplification by neighbour cells of the primary signals released by irradiated cells, or a combination of both hypotheses. These modelling studies help towards detailed understanding of bystander effects and unifying information derived from available data, necessary for drawing implications for low-dose radiation risks.

Istituto Superiore di Sanita made progress in construction of models of adaptive response using the molecular dynamic approach. From the analysis of the literature three classes of the adaptive response mechanisms have been identified, namely: increased DNA repair efficiency, modification of the cell cycle and modulation of the radio-protective compounds concentration. Initially, the limited number of studies concerning the influence of radiation quality as well as the role of cell-cell communication on the adaptive response did not allow derivation of clear cut indications useful for modelling and experiments were planned by the ISS group to get more insights on these specific topics. As a result ISS completed formulation of the equations of the model, and the parameters in the equations were optimized with the aim of improving the agreement between the output of the model and the experimental results. The induction of anti-oxidant enzymes was considered with more attention in the simulation code as adaptive response mechanism, even if the more relevant mechanism remains the modulation of the repair efficiency by which the cells process the initial radiation damage. From the analysis of the literature, information about levels of anti-oxidant enzymes in cells before and after low dose irradiation and their ability to inhibit radical reactions leading to oxidative damage *in vitro* was obtained. The limited number of studies concerning the influence of radiation quality as well as the role of cell-cell communication on the adaptive response did not allow derivation of useful data for modelling. Therefore, further experiments were performed by the ISS experimental group in order to get more insights on these specific topics, which guided further developments of their model (Esposito et al., 2011). The output of this revised model has given suggestion for

the design of new experiments to be performed. Relevant experimental data produced by the project have been used to test the model.

University of Pavia developed different possible approaches and preliminary models using both an analytical equation system and a Monte Carlo code to simulate *in vitro* cell communication following irradiation of sparsely seeded cells. UNIPV developed both analytical (mass-action, with cytokines treated as population averages) and Monte-Carlo approaches. The Monte Carlo code describes the release, diffusion and internalization of candidate signalling molecules - typically cytokines - following *in vitro* exposure of cells that are *not* in close contact, so that communication via gap junctions is not involved. The work was carried out in very close connection with various experimental partners, in particular with *ad-hoc* experimental measurements of time-dependent cytokine concentrations (e.g. IL-6 and IL-8) in the culture medium of sparsely-seeded AG01522 human fibroblasts exposed to different gamma-ray doses (typically: 0, 0.25 and 1 Gy). Both the analytical and Monte Carlo approaches stimulated the design of *"ad hoc"* experiments in order to get inputs that would be useful for model development. In particular, the analytical model has allowed quantification of the role induced by radiation in the mechanisms of cell-to-cell communication for IL-6 molecules. The perturbation induced by radiation has been investigated, in mechanisms - such as the cytokine decay rate - involved in intercellular communication from the point of view of the whole cells and cytokines population. Finally their Monte Carlo code was augmented to include the transport of a second type of molecule in order to reproduce the stimulated (triggered) mechanism of emission (e.g. typical IL-8 signaling scenario).

The work of UNIPV and ISS has been characterised throughout by extensive collaborations between the experimental and modelling groups within and between these two partners (Alloni et al, 2008), testing and developing their bystander and adaptive-response models.

The definition of non-targeted effects also impacts on our understanding of the concept of dose. At the "Conceptualisation of New Paradigm" workshop organised by the NOTE project, held in Galway in 2008 and at a joint DoReMi – NOTE workshop held at the IRPA congress in Helsinki in 2010, these concepts were discussed with a view to defining more clearly non-targeted effects. Much of this was defined by key inputs from Dr. Oleg Belyakov (Baverstock & Belyakov, 2010), Professor Barry Michael and Professor Dudley Goodhead (Goodhead, 2010).

Dose is defined as energy deposited in a unit mass, which means both an understanding of energy deposition and critical targets. Historically radiation effects have come from an appreciation of target theory. This stems from the early concepts of Dessauer (1922) that absorption of radiation is not continuous but is quantized and is defined by Poisson statistics. This concept was extended to the calculation of a target volume within which the required number of absorption events had to occur, with a given probability (Lea 1946). Since then radiation biology has defined nuclear DNA as "the target" with direct energy deposition being the driver of direct DNA damage, especially DNA double-strand break (DSB) formation, and this leading to the cellular consequences of exposure. The definition of non-targeted responses for ionising radiation was first conceptualised by Ward in 2000 but follows from long standing concepts in the photobiology field (Wood & Hutchinson, 1984). The concept of non-targeted effects have been more generally applied to a range of non-linear responses to radiation, including not only bystander signaling, but genomic instability, adaptive responses, gene induction, low dose hypersensitivity and the inverse dose-rate effect. However of these "non-linear" effects, only the bystander response and genomic instability could be defined by the following more robust definition which has been proposed during the NOTE project:

- Targeted effect is one related to whatever in the cell/tissue etc was damaged by the radiation
- Non-targeted effect is related to the radiation damage but not directly to where the damage occurred

Further experimental work will lead to more robust understanding of the processes underlying "non-targeted" responses and their relevance and in particular, an integrative approach taking mechanistic information to relate targeted and non-targeted effects needs to be developed. Studies in radiotherapy will impact on our understanding as concepts of abscopal effects and systemic responses after localised irradiation are rapidly developing. In the longer term, systems biology approaches may allow a reassessment of non-targeted effect contribution to overall responses at the organism level to radiation exposures.

There have been a number of assessments made of cancer and non-cancer risk in the radio-epidemiological data and the possible contributory role of non-targeted effects, in particular two summary papers (Little, 2009, Little, 2010). Excess cancer risks observed in the Japanese atomic bomb survivors and in many medically and occupationally exposed groups exposed at low or moderate doses are generally statistically compatible (Little, 2010). For most cancer sites the dose response in these groups is compatible with linearity over the range observed. The available data on biological mechanisms do not provide general support for the idea of a low dose threshold or hormesis. This large body of evidence does not suggest, indeed is not statistically compatible with, any very large threshold in dose for cancer, or with possible hormetic effects, and there is little evidence of the sorts of non-linearity in response implied by non-DNA-targeted effects (Little, 2010).However, the mechanistic model describing selective induction of apoptosis in oncogenic transformed cells via signaling by surrounding normal cells indicates that intercellular induction of apoptosis may serve as a crucial anticarcinogenic mechanism in vivo, limiting the growth of neoplastic lesions (Kundrat et al, 2011).

3.3 Can ionizing radiation cause non-cancer diseases or beneficial effects at low or intermediate doses?

Epidemiological studies could demonstrate that ionising radiation (IR) as a long-term dose-dependent effect may induce an impairment of the immune system as well as a persistent inflammatory profile that could increase the risks of both cancer and non-cancer diseases. Therefore, a key objective for NOTE was to study whether low and intermediate doses of IR can cause non-cancer diseases or beneficial effects in several experimental systems *in vitro* and *in vivo* to provide qualitative and quantitative data for mathematical modelling, to better understand low dose risks.

Throughout the entire project period, studies on non-cancer effects were divided into 4 specific tasks:

- Mechanisms of cardiovascular diseases induction – *in vitro*
- Mechanisms of cardiovascular diseases induction – *in vivo*
- Inflammatory or anti-inflammatory responses?
- Role of non-targeted effects in developmental defects

All experimental activities were also intended to provide suitable qualitative and quantitative experimental data to be used and tested by partners involved in mathematical modelling of

non-targeted effects of low doses of IR. Throughout the project period there have been especially close interactions between modellers and experimentalists for final data analysis.

Mechanisms of cardiovascular diseases induction – in vitro & in vivo

In recent years, there has been growing epidemiological evidence of excess risk of late occurring cardiovascular disease at much lower radiation doses and occurring over much longer intervals after radiation exposure without a clear cut threshold. However, the epidemiological evidence available so far for non-cancer health effects after exposure to low radiation doses is suggestive rather than persuasive. Furthermore, the mechanisms of radiation-induced vascular disease induction are far away from being understood. It might be that inflammatory responses are involved. Experimental studies by Stewart et al, 2006 could demonstrate that high dose exposure to the cardiovascular system is associated with an earlier onset and accelerated development of macrophage-rich, inflammatory atherosclerotic lesions prone to intra-plaque hemorrhage and may also cause a decrease in myocardial perfusion. Both, macro- and microvascular radiation effects involve the endothelium and pro-inflammatory signalling cascades. If modulation of inflammatory response is arguably the most likely cause of radiation-induced cardiovascular disease after low dose exposure, this also implies a role for non-targeted radiation effects (Hildebrandt, 2010).

The objective of Queens University of Belfast was to determine the responses of endothelial cells and tissue based models to targeted low dose exposure as a surrogate for determining the role of radiation damage to the vasculature in non-cancer diseases. The hypothesis should be tested whether cell-cell signalling plays a major role in the response of endothelial tissue to low dose exposure and that this ultimately may play a role in the development of non-cancer diseases in exposed populations.

The objective of University of Leipzig, Imperial College, AECL Ottawa Heart Research Institute and University of Rostock was to investigate radiation effects on atherosclerosis after low dose rate (LDR) exposure (1 mGy/min, 100 mGy/day, 5 days/week) as compared to high dose rate (about 150 mGy/min) exposure in mice *in vivo*. The hypothesis was to be tested whether low (HDR) dose exposure might modulate (i.e. promote) atherosclerosis progression depending on (a) genetic background and (b) stage of disease at time of exposure, and furthermore whether this might be mediated by modulation of stress and/or inflammatory responses.

Experiments were performed with animals either prone to the disease (ApoE-/-), normal (ApoE+/+), or knockouts heterozygous for TP53 (ApoE-/- TP53 +/-). Mice were exposed to several radiation doses (0, 0.025, 0.05, 0.1, 0.5, 2.0 Gy) at early stage disease (2 months age). Time kinetics of atherosclerosis development and progression should be monitored by markers of stress response and inflammation as well as morphological endpoints (lesion area, number of lesions) at 3 and 6 months after exposure (5 and 8 months age).

Changes in aortic lesion frequency, size and severity, as well as total serum cholesterol levels and the uptake of lesion lipids by lesion associated macrophages were assessed. Statistically significant changes in each of these measures were observed, depending on dose, dose rate and disease stage. In all cases, the results were distinctly non-linear with dose, with maximum effects tending to occur at 25 or 50 mGy.

In general, low doses given at low dose rate at either early or late stage disease were protective, slowing the progression of the disease by one or more of these measures. Most effects appeared

and persisted for months after the single exposures, but some were ultimately transitory. In contrast to exposure at low dose rate, high dose rate exposure at early stage disease produced both protective and detrimental effects, suggesting that low doses may influence this disease by more than one mechanism, and dose rate is an important parameter.

In addition, experimental results obtained by University of Leipzig and University of Rostock demonstrate significant changes, in general increases in peripheral markers of inflammatory response, leukocyte extravasation and pro-thrombotic surface at both 3 and 6 months after radiation exposure. At the moment this needs to be taken carefully, due to the low number of tissue samples (4 animals per data point) and thus the relatively low power of the statistical analysis. However, these data still might indicate that inflammatory changes after low dose exposure can be found locally in the heart as well as systemically in the blood plasma. This finding is in accordance with the observations of a persistent pro-inflammatory state detected in the A-bomb survivors many years after radiation exposure, and there might be a potential relationship of this systemic radiation effects with the development of atherosclerosis and cardiovascular morbidity and mortality. However, there is no strong indication so far that both effects of total body irradiation are causally related.

