PROPOSAL FOR AN INFN EXPERIMENT

2015-17

ETHICS

Pre-clinical experimental and theoretical studies to improve treatment and protection by charged particles

Understanding the underlying action mechanisms on normal cells by charged particles used in medicine to reduce the risks for human health

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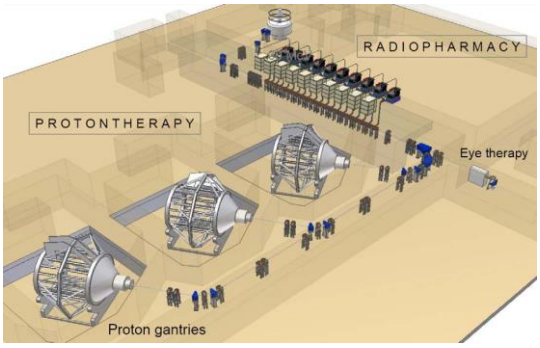
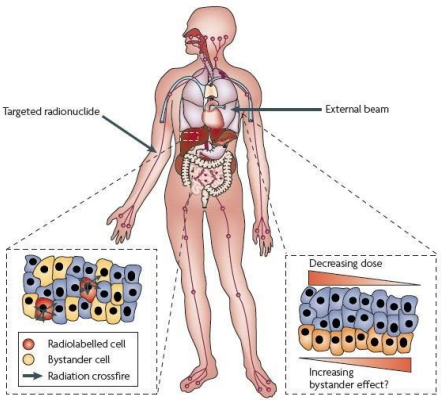
July 2014





CONTENTS

1. PROPOSAL OVERVIEW

Proposal name	ETHICS
Participating INFN Sections	NA,RM1-Gr.coll.Sanità, PV, LNL, AQ-LNGS From 2016: BO, LNF, LNS
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Duration	2015-17

ABSTRACT

Medical applications of charged particles, such as hadrotherapy an radionuclide therapy, involve the exposure of normal cells composing the tissues and organs proximal to the tumour from either external or internal sources. Clinical implementation of biologically-optimised treatment plans and a safer use of cancer cell-targeting radionuclides are hampered by the uncertainties inherent to the radiobiology of healthy tissue response to densely ionising radiations which may lead to increased risks of secondary cancers, hence needing to be urgently addressed. The main objective of this research project is, therefore, to study the basic mechanisms underlying the biological effects brought about by charged particles that are of relevance for the integrity and normal functions of healthy tissues/organs. To achieve such a goal, both in vitro and in vivo experiments are planned at INFN national Laboratories as well at external facilities, involving a vast network of national and international collaborations and in conjunction with theoretical studies and medical physics-based approaches. The action of a number of ions of interest in external beam therapy and internal targeted radionuclide therapy will be investigated, employing a wide array of assays and state-of-the art techniques. The ultimate aim is to help develop strategies that may limit high-LET radiation detrimental consequences for human health while improving their therapeutic benefits.

2. Scientific rationale of the proposed research activity

2.1 Introduction

Treatment by ionising radiation is one of the most successful and common approaches against cancer. Therefore, improvement of its efficacy and reduction of the risks to patients bear a fundamental impact on society. The major modalities of administering radiation dose to the tumour by modern radiotherapy are either by external beams, usually delivered by accelerators, with a growing adoption of ion beams (protons and carbon ions), or through the incorporation of radioisotopes and radiolabelling of tumour seeking-molecules so that local irradiation of cancer cells is achieved. However, irrespective of which of the approaches employing charged particles is used, a number of uncertainties are still in place, essentially as regards the effects on the inevitably exposed healthy cells [1, 2]. How and to what extent such effects translate from the molecular and cellular level to affect normal tissue integrity and organ functionality at the organismal level is still largely undetermined. Sublethally damaged normal cells may represent a risk for secondary cancers as well as for impairment of normal tissue/organ function. Addressing this issue is therefore important to maintain the unarguable advantages that these anti-cancer strategies have brought to patients without posing avoidable risks to human health.

The main uncertainties can be listed as follows:

a. Charged particle effectiveness (RBE) for the induction of sublethal damage in normal cells;
b. Dose distribution inhomogeneity, particularly relevant for low dose and low dose-rate regimes and/or ion irradiation;
c. Interaction between irradiated healthy cells and the tumour microenvironment (e.g. cell signalling, extracellular matrix remodelling, etc.) and the role of stem cells;
d. In vivo relevance of the so-called non-targeted effects (e.g. genomic instability).

All of the issues raised above are interrelated: For example, a non-homogeneous dose distribution within the treatment field can favour non-targeted effects in nearby unirradiated cells [3] while sublethally damaged healthy cells may influence adjacent tumour cells by releasing pro-tumorigenic factors or cross-talking with the tumour microenvironment [4, 5]. In addition, each of the above points is, in turn, dissectible in several aspects whose study can be attained by using different but complementary approaches. Thus, in *vitro* studies are necessary for understanding clinically relevant treatment protocols that improve normal tissue protection at the cellular level. Combined assessment of early and late cellular response including DNA damage in a range of relevant cell lines is therefore needed to provide systematic high-resolution information to develop a rigorous theory of ion radiation action at the cellular and molecular level. The in vivo studies are, conversely, needed as the next step in order to validate and

expand the benchmark findings to more realistic biological systems. Finally, not all quantities involved in radiobiological and medical physics studies are measurable experimentally (e.g. LET), hence tools such as Geant4 and biophysical modellization of ion action at the subcellular level are necessary to model experimental beam lines and predict particle behaviour in the cellular environment compensating for the discontinuous dose delivery pattern inherent to radiation, whether administered externally [6] or internally [7].
In ETHICS these three approaches come together for making pre-clinical benchwork research truly translational.