During the final project period a further extension of the initial work program to analyze cytokines and thrombotic markers in EDTA plasma samples of ApoE-/-, Trp53 +/+ and ApoE+/+, Trp53 +/+ mice, collected at shorter time points after irradiation was carried out by, AECL, UL-MED, and UROS. The intention was that the resulting plasma data should shed more light on the time dependent cytokine response and fill the gap between irradiation at 8 weeks and the first plasma sample taken 3 month after irradiation. Therefore, here EDTA plasma samples were collected 24 hours before, immediately after, 24 hours after, 7 days after, and 28 days after irradiation.

Cytokine levels seem to increase with age and were generally higher in ApoE+/+ mice than in ApoE-/- mice. In the latter some cytokine levels were close to detection limit so that slight increases might be missed. For the ApoE+/+ strain, significant increases of cytokines (IL10, MCP1) were found most frequently in the LDR cohort 28 days after IR. After HDR exposure levels of fibrinogen decreased significantly. For the ApoE-/- strain, sICAM levels increased significantly 7 and 28 days after LDR exposure and tend to increase after HDR exposure. Also TGFβ levels increase 28 days after IR. At earlier time points (before irradiation and 24 h after IR only sporadic changes (IL10, sICAM) were found. Taken together, these findings indicate that IR at both, HDR and LDR induces pro- and anti-inflammatory pathways already at very low doses with strain specific differences.

The results obtained so far contrast with the known, generally detrimental effects of high doses on the progression of this disease in the same mice, and in humans, suggesting that a linear extrapolation of the known increased risk from high doses to low doses may not be appropriate.

Especially within this task, there were ongoing interactions between University of Leipzig, University of Rostock and Imperial College as well as AECL and Ottawa Heart Research Centre to devise and analyse extension experiments using the *ApoE*$^{-/-}$ mouse system to further investigate cardiovascular-related disease endpoints, in particular extra macrophage, leukocyte and other markers of apoptosis and inflammation in the tissue samples to be taken from the mice. These experiments were conducted in the low dose-rate animal irradiator at AECL Chalk River. Analysis of the cytokine and other inflammation marker data was conducted by University of Leipzig/University of Rostock. Statistical analysis of the Canadian data was under the leadership of Imperial College. A number of open literature papers of this very rich data have been

written, and one so far published (Mitchel et al, 2011). Imperial College also made significant progress in mathematical modelling of cardiovascular disease. A paper outlining a candidate mechanism (MCP-1 accumulation in the intima via radiation-induced monocyte cell killing) has been published (Little et al, 2009)A paper assessing population risks of radiation-induced circulatory disease has been completed (Little et al, submitted). This analysis concluded that epidemiological evidence for an association between circulatory disease risk and low doses of ionizing radiation is persuasive, although more epidemiological and experimental research is needed to characterize the association. The analysis is limited by the heterogeneity among analyzed cohorts, particularly for the endpoints other than heart disease, the possibility of confounding in some occupational groups by lifestyle factors, and that trends are generally driven by high dose groups (>0.5 Sv). The models fitted in the meta-analysis were used to estimate excess population risks in a number of countries. Predicted risk ranged from 2.7% per Sv (95% CI 0.9 to 4.4) for France to 9.3% per Sv (95% CI 4.6 to 14.0). These estimates are similar to those of radiation-induced cancer, which range from 4.2% to 5.6% per Sv. This evidence of a radiation-associated risk of circulatory disease at low doses, if confirmed, suggests that overall radiation-related mortality is about twice that currently estimated. There has also been a paper drafted on modelling of radiation-associated capillary and microvascular damage and projected impact on cardiovascular risk. (Little et al, submitted).

Inflammatory or anti-inflammatory responses?

The induction of inflammatory or anti-inflammatory responses after low and moderate doses was studied by University of Leipzig, Hungarian National Institute for Radiobiology and Radiohygiene, Friedrich-Alexander University of Erlangen-Nürnberg, Johann-Wolfgang Goethe University, Frankfurt am Main, and University of Rostock. Human health risks associated with exposure to IR have been based primarily on the assumption that these effects occur in irradiated (targeted) cells. Non-targeted cellular responses to IR have also been demonstrated in the immune system, but their implications on human health are almost completely uncertain. However, these effects, which predominate at the low doses of relevance to radiation protection, needed to be characterised and evaluated in terms of health risks. Therefore, the main question of this task was to analyse quantitatively and qualitatively the effects of low dose irradiation on the immune system and/or inflammatory responses either in mono-culture and co-culture models *in vitro*, *in vivo*, and in *ex vivo* samples. The hypothesis should be tested whether low doses might have a different effect as compared to higher doses, and that this might dose-dependently modulate immune and/or inflammatory responses.

University of Leipzig and University of Rostock showed that ionizing irradiation could act directly on immune cells and may induce bystander effects mediated by soluble factors that are released by the irradiated cells. In this subtask, the direct effect of low dose ionizing radiation (LDIR) on the maturation and cytokine release of human dendritic cells (DCs) and the functional consequences for co-cultured T-cells were analyzed.

Results showed that irradiation of DC-precursors *in vitro* does not influence surface marker expression or cytokine profile of immature DCs nor that of mature DCs after LPS treatment. There was no difference of single dose irradiation vs. fractionated irradiation protocols on the behaviour of the mature DCs. Further, the low dose irradiation did not change the capacity of the DCs to stimulate T-cell proliferation. But the irradiation of the co-culture of DCs and T-cells revealed significantly lower proliferation of T-cells with higher doses. Summarizing the data there was no significant effect of low dose ionizing irradiation on the cytokine profile, surface

marker expression and maturation of human DCs *in vitro* although functional consequences can not be excluded (Jahns et al , 2011).

Hungarian National Research Institute for Radiobiology and Radiohygiene studied the effects of acute exposure to low- and high-dose radiation on the quantitative and functional parameters of the immune system. C57BL/6 mice were irradiated with different doses of γ-radiation (0.01, 0.05, 0.1, 0.5 and 2 Gy) and splenocytes were isolated at various times. Alterations in the distribution and surviving fraction of splenocyte subsets such as CD4+ and CD8+ T lymphocytes, regulatory T cells (Treg), natural killer (NK) cells, dendritic cells (DCs) and B lymphocytes were analyzed by flow cytometry. Apoptosis frequency was quantified by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method 4 h after irradiation. Cytokine expression was investigated by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Low doses decreased apoptosis in the splenocyte subpopulations studied most prominently in NK cells and DCs. Exposure to 2 Gy increased apoptosis in all splenocyte subpopulations; B cells were the most sensitive and NK cells and DCs the least sensitive. The lowest cell numbers were measured 3 days after irradiation, with minor changes by day 7. CD8+ and B cells were rather resistant to low doses but were very sensitive to 2 Gy, while NK cells, DCs and Treg cells were much more resistant to high doses. Expression of the T-helper 1 (Th1)- and helper 2 (Th2)-type cytokines decreased after low doses and increased after high doses. Interleukin 6 (IL-6) reacted at early times and IL-10 at later times. IL-5 levels were consistently elevated.

These data highlight the differences in the responses of different splenocyte subpopulations to low- and high-dose radiation (Bogdándi et al, 2010).

In the last two project years, total body irradiation of healthy mice was performed and the dynamics of immune responses was checked at various time points after irradiation from the peripheral blood or spleen of irradiated animals. The aim of this task was to compare the effect of LDIR on the immune reactions of healthy animals and animals with a compromised immune system. Immune compromising was modelled by using mice bearing large, subcutaneously growing B16 tumors, known to induce strong immune suppression.

The immune parameters of tumor bearing mice were severely altered compared to healthy mice and these parameters are becoming worse with tumor progression. Total body irradiation of tumor bearing mice, if performed at an earlier stage of tumor progression might reduce tumor induced immune suppression (increase in the percentage of CD4, CD8 cells, increase in the cytokine expression levels). These effects are present both after low and high doses. Further investigations concerned comparison of immune alterations after low and high dose irradiation in healthy and immune compromised mice. The investigators could provide some experimental evidence for an improved antitumor immune activating capacity of DCs irradiated with high doses. However, such an effect was not detected after LDIR.

Friedrich-Alexander University of Erlangen-Nürnberg showed, that irradiation of phorbol 12-myristate 13-acetate (PMA) activated granulocytes with doses below 0.3 Gy resulted in a highly significant decrease in activation induced cell death (AID) when cells were irradiated with 0.1 Gy in comparison to non-irradiated cells. Similar to 0.1 Gy, irradiation with 0.5 Gy led to a significant decrease in AID, while 0.3 Gy increased AID in PMA activated granulocytes. These results once more revealed a discontinuous dose dependency. The latter was also observed for Akt 1/2 expression in granulocytes after moderate dose of X-ray (Gaipl et al, 2009). A number of extracellular stimuli have been described to activate granulocytes and to foster AID under inflammatory conditions. Depending on distinct stimuli, AID of granulocytes has been shown to

be accelerated or slowed down. The objective for the last project period was to investigate the effects of LDIR on cell death of granulocytes that have been activated with other stimuli than PMA.

The low and moderate dose effects on the phagocytosis of apoptotic lymphocytes by irradiated monocytes-derived macrophages revealed again a biphasic dose dependency. A significantly increased phagocytosis was present when macrophages were irradiated with 0.1, 0.3, or 0.7 Gy. The inflammatory phenotype of activated macrophages was further determined in an *in vitro* cell culture model with THP-1 cells (acute monocytic leukemia monocytes). The secretion of the inflammatory cytokine IL-1β of activated macrophages is a crucial step in the propagation and resolution of inflammation. FAU has previously demonstrated that low/moderate dose irradiation down regulates the secretion of IL-1β in a discontinuous manner, probably induced by affecting the PI3K-NFκB pathway. Those results further contributed to understand how low dose IR exposure might induce an anti-inflammatory phenotype of macrophages and granulocytes. The objective for the last project period was to reproduce the results and to analyse inflammatory signalling molecules in macrophages after irradiation with low X-ray doses. A review paper dealing with the immune modulatory properties of phagocytes and phospholipids in the outer membrane of dying cells was published by FAU (Chaurio et al, 2009).

Local irradiation may lead to systemic effects, the so called abscopal effects. FAU did explore whether local irradiation influences the systemic cellular immune response. For this purpose, an in vivo model to monitor the delayed type hypersensitivity reaction (DTH) in mice after local or systemic irradiation has been established. The objective was to analyse the impact of low and moderate dose irradiation on the systemic immune reaction in this established DTH mouse model.

First experiments with the moderate dose of 0.5 Gy showed that whole body irradiation is capable to improve the course of beginning polyarthritis (Frey et al., 2009). Those experiments were performed *in vivo* with human TNF transgenic mice (hTNF-tg) mice. The mice express the human cytokine TNF-alpha and develop chronic polyarthritis at an age of about 5 weeks. Future work will focus on the effects of lower doses of X-ray in this inflammatory mouse model and on detailed analyses of the molecular basis of those immune modulatory properties of LDIR.

The results achieved during the whole project duration shed more light on the molecular effects induced in activated immune cells by low and moderate dose IR exposure.

Further insight in the influence of low or moderate dose IR exposure on the interrelationship of inflammatory cells arises from studies on it's effect on CCL20 chemokine secretion and polymorphonuclear neutrophil (PMN)/endothelial cell (EC) adhesion performed at Johann Wolfgang Goethe-University. CCL20 chemokine secretion was found to be exclusively induced by a direct cell-cell contact between PMN and EC in a tumor necrosis factor (TNF)-α dependent manner. Furthermore, irradiation with doses between 0.5 and 1 Gy resulted in a significant reduction of CCL20 release which was dependent on the cytokine transforming growth factor beta 1 (TGF-β_1). Moreover, the decrease of CCL20 parallels a significant reduction in PMN/EC adhesion (Rödel et al, 2008).

In a search attempt for putative novel main regulatory mediators of low or intermediate dose irradiation a genome wide screening using Affymetrix Human Genome U133 Plus 2.0 array technology was performed on EA.hy 926 EC. Using GOstat pathway over-representation analy-

sis, among others a variety of genes that are differentially regulated have been observed. These genes comprise proteins either involved in cell adhesion (e.g. inter-cellular adhesion molecule 1: ICAM-1), negative regulation of cellular processes (e.g. TGFß1), immune system processes (e.g. heat schock protein 70: HSP70) regulation of transcription (e.g. activator protein 1: AP-1) and apoptosis (e.g. X-linked inhibitor of apoptosis protein: XIAP). Notably, these data confirm the involvement of a variety of factors already described or expected to contribute to the regulation of immune cell properties by low and intermediate dose irradiation (Rödel et al, 2007).)

For the validation of selected factors and to further explore underlying molecular pathways, electrophoretic mobility shift assays (EMSA) and luciferase reporter assays revealed a biphasic profile of DNA-binding and transcriptional activity of the transcription factor AP-1 in EA.Hy 926 EC following irradiation with 0.3 and 2 Gy (Rödel et al, 2009). Furthermore, XIAP expression was analysed by quantitative real time-PCR and Western-blot analysis from activated EA.Hy.926 EC, and the functional impact of XIAP on apoptosis induction and EC function was characterised using specific siRNA oligonucleotides.

LD-RT of the activated EA.Hy.926 EC induced XIAP expression in a discontinuous manner with a relative maximum at 0.5 Gy and 3 Gy which parallels a discontinuity in apoptosis induction and caspase 3/7 activity. siRNA-mediated attenuation of XIAP resulted in an increased rate of apoptosis, a hampered NF-κB transcriptional activity and a diminished secretion of TGF-β1. As compared to control-siRNA treated cells, adhesion of PBMC to EC was increased in XIAP depleted EA.Hy.926 EC. In conclusion, these experimental data demonstrate that LD-RT induces a discontinuous dose dependency of XIAP expression in activated EA.hy.926 EC and thereby modulates NF-κB activity, TGF-β1 expression and PMBC/EC adhesion (Rödel et al, 2010).

Role of non-targeted effects in developmental defects

Recent studies have shown that irradiation of a single cell, the zygote or 1-cell embryo of various mouse strains, could lead to congenital anomalies in the fetuses. In the Heiligenberger strain, a link between the radiation-induced congenital anomalies and the development of a genomic instability was also suggested. Moreover, further studies showed that in that strain, both congenital anomalies and genomic instability could be transmitted to the next generation. The aim of the experiments performed by SCK-CEN was to investigate whether such non-targeted transgenerational effects could also be observed in two other radiosensitive mouse strains (CF1 and ICR), using lower radiation doses.

Irradiation of the CF1 and ICR female zygotes with 0.2 or 0.4 Gy did not result in a decrease of their fertility after birth, when they had reached sexual maturity. Moreover, females of both strains that had been X-irradiated with 0.2 Gy exhibited higher rates of pregnancy, less resorptions and more living fetuses. Additionally, the mean weight of living fetuses in these groups had significantly increased.

Exencephaly and dwarfism were observed in CF1 fetuses issued from control and X-irradiated females. In the control group of that strain, polydactyly and limb deformity were also found. The yields of abnormal fetuses did not differ significantly between the control and X-irradiated groups. Polydactyly, exencephaly and dwarfism were observed in fetuses issued from ICR control females. In addition to these anomalies, gastroschisis, curly tail and open eye were observed at low frequencies in ICR fetuses issued from X-irradiated females. Again, the frequencies of abnormal fetuses found in the different groups did not differ significantly.

In both CF1 and ICR mouse strains, irradiation of female zygotes did not result in the development of a genomic instability in the next generation embryos. Overall, the results suggest that, at the moderate doses used, developmental defects observed after X-irradiation of female zygotes of these two sensitive mouse strains should not be transmitted to the next generation. Paradoxically, other studies would be needed to address the question of a potential increase of fertility after doses lower than 0.2 Gy in both strains (Jacquet et al, 2010).

To expand this work SCK-CEN, University of Leipzig and University of Rostock have started to analyse the role of exposure to low dose radiation to induce delayed adult behavioural defects. Long-term effects of ionising radiation on brain development are of concern for radiological protection as the mammalian embryos and fetus are highly radiosensitive (ICRP Publication 90, 2003).The brain is the final result of a series of well timed consecutive or concomitant waves of cellular proliferation, migration, and differentiation. Low dose irradiation during pregnancy could selectively disturb these events without induction of cell death. The aim of this task was to identify molecular and pro-inflammatory responses induced in the developing mouse brain by low doses of ionizing radiation during neurogenesis, and that could contribute to neurological defects and delayed cognitive disorders in the mature adult brain.

To analyse the profile of gene expression in the brain, mouse embryos were irradiated with low doses of X-rays (0.1 and 0.2 Gy) on days 10 or 11 of gestation. For each day and dose of irradiation, total RNA was extracted from a minimum of 3 individual brain samples isolated out from 3 different females. Pathway analysis of the different expression clusters showed genes involved mostly in the AKT/PI3K signalling pathway and the Ras/ERK pathway, genes that are involved in nervous system development and function, neurogenesis, synaptogenesis, neurite formation. Two other main actors were identified: FGF2 (fibroblast growth factor 2) which is involved in several diseases such as neoplasia, cancer, neurodegeneration, skin cancer, immunodeficiency, inflammation, and NOG (Noggin) which is involved in numerous developmental processes, such as neural tube fusion and joint formation. Over-expression of such genes would compromise the normal development of the brain. Proteomic analysis was also performed and key up-and down regulated proteins selected for further analysis.

No major inflammatory proteins appeared to be modulated upon in utero exposure of the brain to a dose of 0.2 Gy of X-ray and no overlap between transcriptomic and proteomic analyses. Based on the transcriptomic data, modulation of PI3K/AKT and NF-κB pathwas supported strongly our hypothesis of inflammatory reponse in the brain. Modulation of these genes and related pathway members at the transcriptional level indicate high inflammatory response in the brain following in utero exposure of the embryo to low doses of ionising radiation. In further work on the function of neuronal cells *in vitro* and by using gene expression and immunofluorescent staining partners were able to demonstrate the effect of low doses of radiation on the neuronal network.

Risks of non-malignant diseases

There are also excess risks of various types of non-malignant disease in the Japanese atomic bomb survivors and in other groups. In particular, elevated risks of cardiovascular disease, respiratory disease and digestive disease are observed in the A-bomb data. In contrast with cancer, there is much less consistency in the patterns of risk between the various exposed groups; for example, radiation-associated respiratory and digestive diseases have not been seen in these other (non A-bomb) groups. Cardiovascular risks have been seen in many exposed populations, particularly in medically exposed groups, but in contrast with cancer there is much less consistency in risk between studies: risks per unit dose in epidemiological studies vary over at least two orders of magnitude, possibly a result of confounding and effect modification by well

known (but unobserved) risk factors (Little, 2010). However, there is much more homogeneity in risk if circulatory disease subtypes are evaluated, and attention is restricted to moderate and low dose exposed groups (Little et al, submitted). Epidemiological evidence for an association between circulatory disease risk and low doses of ionizing radiation is persuasive, although more epidemiological and experimental research is needed to characterize the association. The analysis is limited by the heterogeneity among analyzed cohorts, particularly for the endpoints other than heart disease, the possibility of confounding in some occupational groups by life-style factors, and that trends are generally driven by high dose groups (>0.5 Sv) (Little et al, submitted). Inflammatory processes are thought to be the most likely mechanism by which radiation could modify the atherosclerotic disease process. If there is to be modification by low doses of ionising radiation of cardiovascular disease through this mechanism, a role for non - targeted effects cannot be excluded (Little, 2010).

3.4 Do non-targeted effects contribute to the individual variability in radiation response?

An important question for radiation protection is the extent of individual variability in radiation risk. To consider protection principles, it would be good to know if there are individuals or groups of people that are more susceptible to radiation effects, to know how large this fraction of population is and to know, how large the variation is. In addition to classical radiation biological effects, such individual variability could also be linked to non-targeted responses.

At the start of the project, non-targeted effects had been demonstrated in some, but not all, cells and in some, but not all, genotypes. Neither was it known whether all endpoints reflect any commonality of underlying mechanism.

The objective of NOTE was to investigate inter-individual genotype differences between cells of the different type from different individuals. Another main objective was to investigate mechanisms, such as DNA repair capacity and cell cycle control, by which the genetic factors influence the induction of non-targeted effects. The overall work plan relevant for individual variation addressed three main questions:

- Why is the cellular response to irradiation or bystander conditions *in vivo* influenced by genetic background?
- Could similar genetic variations be operable *in vitro* and explain the differences observed between cell lines in terms of non-targeted effects?
- Which mechanisms contribute to the induction and perpetuation of non-targeted effects? (This issue is discussed in more detail at the mechanisms section above)

To address these questions, we studied human primary fibroblasts from radiation sensitive and resistant persons, compared two mouse strains of varying sensitivity, studied if LET has any influence on expression of genomic instability and studied the role of DNA repair genes and cell cycle checkpoint responses.

The Hungarian National Research Institute for Radiobiology and Radiohygiene established primary fibroblast cultures from the skin biopsies of cancer patients. Radiation sensitivity of the fibroblasts was determined by clonogenic assay, with the aim to study differences in non-targeted responses to radiation in cells with a range of genetic backgrounds using different biological endpoints. This included studies on the relationship of delayed effects to the radiation sensitivity of the cells, and the evaluation of the relative importance of effectors and target cells in bystander responses. Two primary human fibroblasts with different radiation sensitivity

were exposed to a range of γ-radiation doses (up to 2.0 Gy), delayed effects were studied in both irradiated and bystander cells. Cell killing was increased in sensitive cell lines compared to resistant ones (43-46% and 8-10%, respectively). The induction of micronuclei frequency was also higher in the sensitive cells. Interestingly in the bystander populations of the sensitive cells there was significant induction in micronuclei even at low doses (10 and 40mGy), however there was no change in the frequency of bi-nucleated cells in this group. These results suggest that the bystander response is not influenced by the radiation sensitivity of the cells.

In order to study potential mechanisms which modify non-targeted responses investigations focused on the question: Are bystander responses influenced by donor or recipient cells? Normal and radio-sensitive human fibroblast cell lines were used to study bystander effects with media from irradiated sensitive cells transferred to normal cells and vice versa. Results showed that bystander response is determined by the donor cells. In addition the role of specific cytokines IL6, IFN-gamma and TNF-alpha in mediation of bystander response was evaluated using adenoviral expression of potential candidates. The data showed no involvement of these cytokines in bystander effects with these cells. Further to this, in order to find a more sensitive biological endpoint to analyze non-targeted effects, the Hungarian group studied deletions in the mitochondrial (mt) genome by real time PCR both in directly irradiated and bystander sensitive and normal cells. The application of real time PCR makes the results fully quantitative. Cells were irradiated with 0.1 and 2.0 Gy gamma-rays and deleted mtDNA was measured 72 hours after irradiation. Significantly elevated mtDNA deletion rates were observed at and above 0.1Gy. Long-term follow up showed that in normal cells the level of mtDNA deletions decreased, approaching the control level 5-6 weeks after irradiation. In contrast, the radiosensitive cell lines showed a second elevated peak around 8 weeks after irradiation. This suggests that radiation sensitive cells are more prone to delayed effects.

Previous results from the Hungarian National Institute for Radiobiology and Radiohygiene were successfully reproduced at Oslo University Hospital with gamma radiation and additionally were compared to their results from alpha radiation (Dahle et al, 2011) using a 238Pu-based alpha particle irradiator constructed specifically for this project (Tisnek et al, 2009). Oslo University Hospital analysed gene expression data from control, irradiated and bystander populations using a range of approaches. Irradiated immortalized human fibroblasts showed significant gene expression changes such as induction of signalling and apoptosis genes and the gradual formation of a cellular immune response. A large portion of the genes which were differentially expressed early after irradiation (4 hours), consisted of soluble factors belonging to the extracellular region. Among those genes were GDF15 (also found by the Hungarian group), CCL2, and IGFBP3 which could be implicated with the bystander effect. No major changes in gene expression were observed in bystander fibroblasts at the single gene level; however, transient enrichment in ribosome, RNA degradation and oxidative phosphorylation pathways was seen in bystander fibroblasts 6 hours after medium transfer. These findings confirm the involvement of mitochondria in the bystander effect and bring new evidence on the implications that the radiation induced bystander effect may have on global protein synthesis.

Previous results from the Oxford Brookes University group showed that chromosomal instability was induced for alpha particle (high LET) but not after 3.0 Gy X-ray (high dose, low LET). As part of the NOTE project, Oxford Brookes focused on the induction of genomic instability after low dose low LET exposure. Specifically, the objective of this work was to study the molecular differences in genetic dependency of radiation-induced delayed genomic instability, following low doses of low LET X-ray radiation. Bone marrow cells from CBA/CaH (radio-sensitive) and C57BL/6 (radio-resistant) mice were exposed *in vitro* to a range of low dose X-irradiation. The

role of particular proteins for DNA damage recognition, apoptotic function (e.g. ATM, Bcl-2 and Bax) and the potential role for caspases in the observed genetic differences were also investigated.

Cells were more sensitive for cell killing in CBA compared to C57. In the surviving cells, chromosomal damage was induced in both strains of mice following low doses of low LET irradiation (<0.1 Gy). The spectrum of damage differed between strains with C57 showing more complex types of damage. Assessment of the level of apoptosis in these cells also showed genotype differences. Overall results show an inverse relationship between induction of delayed chromosome instability and apoptosis. Protein levels demonstrated strain specific differences in ATM activation following irradiation. Similar strain specificity in relative concentration of Bcl-2 family proteins was also observed. The balance of these proteins can dictate cell fate and could explain the different trends in levels and timing of apoptosis observed between CBA and C57. To gain further insight into strain specific difference in the apoptotic pathway, the role of caspases was studied through the use of a pan-caspase inhibitor (Z-VAD). A significant reduction in levels of apoptosis in CBA was observed in irradiated cells pre-treated with Z-VAD, but not in C57. Thus, C57 may utilise a caspase-independent apoptotic pathway as cells were still removed from the population when caspases were inhibited. In summary, low dose low LET X-ray in general induces genomic instability, with strain specific differences in survival, chromosome damage and apoptosis. In particular focusing on the protein and caspase inhibition data, results suggest that there is a genetic component affecting the threshold for induction of apoptosis and chromosomal instability.

Previous work within the Oxford Brookes group showed similar types and levels of induction of delayed chromosomal instability in irradiated and bystander primary human foetal lung fibroblasts (radio-sensitive) following both high and low LET radiation. However, no investigation had been conducted into the initial lesions and subsequent perpetuation through to delayed time points in both populations. As such, Oxford Brookes University investigated the relationship between early damage induction and long term effects (genomic instability) in both irradiated and bystander cells, as well as establishing possible mechanistic links between these two populations. The role of genetic sensitivity was also examined through the use of primary foetal lung human fibroblasts known to show different radiation responses: HF-19 (radio-sensitive) and HF-12 (radio-resistant). Samples were taken immediately, at medium term and delayed time-points post X-irradiation, to assess initial, perpetuated and delayed *de novo* events typifying the genomic instability phenotype. The data demonstrated that low dose radiation (down to 0.1 Gy) can induce genomic instability in bystander populations in sensitive cells, whilst radio-resistant cells appeared refractory to induction of instability in bystander populations. These differences in response suggest that HF-12 may respond less to external damage signalling or the induction of the signalling molecule/s, whilst HF-19 is more responsive to external signals leading to bystander responses.

The role of DNA repair and checkpoint responses were studied by University of Duisburg Essen, Oxford Brookes University and STUK. The objective for this task for University of Duisburg Essen was to study the role of DNA repair and checkpoint responses in non-targeted radiation effects. Work involved establishment of homologous recombination repair (HRR) and non-homologous end-joining (NHEJ) plasmid based assays for measuring the capacity of cells to carry out DSB repair and to study the direct impact of ionising radiation on the efficiency of these DSB repair pathways. In addition cell cycle analysis was performed to investigate checkpoint activation using non-irradiated cells. The cell lines used were shown to elicit an adaptive response and/or a bystander effect in the plasmid based assays. Effects in targeted cells were

also measured at the same time and used to gauge the effect in non-targeted cells. University of Duisburg Essen worked at the molecular level to analyse aspects of this DNA damage response. Cell cycle dependent induction of DSBs in bystander cells after medium transfer was observed, which were preferably repaired by homologous recombination repair (Klammer, 2010). These results provide important mechanistic information on the cellular processes involved in bystander responses. Evidence that damage induction in bystander cells is mediated by oxidative stress as the response could be significantly reduced in the presence of an effective scavenger of reactive oxygen species. Checkpoint response was examined in non-targeted cells and showed no detectable cell cycle checkpoint induction in bystander cells using flow cytometry methodology. An alternative method to evaluate DNA damage induction was established by visualising formation of γ-H2AX foci (Kinner, 2008). Using this approach it was possible to show that adaptive response in bystander cells is associated with damage induction specifically in G2 phase cells (Klammer, 2010). Thereby also indicating that cell cycle analysis by flow cytometry lacks the sensitivity required to measure such effects.

The role of genetic heterogeneity in the ATM (Ataxia telangiectasia mutated) gene with respect to the individual variation of non-targeted responses to ionizing radiation was studied by STUK and Oxford Brookes University. The main aim of this study was to investigate the role of genetic heterogeneity with respect to the individual variation of non-targeted response to ionization radiation. This study also addressed the issue of low dose effects using X-rays. The ATM gene was used as a model for characterizing the role of repair genes in the non-targeted effect. The experimental system was based on a co-culture system using TK6 cell line where irradiated cells were able to communicate with unirradiated cells immediately post-irradiation. Initial pilot studies showed evidence of a bystander response in the parental TK6 line in some experiments measuring viability but not for chromatid or chromosome aberrations. Further work showed no evidence for bystander responses in the ATM wild-type or mutant strains.

Summary of main conclusions on individual variability

Could similar genetic variations be operable in vitro and explain the differences observed between cell lines in terms of non-targeted effects?

Oxford Brookes University and University of Dundee both utilised bone marrow from the same strains of mice (CBA/CaH and C57BL/6) and used *in vitro* versus *in vivo* approaches, respectively. Conclusions from the *in vitro* studies using low dose X-ray were in agreement with the *in vivo* results to a certain extent. Common observations between *in vitro* and *in vivo* studies include:

- Production of signals (cytokines in the *in vitro* system) involved in non-targeted effects are genotype-dependent
- Responses to signals are genotype-dependent
- Signalling pathways involving reactive oxygen/nitrogen species and cytokines are commonly implicated in non-targeted mechanisms.

In addition, both *in vitro* and *in vivo* studies identified that the apoptotic pathway and related proteins are significant factors underlying differences in radio-sensitivity phenotypes.

The main difference between *in vitro* and *in vivo* studies regarded low dose threshold of detection of non-targeted responses. For example in the *in vivo* studies, chromosomal instability was not able to be detected below 0.5 Gy, whereas *in vitro* studies, instability was observed even in

doses as low as 0.1 Gy X-ray. This *in vitro* 0.1 Gy threshold was also observed in radio-sensitive primary human fibroblasts.

Which mechanisms contribute to the induction and perpetuation of non-targeted effects?

With regards to the *in vitro* mouse haemopoietic model system, strain specific differences in recognition of damage (ATM) were observed and also associated apoptotic pathways. This may contribute towards the observed inverse relationship between induction of chromosomal damage and clearance of damaged cells by apoptosis. These responses are certainly strain dependent in the low dose low LET region. For the human primary fibroblast model system, there were no specific lesions typifying a particular time point. Mechanistic related studies using gene array with human fibroblasts showed that there was transient enrichment in gene expression in RNA degradation and oxidative phosphorylation pathways in bystander populations at early time points. Regarding repair mechanisms, it was shown the DSBs in bystander cells were preferably repaired by homologous recombination repair (Klammer *et al.* 2010 Cancer Research).

3.5 Does the research in NOTE warrant a change in the current paradigms of radiation biology or radiation risk?

One of the main objectives of NOTE was to contribute to the conceptualisation of a new paradigm that would cover both the classical direct (DNA targeted) and non-targeted (indirect) effects. The classical paradigm of radiobiology was based on the concept that all radiation effects on living matter are due to direct action of radiation on cellular DNA, in particular due to cell sterilisation and mutation or larger scale (chromosomal) changes in DNA. The discovery of non-DNA targeted and delayed effects has challenged this concept. At the start of the NOTE project, there were observations on a number of biological phenomena in irradiated cell cultures and tissues and progeny of irradiated cells that did not fit the classical radiation paradigm (Figure3). Subsequently, research in NOTE and elsewhere have addressed the mechanism of these biological phenomena judging which of them arise as a consequence of DNA damage or have a non-DNA-targeted origin.

NOTE started systematic research to test the validity of DNA paradigm and discussing the need and conceptualization of a new paradigm. For shaping the discussion, it was defined that there are three levels of paradigm. The paradigm of radiation biology sets our understanding on mechanisms of radiation action on cells and tissues. The paradigm of radiation risk is the quantitative and qualitative understanding of radiation-induced health effects. The radiation protection paradigm is the pragmatic system for the protection of humans and biota. The scientists in NOTE shared the view that the science is mature enough to start assessing the implications of the non-targeted effects in our understanding of radiation action on cells and tissues and potential health risks. A milestone for this was the international workshop arranged by NOTE in Ireland on the 13th-14th September 2008. A number of novel non-DNA targeted effects were considered including cell signalling, epigenetic effects and the role of phenotype (tissue microenvironment). A special issue of Mutation Research (Vol. 687) recently reported several papers that were presented in the Galway workshop as well as selected other contributions which address this issue. Whilst the experimental evidence generated within and outside the consortium provides compelling evidence that these effects occur experimentally *in vitro* and (to some ex-

tent) *in vivo*, further animal experiments and epidemiological studies on non-cancer effects at low doses are needed to assess implications for radiation risk.

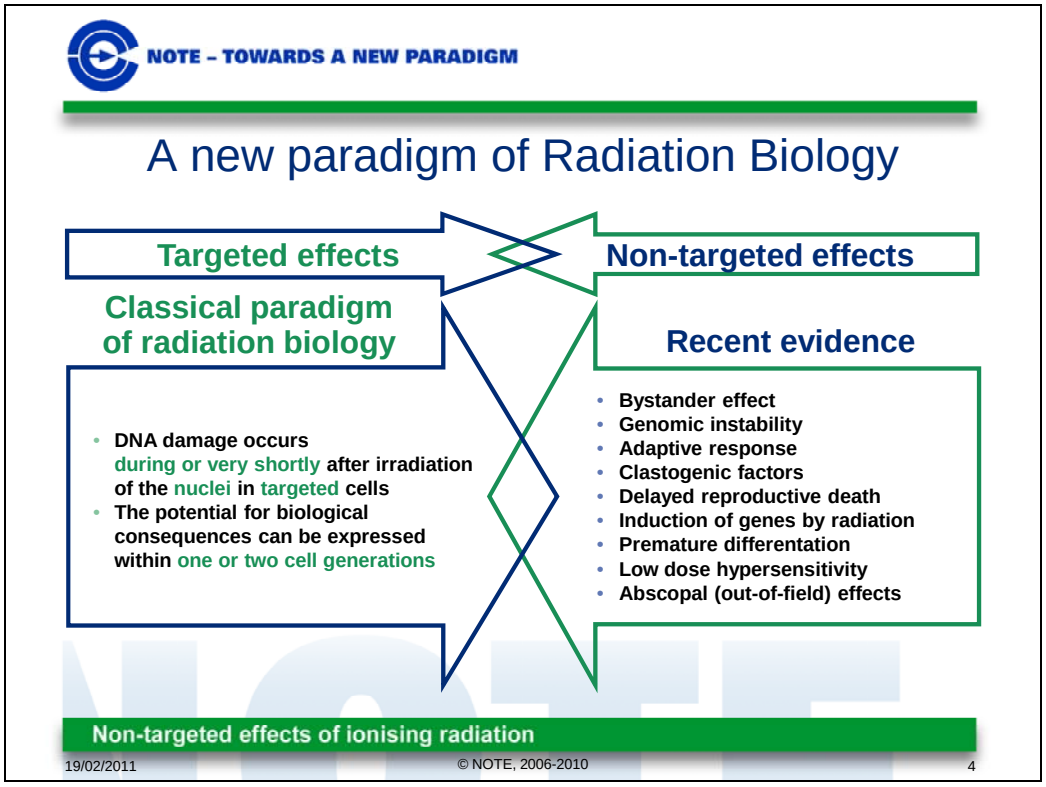

Figure 3. At the start of the NOTE project, there were observations on a number of biological phenomena in irradiated cell cultures and tissues and progeny of irradiated cells that did not fit the classical radiation biological paradigm. A range of responses could be considered non-targeted radiation effects.

Non-DNA targeted effects arise in cells that have received damage signals produced by irradiated cells (radiation-induced bystander effects) or that are the non-clonal descendants of irradiated cells (radiation-induced genomic instability). Radiation-induced genomic instability is characterized by a number of delayed adverse responses including chromosomal abnormalities, gene mutations and cell death. Similar effects, as well as responses that may be regarded as protective or adaptive, have been attributed to bystander mechanisms.

At present, the reason why non-DNA targeted effects are seen in some, but not all, cells and genotypes, is not understood nor is it known whether all endpoints reflect a common set of underlying mechanisms. A number of *in vitro* studies have pointed to an association with free radicals/oxidative processes, inter-cellular signalling and the production of cytokines. *In vivo*, major causes of "spontaneous" DNA damage are associated with oxidative stress and such processes have also been implicated in the tissue responses to irradiation. Mechanistically these are also features of inflammatory responses that may be protective or damaging, depending on context.

Whilst many published studies have concentrated on damaging responses of non-DNA targeted effects a limited number of studies have drawn attention to the possibility of protective res-

ponses it present the determinants of whether a non-DNA targeted effect will occur and if it would be damaging or protective are not known. One of the major questions is whether effects demonstrated *in vitro* can be extrapolated to the *in vivo* situation where additional protective mechanisms are important. However, the observations so far highlight the complexity of non-DNA targeted effects and there appears to be no single mechanism for non-DNA targeted effects.

Genomic instability occurs in the progeny of irradiated cells at a frequency that is several orders of magnitude greater than would be expected for mutation in a single gene, implying that a more complex, multigenic phenomenon may underlie the phenotype. The expression of genomic instability can depend on the genotype of the irradiated cell/animal with considerable inter-individual variation even in those genotypes that may generally express high levels of instability. Similarly there is evidence to suggest that the relationship between genomic instability and radiation dose is non-linear, but is maximally induced at the lowest doses investigated, including for instance a single alpha particle traversal to a single cell nucleus in a population.
At the start of the project, there was a strong body of evidence which didn't fit with the classical radiation biology paradigm of DNA being the primary target of radiation damage, with damage being proportional to the energy deposited/dose received. A range of responses that could be considered non-targeted radiation effects is shown in Figure 3. During the project the definition of non-targeted effects has been reassessed. In particular, the definition of non-targeted effects was discussed at the "Conceptualisation of New Paradigm" workshop organised by the NOTE project, held in Galway in 2008 and at a joint DoReMi – NOTE workshop held at the IRPA congress in Helsinki in 2010.

Dose is defined as energy deposited in a unit mass, which means both an understanding of energy deposition and critical targets. Historically radiation effects have come from an appreciation of target theory. This stems from the early concepts of Dessaur (1922) that absorption of radiation is not continuous but is quantized and is defined by Poisson statistics. This concept was extended to the calculation of a target volume within which the required number of absorption events had to occur, with a given probability (Lea 1946). Since then radiation biology has defined nuclear DNA as "the target" with direct energy deposition being the driver of direct DNA damage, especially DNA dsb formation, and this leading to the cellular consequences of exposure. The definition of non-targeted responses for ionising radiation was first conceptualised by Ward in 2000 but follows from long standing concepts in the photobiology field (Wood & Hutchinson, 1984). The concept of non-targeted effects have been more generally applied to a range of non-linear responses to radiation, including not only bystander signaling, but genomic instability, adaptive responses, gene induction, low dose hypersensitivity and the inverse dose-rate effect. However of these "non-linear" effects, only the bystander response and genomic instability could be defined by the following more robust definition which has been proposed during the NOTE project:

- Targeted effect is one related to whatever in the cell/tissue etc was damaged by the radiation
- Non-targeted effect is related to the radiation damage but not directly to where the damage occurred

The situation with adaptive responses maybe more complex. It can be argued that a radiation-induced adaptive response where a small radiation priming dose leads to adaptation to a subsequent challenge dose does not meet the criteria for being classified as a non-targeted re-

sponse. However, if a bystander signal per se can trigger adaption in cells to subsequent radiation exposure, this could be a potential non-targeted response. Further experimental work will lead to more robust understanding of the processes underlying "Non-targeted" responses and their relevance and in particular, an integrative approach taking mechanistic information to relate targeted and non-targeted effects needs to be developed

Mathematical modelling of non-DNA targeted effects, in collaboration with experimental investigation, has highlighted novel mechanisms that can be experimentally tested. Mathematical modelling also facilitates integration with models of DNA-targeted effects, and will allow determination of the role they may play in low-dose risk.

The NOTE consortium has identified three main questions for radiation protection:

1. Is there a deviation from a linear non-threshold dose response for cancer risk at low doses, and how much of this can be accounted for by non-DNA targeted effects?
2. Can ionising radiation cause non-cancer diseases at low and intermediate doses?
3. How large are the differences in individual susceptibility to ionising radiation?

Non-DNA targeted effects could have a major impact on radiation protection should there be enough evidence on health endpoints not taken into account (such as cardiovascular diseases, for which there is now a considerable body of human data) or reliable findings of thresholds for health effects e.g., cancer due to epigenetic mechanisms. While the mechanisms for radiation-induced circulatory disease are still unclear, inflammatory processes are thought to be the most likely mechanism by which radiation could modify the atherosclerotic disease process. If there is a modification by low doses of ionising radiation of cardiovascular disease through this mechanism, a role for non-DNA-targeted effects cannot be excluded (Little, 2010). Meta-analysis of a radiation-associated risk of circulatory disease at low doses, if confirmed, suggests that circulatory disease contributes at least as much as cancer to radiation-induced mortality, so that overall radiation-related mortality is about twice that currently estimated (Little et al, submitted).

Based on the research carried out in NOTE and other recent studies, we propose a new paradigm of radiation biology that would cover both the classical radiation effects that are explained by the DNA targeted effects as well as non-targeted effects that are explained by epigenetic changes due to radiation exposure as well as effect of tissue micro environment (Figure 4). This new paradigm would explain both cancer and non-cancer effects. It would also facilitate the solution of the conceptual and terminological issues related to definition of stochastic and deterministic effects in the situation where lower and lower doses show association to non-cancer effects such as cardiovascular diseases and lens opacities. Details of the definitions of non-targeted effects will be subject of a symposium at the upcoming 2011 ICRR meeting, which hopefully contribute to the establishment of the new paradigm.

NOTE has contributed to the understanding of mechanisms of radiation actions at low and intermediated doses and the formulation of a new radiobiological paradigm. However, it is evident that continued research efforts are needed on the role of non-DNA targeted effects to obtain definitive answers on low dose risk. Research on non-targeted effects are included in the Strategic Research Agendas of DoReMi and MELODI and should be further supported from the Euratom program.

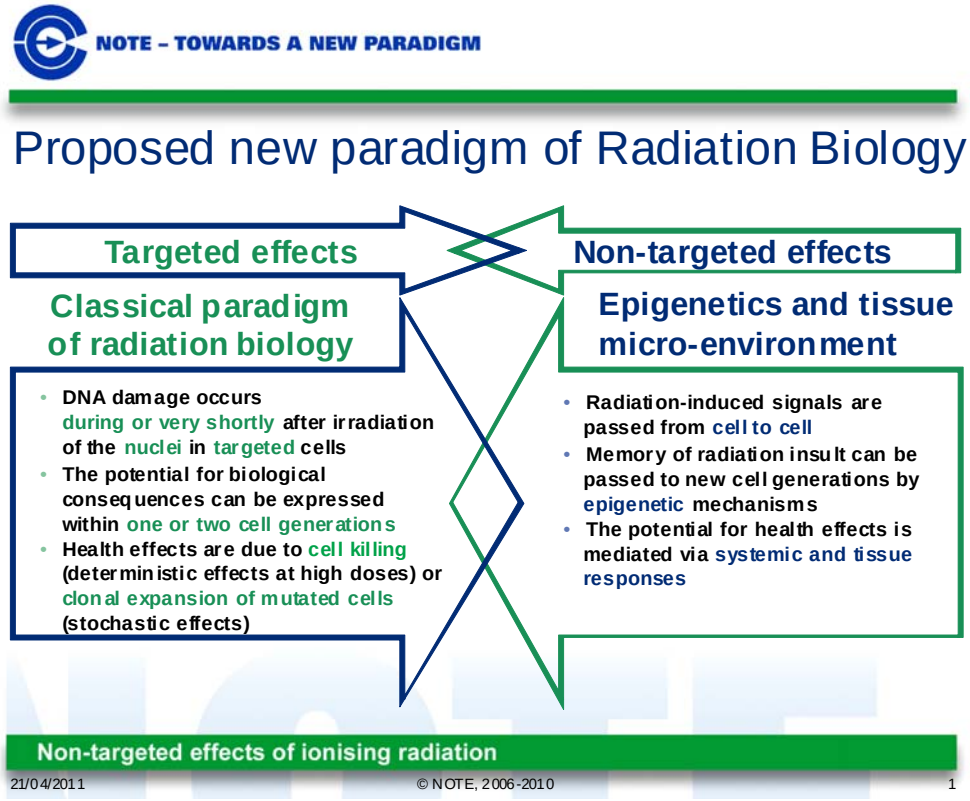

Figure 4. The proposed new paradigm of radiation biology covers both the classical radiation effects that are explained by DNA targeted effects as well as non-targeted effects induced by epigenetic changes or the influence of tissue microenvironment.

3.6 How did NOTE project contribute to the integration of European Research Area and training of scientists?

The NOTE project was an interdisciplinary action integrating expertise from various scientific fields. The inherent international dimension of the project also brings important benefits through the reciprocal transfer of the research experience in area of radiation protection related research. This interdisciplinary approach strengthens the foundations of the European research through collaboration with high rank experts at institutions in EU and on international level.

As Integrated Project (IP), NOTE brought together scientists of a variety of disciplines, like radiation biology, medicine, physics and chemistry, epidemiology and modelling. A dedicated work package on integration activities formed a key part of the NOTE program facilitating the development of infrastructures which were necessary for the successful attainment of scientific aims, creating new collaborative links and supporting the training of young researches. The access to infrastructures involves predominantly microbeam access and the associated running costs and some additional development work of other radiation facilities. The objective was also to provide training opportunities and to promote integration activities and mobility for members of NOTE. Means for building the European Research Area and connecting with strong research groups in third countries were provided by:

- development and better use of existing radiobiological infrastructures;
- strengthening collaborative RTD activity;
- supporting training and mobility of scientists and
- educating the next generation of European radiobiologists on non-targeted effects.

Infrastructure development for biological experiments was facilitated by NOTE. Initially Gray Cancer Institute provided both a charged particle microbeam capable of delivering protons helium-3 and helium-4 ions for NOTE experiments. This was capable of delivering individually counted ions with 1-2 μm precision for both cell and tissue model studies and was used by both STUK and MRC/Oxford Brookes University along with the group at Gray Cancer Institute. A soft X-ray microbeam was also available. On the closure of Gray Cancer Institute, the particle microbeam was decommissioned. The soft X-ray microbeam was transferred to Queens University of Belfast and upgraded to be able to deliver carbon-K, aluminium-K and titanium-K X-rays at spot sizes to down to 1μm. A ^{241}americium alpha-particle source was also commissioned for localised irradiation with charged particles. Both these sources have been utilised for NOTE studies. A charged particle microbeam (LIPSION) was also further developed at University of Leipzig using protons. This was upgraded to include a new irradiation chamber, new online microscope and automated cell recognition software. A fiducial marker system was developed for offline imaging and analysis. Calibration experiments were performed and irradiations carried out for the STUK group. A training workshop was also organised for NOTE users willing to access the microbeam. Access of STUK team to Gray Cancer Institute charged particle microbeam as well as the LIPSION facility was supported by NOTE. The facility was also used for characterization and classification of molecular lesions progression in irradiated and bystander cells following low LET radiation in collaboration with MRC.

A broad-beam alpha particle source was developed at STUK. Consisting of a ^{238}Pu source a collimator assembly was installed, along with computerised stage and particle detection and testing performed. Further work modified the source from a broad-beam configuration of 34 mm diameter to a narrow beam source of 100 μm by 15 mm with a dose-rate of 200 mGy s^{-1} and this has been fully calibrated and tested.

An improved version of the existing α-particle irradiator has been developed at the ISS for bystander effect studies using the co-culture approach. The stainless steel chamber, small enough to be inserted into a cell incubator for irradiation in physiological conditions, has been equipped with ^{244}Cm or ^{241}Am sources delivering a dose rate of 2.8 or 88.6 mGy/min, respectively. Mylar®-based Petri dishes of 56 mm diameter were especially designed to house adaptors for permeable membrane inserts that reproduce the geometry of commercial cell culture insert companion plates. For both sources uniformity of α-dose was better than ±7 % and the photon dose calculated at the cell entrance was negligible compared to the α-particle dose (Esposito et al., 2009).

A key development during the project was a series of internal calls which led to the funding of 4 new sub projects within NOTE, contributing to the characterisation of clastogenic factors in human plasma, studies on gene expression and proteomic changes in the developing mouse brain, testing for a role of ATM status on bystander responses in lymphoblastoid cell cultures and provision of data and development of models for analysis of 3-D bystander signalling.

Short term training projects were supported to allow students to work in NOTE partner laboratories contributing to projects and getting training in low dose radiation biology. Four projects

were supported during the project at University of Leipzig, STUK, Queens University of Belfast and Oxford Brookes University.

Through internal calls, two training courses were organized. Protocols and Pitfalls in the Study of Non-targeted Effects of Radiation, 15-16 September 2007, on Crete, Greece (in connection with the 1st annual meeting)."Mathematical Methods in Radiation Biology" was organised on 1-2 July 2009 in Rome, Italy (in connection of 3rd annual meeting)

Important part of the project is communication with scientific societies, stakeholders in radiation protection and the general public. There are several end-users for the results obtained in the NOTE projects: the scientific community, the radiation protection community, the decision makers and the general public. Dissemination and exploitation activities of the project are extended to bring a message to all of them. Different means of communication are used for each of these target groups. To address the scientific community, the results are published in peer reviewed journals and presented in international scientific conferences.

NOTE website can be found at www.note-ip.org. It has both a public part accessible to everybody as well as secure part for consortium members, Advisory Committee and the EC. The NOTE website has been the main channel for distribution of information and it has been continuously updated by STUK and including all relevant material regarding the project. The public NOTE website is an integrated part of the NOTE website intended for dissemination and popularisation of the knowledge, obtained during the project. The general public has also been addressed via information by NOTE Newsletters (electronical newsletters sent to subscribers) and press releases. NOTE website was available for the public throughout the project duration and beyond (until year 2013).

Two major workshops have been arranged during the project: one discussing the "Conceptualisation of the New Paradigm" (held on 13-14 September 2008 in Galway, Ireland) and one targeted to the radiation protection community, discussing the "Relevance of Non-targeted Effects for Radiation Protection" (held on 14 June 2010 in Helsinki, Finland). Scientific advancements of new areas include a series of Systems Radiation Biology workshops, jointly arranged with other Euratom projects and US DOE Low Dose programme (years 2007, 2008, 2009, 2010). Another important event has been the workshop on Science and Values in Radiation Protection, jointly arranged by OECD/NEA Committee for Radiation Protection and Public Health (CRPPH) and STUK in January 2008.

By May 2011, there have been altogether 114 scientific peer-reviewed publications published (see the List of Publications). The listing of the NOTE-related publications with a possible link to abstract or full paper is provided at public NOTE website. On the basis of papers in press and manuscripts submitted or in preparation, more than 40 additional articles are expected after the end of project.

List of publications

Ahuja, A.K., Barber, R.C., Hardwick, R.J., Weil, M.M., Genik, P.C., Brenner, D.J. and Dubrova, Y.E. (2008): The effects of Atm haploinsufficiency on mutation rate in the mouse germ line and somatic tissue. *Mutagenesis*, 23:5, 367-70.

Alloni D., Campa A., Belli M., Esposito G., Facoetti A., Friedland M., Liotta M., Mariotti L., Paretzke H.G. and Ottolenghi A. (2010): A Monte Carlo study of the radiation quality dependence of DNA fragmentation spectra. *Radiat Res 173; 3: 263-271*.

Alloni, D., Antonelli, F., Ballarini, F., Belli, M., Bertolotti, A., Campa, A., Dini, V., D` Ercole, L., Esposito, G., Facoetti, A., Friedland, W., Giovannini, C., Grande, S., Guidoni, L., Liotta, M., Lisciandro, F., Luciani, A.M., Mantovani, L., Mariotti, L., Molinelli, S., Nano, R., Ottolenghi, A., Palma, A., Paretzke, H.G., Pasi, F., Raffaele, L., Rosi, A., Sapora, O., Scannicchio, D., Simone, G., Sorrentino, E., Tabocchini, M. A. and Viti, V. (2008): Charged particle effects: Experimental and theoretical studies on the mechanisms underlying the induction of molecular and cellular damage and the modulation of intercellular signalling. *Il Nuovo Cimento C* 31:1, 21-38.

Audette-Stuart, M., Kim, S.B., McMullin, D., Festarini, A., Yankovich, T.L., Carr J. and Mulpuru, S. (2011): Adaptive Response in Frogs Chronically Exposed to Low Doses of Ionizing. *Radiation in the Environment, Journal of Environmental Radioactivity* Jun;102(6):566-73 (Epub 2011 Apr 16).

Ballarini, F., Alloni, D., Facoetti and A., Ottolenghi, A. (2008): Heavy-ion effects: From track structure to DNA and chromosome damage *New J Phys*, 10, 1-17, art. no. 075008.

Ballarini, F., Alloni, D., Facoetti, A., Mairani, A., Nano, R. and Ottolenghi, A. (2006): Modelling radiation-induced bystander effect and cellular communication. *Radiat Protect Dosim*, 122:1-4, 244-51.

Ballarini, F., Alloni, D., Facoetti, A., Mairani, A., Nano, R. and Ottolenghi, A. (2007): Radiation risk estimation: Modelling approaches for “targeted” and “non-targeted” effects. *Adv Space Res* 40:9, 1392-1400.

Ballarini, F., Facoetti, A., Mariotti, L., Nano, R. and Ottolenghi, A. (2009): Cellular communication and "non-targeted effects": Modelling approaches. *Advances in Space Res 44; 8: 917-925*.

Barber, R.C., Hardwick, R.J., Shanks, M.E., Glen, C.D., Mughal, S.K., Voutounou, M. and Dubrova, Y.E. (2009): The effects of *in utero* irradiation on mutation induction and transgenerational instability in mice. *Mutat Res Fund Mol Mech Mutag,* 664**:**1, 6-12.

Baverstock K. and Belyakov O. (2010): Some important questions connected with non-targeted effects. *Mutat Res 687; 84-88*.

Bogdándi E.N., Balogh A., Felgyinszki N., Szatmári T., Persa E., Hildebrandt G., Sáfrány G. and Lumniczky K. (2010): Effects of low-dose radiation on the immune system of mice after total-body irradiation. *Radiation Research* 174:4, 480-489.

Burdak-Rothkamm, S. and Prise, K.M. (2009): New molecular targets in radiotherapy: DNA damage signalling and repair in targeted and non-targeted cells. *European Journal of Pharmacology* 625, 151-155.

Burdak-Rothkamm, S., Rothkamm, K. and Prise, K.M. (2008): ATM acts downstream of ATR in the DNA damage response signaling of bystander cells. *Canc Res*, 68:17, 7059-65.

Burr K., Robinson J., Rastogi S., Boylan M., Coates P., Lorimore S. and Wright E. (2010): Radiation-induced delayed bystander-type effects mediated by hemopoietic cells. *Radiation Research 173; 760-768.*

Burr, K.L., van Duyn-Goedhart, A., Hickenbotham, P., Monger, K., van Buul, P.P. and Dubrova, Y.E. (2007): The effects of MSH2 deficiency on spontaneous and radiation-induced mutation rates in the mouse germline. *Mutat Res*, 617:1-2, 147-51.

Chapman, K., Kelly, J., Lee, R., Goodwin, E. and Kadhim, M. (2008): Tracking genomic instability within irradiated and bystander populations. *J Pharm Pharmacol* 60:8, 959-68.

Dahle, J., Kalanxhi, E., Tisnek N. (2011) Dosimetry of a ^{238}Pu-based a-Particle irradiator and its biological application in a study of the bystander effect. *Anticancer Res* (in press)

Davies, B.G., Hussain, A., Ring, S.M., Birch, J.M., Eden, T.O., Reeves, M., Dubrova, Y.E. and Taylor, G.M. (2007): New germline mutations in the hypervariable minisatellite CEB1 in the parents of children with leukaemia. *Br J Canc*, 96:8, 1265-71.

Derradji, H., Bekaert, S., De Meyer, T., Jacquet, P., Abou-El-Ardat, K., Ghardi, M., Arlette, M. and Baatout, S. (2008): Ionizing radiation-induced gene modulations, cytokine content changes and telomere shortening in mouse fetuses exhibiting forelimb defects. *Dev Biol*. 322, 302-13.

Dieriks B., De Vos W., Derradji H., Baatout S., and Van Oostveldt P. (2010): Medium-mediated DNA repair response after ionizing radiation is correlated with the increase of specific cytokines in human fibroblasts. *Mutat Res 687; 40-48.*

Esposito, G., Antonelli, F., Belli, M., Campa, A., Simone, G., Sorrentino, E. and Tabocchini, M.A. (2009): An alpha-particle irradiator for radiobiological research and its implementation for bystander effect studies. *Radiat Res*. 172; 5: 632-642.

Esposito, G., Campa, A., Pinto, G., Simone, G., Tabocchini, M.A., Belli, M. (2011): Adaptive Response: Modelling and Experimental Studies. *Radiation Protection Dosimetry* 143 (2-4), 320-324, 2011.

Facoetti, A., Ballarini, F., Cherubini, R., Gerardi, S., Nano, R., Ottolenghi, A., Prise, K.M., Trott, K.R. and Zilio, C. (2006): Gamma ray-induced bystander effect in tumour glioblastoma cells: a specific study on cell survival, cytokine release and cytokine receptors. *Radiat Protect Dosim*, 122:1-4, 271-4.

Facoetti, A., Mariotti, L., Ballarini, F., Bertolotti, A., Nano, R., Pasi, F., Ranza, E. and Ottolenghi, A. (2009): Experimental and theoretical analysis of cytokine release for the study of radiation-induced bystander effect. *Int J Radiat Biol*. 85;8: 690-699.

Frey, B., Gaipl, U., Sarter, K., Zaiss, M., Stillkrieg, W., Rödel, F., Schett, G., Herrmann, M., Fietkau, R. and Keilholtz, L. (2009): Whole body low dose irradiation improves the course of beginning polyarthritis in human TNF-transgenic mice. *Autoimmunity* 42:4, 346-8.

Friedland, W., Kundrát, P. (2011): Track structure calculations on hypothetical subcellular targets for release of cell killing signals in medium transfer experiments. *Radiation Protection Dosimetry 143; 325-329: Proceedings of the 15th Microdosimetry Symposium*.

Friedland, W., Paretzke, H.G., Ballarini, F., Ottolenghi, A., Kreth, G. and Cremer, C. (2008): First steps towards systems radiation biology studies concerned with DNA and chromosome structure within living cells. *Radiat Environ Biophys*, 47:1, 49-61.

Gaipl, U.S., Meister, S., Lödermann, B., Rödel, F., Fietkau, R., Herrmann, M., Kern, P. M. and Frey, B. (2009) Activation-induced cell death and total Akt content of granulocytes show a biphasic course after low-dose radiation. *Autoimmunity* 42:4, 340-2.

Glaviano, A., Mothersill, C., Case, C.P., Rubio, M.A., Newson, R. and Lyng, F. (2009): Effects of hTERT on genomic instability caused by either metal or radiation or combined exposure. *Mutagenesis* 24:1, 25-33.

Gow M.D., Seymour C.B., Ryan L.A. and Mothersill C.E. (2010): Induction of bystander response in human glioma cells using high-energy electrons: A role for TGF-β1. *Radiation Research* 173; 769-778.

Gow, M.D., Seymour, C.B., Byun, S.H. and Mothersill, C.E. (2008): Effect of dose rate on the radiation-induced bystander response. *Phys Med Biol*, 53:1, 119-32.

Hardwick, R.J., Tretyakov, M.V. and Dubrova, Y.E. (2009): Age-related accumulation of mutations supports a replication-dependent mechanism of spontaneous mutation at tandem repeat DNA loci in mice. *Mol Biol Evol 26:11; 2647-2654.*

Hatch, T., Derijck, A.A., Black, P.D., van der Heijden, G.W., de Boer, P. and Dubrova, Y.E. (2007): Maternal effects of the scid mutation on radiation-induced transgenerational instability in mice. *Oncogene*, 26:32, 4720-4.

Hegyesi H, Sándor N, Schilling B, Kis E, Lumniczky K and Sáfrány G. (2011) Differentially expressed genes associated with low-dose gamma radiation: Growth Differentiation Factor (GDF-15) as a radiation response gene and radiosensitizing target. *Radiation Damage in Biomolecular System (ed. Gustavo Garcia), Springer Verlag* pp. 00-04.

Hildebrandt G. (2010): Non-cancer diseases and non-targeted effects. *Mutat Res* 687; 73-77.

Jacquet P., Buset J., Neefs M., Vankerkom J., Benotmane M.A., Derradji H., Hildebrandt G. and Baatout S. (2010): Transgenerational developmental effects and genomic instability after X-irradiation of preimplantation embryos: Studies on two mouse strains. *Mutat Res* 687; 54-62.

Jacquet, P., Buset, J., Neefs, M. and Vankerkom, J. (2008): Studies on the adaptive response in mouse female germ cells X-irradiated in vitro at two different stages of maturation. *In Vivo*, 22:2, 179-86.

Jahns, J., Anderegg, U., Saalbach, A., Rosin, B., Patties, I., Glasow, A., Kamprad, M., Scholz, M., and Hildebrandt G. Influence of low dose irradiation on differentiation, maturation and T-cell activation of human dendritic cells. *Mutat Res 709-710*; 32-39.

Kadhim M., Lee R., Moore S., Macdonald D., Chapman K. Patel G. and Prise K. (2010): Genomic instability after targeted irradiation of human lymphocytes: Evidence for inter-individual differences under bystander conditions. *Mutation Research* 688; 91-44.

Kinner, A., Wu, W., Staudt, C. and Iliakis, G. (2008): Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res* 36:17, 5678-94.

Kis, E., Szatmari, T., Keszei, M., Farkas, R., Esik, O., Lumniczky, K., Falus, A. and Safrany, G. (2006): Microarray analysis of radiation response genes in primary human fibroblasts. *Int J Radiat Oncol Biol Phys*, 66:5, 1506-14.

Klammer, H., Kadhim, M. and Iliakis, G. (2010): Evidence of an Adaptive Response Targeting DNA Nonhomologous End Joining and Its Transmission to Bystander Cells. *Cancer Research* 70:8498-8506.

Kundrát, P., Friedland, W., Jacob, P. (2011): Modelling of intercellular induction of apoptosis in oncogenic transformed cells and radiation effects on the phenomenon. *Radiation Protection Dosimetry143; 549-553: Proceedings of the 15th Microdosimetry Symposium.*

Larisch W., Koal, T., Werner, R., Hohlweg, M., Reinert, T., Butz, T.: Proton Beam Writing of microstructures in Agar gel for patterned cell growth. *Nucl. Instr.& Methods B* DOI 10.1016/j.nimb.2011.02.041.

Lindholm C., Acheva A. and Salomaa S. (2009): Clastogenic plasma factors: a short overview. Epub 2009 Dec 18. Radiation and Environmental Biophysics 2009.

Little M.P. (2010): Do non-targeted effects increase or decrease low dose risk in relation to the linear-non-threshold (LNT) model?. *Mutat Res 687; 17-27.*

Little M.P., Heidenreich W. and Li G. (2010): Parameter identifiability and redundancy: Theoretical considerations. *PLoS ONE* 5:1.

Little M.P., Tawn E.J., Tzoulaki I., Wakeford R., Hildebrandt G., Paris F., Tapio S. and Elliott P. (2010): Review and meta-analysis of epidemiological associations between low/moderate doses of ionizing radiation and circulatory disease risks, and their possible mechanisms. *Radiat Environ Biophys 49; 139-153.*

Little, M.P. (2008): Leukaemia following childhood radiation exposure in the Japanese atomic bomb survivors and in medically exposed groups. *Radiat Prot Dos 132; 2: 156-165*.

Little, M.P. (2009): Cancer and non-cancer effects in Japanese atomic bomb survivors. *J Radiol Prot* 29, A43-59.

Little, M.P. (2009): Heterogeneity of variation of relative risk by age at exposure in the Japanese atomic bomb survivors. *Radiat Environ Biophys* 48, 253-262.

Little, M.P. (2010): Cancer models, genomic instability and somatic cellular Darwinian evolution. *Biology Direct 5;19.*

Little, M.P., Gola, A. and Tzoulaki, I. (2009): A model of cardiovascular disease giving a plausible mechanism for the effect of fractionated low-dose ionizing radiation exposure. *PLoS Comput Biol*. 5:10.

Little, M.P., Gola, A., Tzoulaki, I., Vandoolaeghe, W. (2009): Variant assumptions made in deriving equilibrium solutions to "A model of cardiovascular disease giving a plausible mechanism for the effect of fractionated low-dose ionizing radiation exposure" *PLoS Computational Biology* 5(10) e1000539). arXiv:0912.5120.

Little, M.P., Heidenreich, W.F. and Li, G. (2009): Parameter identifiability and redundancy in a general class of stochastic carcinogenesis models. PLoS ONE 4:12.

Little, M.P., Heidenreich, W.F., Moolgavkar, S.H., Schöllnberger, H. and Thomas, D.C. (2008): Systems biological and mechanistic modelling of radiation-induced cancer. *Radiat Environ Biophys,* 47:1, 39-47.

Little, M.P., Tawn, E.J., Tzoulaki, I., Wakeford, R., Hildebrandt, G., Paris, F., Tapio, S. and Elliott, P. (2008): A systematic review of epidemiological associations between low and moderate doses of ionizing radiation and late cardiovascular effects, and their possible mechanisms. *Radiat Res*, 169:1, 99-109.

Little, M.P., Wakeford, R., Lubin, J.H. and Kendall, G.M. (2010): The statistical power of epidemiological studies analyzing the relationship between exposure to ionizing radiation and cancer, with special reference to childhood leukemia and natural background radiation. *Radiation Research* 174, 387-402.

Little, M.P., Wakeford, R.,Tawn, E.J., Bouffler, S.D. and Berrington de Gonzalez, A. (2009): Risks associated with low doses and low dose rates of ionizing radiation: Why linearity may be (almost) the best we can do. *Radiology* 251:1, 6-12.

Little, M.P., Vineis. P. and Li, G. (2008): A stochastic carcinogenesis model incorporating multiple types of genomic instability fitted to colon cancer data. *J Theor Biol* 254*:*2, 229-238 *and erratum to the article in J Theor Biol* 255:2, 268*.*

Liu, Z., Byun, S.H., McNeill, F.E., Mothersill, C.E., Seymour, C.B. and Prestwich, W.V. (2007): Fluence and dose measurements for an accelerator neutron beam. *Nuclear Instruments and Methods in Physics Research B* 263:1, 326-8.

Liu, Z., Mothersill, C.E., McNeill, F.E., Lyng, F.M., Byun, S.H., Seymour, C.B. and Prestwich, W.V. (2006): A dose threshold for a medium transfer bystander effect for a human skin cell line. *Radiat Res*, 166:1 Pt 1, 19-23.

Liu, Z., Prestwich, W.V., Stewart, R.D., Byun, S.H., Mothersill, C.E., McNeill, F.E. and Seymour, C.B. (2007): Effective target size for the induction of bystander effects in medium transfer experiments. *Radiat Res*, 168:5, 627-30.

Lorimore, S.A., Chrystal, J.A., Robinson, J.I., Coates, P.J. and Wright, E.G. (2008): Chromosomal instability in unirradiated hemaopoietic cells induced by macrophages exposed *in vivo* to ionizing radiation. *Canc Res*68: (19) 8122-26.

Lyng, F.M., Howe, O., McClean, B.: Reactive oxygen species induced release of signalling factors in irradiated cells triggers membrane signalling and calcium influx in bystander cells. *International Journal of Radiation Biology* - early online, pp. 1–13.

Maguire, P., Mothersill, C., McClean, B., Seymour, C. and Lyng, F.M. (2007): Modulation of radiation responses by pre-exposure to irradiated cell conditioned medium. *Radiat Res*, 167:4, 485-92.

Mariotti, L., Facoetti, A., Alloni, D., Bertolotti, A., Ranza, E. and Ottolenghi, A. (2010): Effects of ionizing radiation on cell-to-cell communication. *Radiation Research* 174:3, 280-289.

Mariotti, L., Facoetti, A., Bertolotti, A., Ranza, E., Alloni, D. and Ottolenghi, A. (2010): Radiation induced perturbation of cell-to-cell signaling and communication. *Radiation Protection Dosimetry* 143; 294-300.

Miccoli, L., Burr, K.L., Hickenbotham, P., Friedberg, E.C., Angulo, J.F. and Dubrova, Y.E. (2007): The combined effects of xeroderma pigmentosum C deficiency and mutagens on mutation rates in the mouse germ line. *Canc Res*, 67:10, 4695-9.

Mitchel, R.E.J., Hasu, M., Bugden, M., Wyatt, H., Little, M.P., Gola, A., Hildebrandt, G., Priest, N.D., Whitman, S.C. (2011): Low dose radiation exposure and atherosclerosis in ApoE-/- mice. *Radiation Research* May; 175(5):665-76.

Mothersill C. and Seymour C. (2010): Eco-system biology - From the gene to the stream. *Mutat Res 687; 63-66.*

Mothersill C., Saroya R., Smith R.W., Singh H. and Seymor C.B. (2010): Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments. *Radiat Res 174; 1: 119-123*.

Mothersill C., Smith R.W., Hinton T.G., Aizawa K., Seymour C.B. (2009): Communication of radiation-induced signals in vivo between DNA repair deficient and proficient medaka (Oryzias latipes). *Environmental Science and Technology* 43(9):3335-42.

Mothersill C., Smith R.W., Saroya R., Denbeigh J., Rowe B., Banevicius L., Timmins R., Moccia R. and Seymour C.B. (2010): Irradiation of rainbow trout at early life stages results in legacy effects in adults. *International Journal of Radiation Biology* Oct 86(10):817-28.

Mothersill, C. and Seymour, C. (2009): Implications for environmental health of multiple stressors. *J Radiol Prot* 29:2A, A21-8.

Mothersill, C., Bucking, C., Smith, R.W., Agnihotri, N., Oneill, A., Kilemade, M. and Seymour, C.B. (2006): Communication of radiation-induced stress or bystander signals between fish in vivo. *Environ Sci Tech*, 40:21, 6859-64.

Mothersill, C., Moran, G., McNeill, F., Gow, M.D., Denbeigh, J., Prestwich, W. and Seymour, C.B. (2007): A role for bioelectric effects in the induction of bystander signals by ionizing radiation? *Dose-Response* 5:3, 214-29.

Mothersill, C., Salbu, B., Heier, L.S., Teien, H.C., Denbeigh, J., Oughton, D., Rosseland, B.O. and Seymour, C.B. (2007): Multiple stressor effects of radiation and metals in salmon (Salmo salar). *J Environ Radioact*, 96:1-3, 20-31.

Mothersill, C., Smith, R.W. and Seymour, C.B. (2009): Molecular tools and the biology of low-dose effects. *Bioscience* 59:8, 649-55.

Mothersill, C., Smith, R.W., Agnihotri, N. and Seymour, C.B. (2007): Characterization of a radiation-induced stress response communicated in vivo between zebrafish. *Environ Sci Tech*, 41:9, 3382-7.

Nugent S., Mothersill C.E., Seymour C., McClean B., Lyng F.M. and Murphy J.E. (2010): Altered mitochondrial function and genome frequency post exposure to radiation and bystander factors. *International Journal of Radiation Biology* Oct;86(10):829-41.

O`Dowd, C., Mothersill, C.E., Cairns, M.T., Austin, B., McClean, B., Lyng, F.M. and Murphy, J.E. (2006): The release of bystander factor(s) from tissue explant cultures of rainbow trout (Onchorhynchus mykiss) after exposure to gamma radiation. *Radiat Res*, 166:4, 611-7.

O`Neill-Mehlenbacher, A., Kilemade, M., Elliott, A., Mothersill, C. and Seymour, C. (2007): Comparison of direct and bystander effects induced by ionizing radiation in eight fish cell lines. *Int J Radiat Biol*, 83:9, 593-602.

Poon, R.C., Agnihotri, N., Seymour, C. and Mothersill, C. (2007): Bystander effects of ionizing radiation can be modulated by signaling amines. *Environ Res*, 105:2, 200-11.

Prise, K.M. (2008): Bystander effects and radionuclide therapy. *In "Targeted Radionuclide Tumour Therapy: Biological Aspects" (Stigbrand,T., Carlsson, J. and Adams, G. eds),* 311-9. Springer-Verlag, Berlin.

Prise, K.M. and O'Sullivan, J.M (2009): Radiation-induced bystander signalling in cancer therapy. *Nature Reviews Cancer* 9, 351-360.

Prise, K.M., Schettino G. (2011): Microbeams in Radiation Biology: Review and critical comparison. *Radiation Protection Dosimetry* 143, 2–4, 335–339.

Prise, K.M., Schettino, G., Vojnovic, B., Belyakov, O. and Shao, C. (2009) Microbeam studies of the bystander response. *J Radiat Res* 50:Suppl. A, A1-A6.

Ranza, E., Facoetti, A., Morbini, P., Benericetti, E. and Nano, R. (2007): Exogenous platelet-derived growth factor (PDGF) induces human astrocytoma cell line proliferation. *Anticancer Res*, 27:4B, 2161-6.

Rothermel, M., Butz, T. and Reinert, T. (2009): Rearranging a nanoprobe: Line foci, grid shadow patterns and performance tests. *Nuclear Instruments and Methods in Physics Research* B 267, 2017.

Ryan, L.A., Seymour C.B. and Mothersill, C.E. (2009): Investigation of Non-Linear Adaptive Responses and Split Dose Recovery Induced by Ionizing Radiation in Three Human Epithelial Derived Cell Lines. *Dose Response* 7:4, 292-316.

Ryan, L.A., Seymour, C.B., O'Neill-Mehlenbacher, A. and Mothersill, C.E. (2008): Radiation-induced adaptive response in fish cell lines. *J Environ Radioact*, 99:4, 739-47.

Ryan, L.A., Smith, R.W., Seymour, C.B. and Mothersill, C.E. (2008): Dilution of irradiated cell conditioned medium and the bystander effect. *Radiat Res*, 169:2, 188-96.

Rödel F., Frey B., Capalbo G., Gaipl U., Keilholz L., Voll R., Hildebrandt G. and Rödel C. (2010): Discontinuous induction of X-linked inhibitor of apoptosis in EA.hy.926 endothelial cells is linked to NF-jB activation and mediates the anti-inflammatory properties of low-dose ionising-radiation. *Radiotherapy and Oncology* 97:2, 346–351.

Rödel, F., Hofmann, D., Auer, J., Keilholz, L., Röllinghoff, M., Sauer, R. and Beuscher, H.U. (2008): The anti-inflammatory effect of low-dose radiation therapy involves a diminished CCL20 chemokine expression and granulocyte/endothelial cell adhesion. *Strahlenther Onkol*, 184:1, 41-7.

Rödel, F., Keilholz, L., Herrmann, M., Sauer, R. and Hildebrandt, G. (2007): Radiobiological mechanisms in inflammatory diseases of low-dose radiation therapy. *Int J Radiat Biol*, 83:6, 357-66.

Rödel, F., Keilholz, L., Herrmann, M., Weiss, C., Frey, B., Voll, R., Gaipl, U. and Rödel, C. (2009): Activator protein 1 shows a biphasic induction and transcriptional activity after low dose X-irradiation in EA.hy.926 endothelial cells. *Autoimmunity* 42:4, 343-5.

Salbu, B., Denbeigh, J., Smith, R.W., Heier, L.S., Teien, H.C., Rosseland, B.O., Oughton, D., Seymour, C.B. and Mothersill, C. (2008) Environmentally relevant mixed exposures to radiation and heavy metals induce measurable stress responses in Atlantic salmon. *Environ Sci Tech*, 42:9, 3441-6.

Salomaa S., Wright E., Hildebrandt G., Kadhim M.A., Little M.P., Price K. and Belyakov O. (2010): Editorial - Non-DNA targeted effects. *Mutat Res 687; 1-2.*

Saroya, R., Smith R., Seymour, C.B. and Mothersill, C. (2009): Injection of reserpine into zebrafish prevents fish to fish communication of radiation-induced bystander signals; confirmation in vivo of a role for serotonin in the mechanism. *Dose response* Dec 21;8(3):317-30.

Schettino, G., Al Rashid, S.T. and Prise, K.M. (2010): Radiation microbeams as spatial and temporal probes of subcellular and tissue response. *Mutation Research* 704, 68-77.

Schettino, G., Ghita, M., Richard, D.J., Prise, K.M. (2011): Spatio-temporal investigations of DNA damage repair using microbeams. *Radiation Protection Dosimetry* 143, 2–4, 340–343.

Shanks, M., Riou, L., Fouchet, P. and Dubrova, Y.E. (2008): Stage-specificity of spontaneous mutation at a tandem repeat DNA locus in the mouse germline. *Mutat Res Fund Mol Mech Mutagen*

641:1-2, 58-60.

Shao, C., Folkard, M. and Prise, K.M. (2008): Role of TGF-beta1 and nitric oxide in the bystander response of irradiated glioma cells. *Oncogene*, 27:4, 434-40.

Singh, H., Saroya, R., Smith, R., Mantha, R., Guindon, L., Mitchel, R., Seymour, C. and Mothersill,C. (2011): Radiation-induced bystander effects in mice given low doses of radiation in vivo. *Dose Response (pre-press).*

Smith R.W., Wang J., Mothersill C.E., Hinton T.G., Aizawa K. and Seymour C.B. (2011): Proteomic changes in the gills of wild-type and transgenic radiosensitive medaka following ex-posure to direct irradiation and to X-ray induced bystander signals. *Biochim Biophys Acta* Feb; 1814(2):290-8.

Smith, R.W., Wang, J., Bucking, C.P., Mothersill, C.E. and Seymour, C.B. (2007): Evidence for a protective response by the gill proteome of rainbow trout exposed to X-ray induced bystander signals. *Proteomics*, 7:22, 4171-80.

Tartier, L., Gilchrist, S., Burdak-Rothkamm, S., Folkard, M. and Prise, K.M. (2007): Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells. *Canc Res,* 67:12, 5872-9.

Tisnek, T., Kalanxhi, E., Serkland, C.W., Iversen, J., Belyakov, O. and Dahle, J. A. (2009): ^{238}Pu irradiator for exposure of cultured cells with alpha-radiation: Construction, calibration and dosimetry. *Appl Radiat Isot* 67: 1998-2002.

Vines, A.M., Lyng, F.M., McClean, B., Seymour, C. and Mothersill, C.E. (2008): Bystander signal production and response are independent processes which are cell line dependent. *Int J Radiat Biol*, 84:2, 83-90.

Vines, A.M., Lyng, F.M., McClean, B., Seymour, C. and Mothersill, C.E. (2009): Bystander effect induced changes in apoptosis related proteins and terminal differentiation in in vitro murine bladder cultures. *Int J Radiat Biol* 85:1, 48-56.

Wright E. (2010): Manifestations and mechanisms of non-targeted effects of ionizing radiation. *Mutat Res 687; 28-33.*

Zhang, Y., Zhou, J., Baldwin, J., Held, K.D., Prise, K.M., Redmond, R.W. and Liber, H.L. (2009): Ionizing radiation-induced bystander mutagenesis and adaptation: Quantitative and temporal aspects. *Mutation Research* 671, 20-25.

Zyuzikov N., Coates P., Parry J., Lorimore S. and Wright E. (2011): Lack of non-targeted effects in murine bone marrow after low-dose in vivo X-irradiation. *Radiation Research* 175, 322–327.