2.2 State of the art

The cellular response to ionising radiation reflects the energy deposition pattern at the nanoscale level [8]. Severity of DNA damage depends on spatio-temporal lesion proximity and repairability [9], hence it is not a constant value but depends on physical (particle type, LET, dose, dose rate) and biological (cell type, oxygenation status, repair capacity) parameters. Charged particles exhibit a peculiar pattern of energy deposition along and around their track, as described by the Bragg curve, which makes them unique among all the environmental and man-made mutagen and carcinogen agents, including, of course, low-LET radiations.

Inasmuch as the particle beam inverse dose-depth profile is the physical pillar of their use in hadrontherapy since ions exhibit a therapeutically advantageous peak to plateau RBE ratio, which allows considerable dose sparing to the normal tissue compared to photons, there are still challenges such as a considerable uncertainty surrounding normal tissue and organ late effects for these particles [10, 11]. Similar issues exist as for normal tissue exposure in the case of internal irradiation by radionuclides, which is often characterised by low dose-rate regimes and a poor dosimetric characterization [12].

A good example of a hitherto overlooked potential threat to normal tissue homeostasis and organ function is represented by a phenomenon known as Stress-Induced Premature Senescence (SIPS) that has been increasingly attracting attention since it has been shown that low doses of high-LET radiation, and even at very low dose rate, hence scenarios common to external and internal irradiation by charged particles, seem to be very effective at causing cells to prematurely enter senescence [13]. This has raised questions as to the relevance of SIPS in hadrontherapy [14]. The existence of the so-called Senescence-Associated Secretory Phenotype (SAPS), triggered by low doses, points to a crucial role for endothelial senescent cells (Fig.1), which may favour tumour cell proliferation, although cell growth inhibition has also been reported [15, 16]. Being SIPS induced so effectively after exposure to lower doses of radiation, it bears consequences for understanding human cancer risk also to radiation exposure of environmental importance). In clinical scenarios, particularly at low doses future work needs to understand the relevance of radiation quality, dose and dose rate in initiating SIPS in normal cells

and the long-term tissue damage and pathological alterations that may arise as a consequence [17].

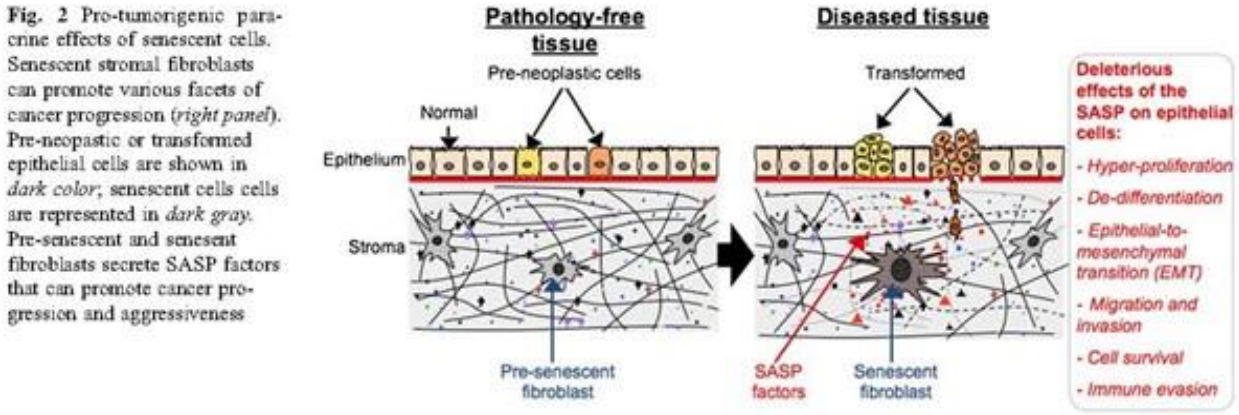

Fig. 1: Example of the complex, potentially carcinogenic interplay between prematurely senescing endothelial cells, normal epithelial cells and mesenchymal stem cells [16]

Similar to hadrontherapy, targeted radionuclide therapy has unique promise as a vehicle for personalized treatment of cancer, because both the targeting vehicle and the radionuclide can be tailored to the individual patient. However, conventional perspectives on the response of tissues to radiation may not adequately describe and predict the effects of targeted radiotherapeutics on tumor and normal tissues [18, 19]. Because biologically targeted radiopharmaceutical therapy is generally characterized by low dose rates, the suggested hypersensitivity of mammalian cells to low-dose radiation may play a role [20]. Non-targeted radiation effects (e.g. bystander) also could have a profound effect on targeted radionuclide therapy [21, 22]. In this process, cells not directly hit by radiation can be killed efficiently through an indirect but as yet unidentified mechanism. This is contrary to conventional radiation biological wisdom, which considers cell death to be a direct consequence of radiation traversal and energy deposition. These findings may have implications for targeted radionuclide therapy because if bystander phenomena could be harnessed, it could help compensate for variability in radiation dose deposition which is the bane of targeted radionuclide therapy, that is point b) in the previous paragraph [12]. Although most work to date has been done with external beam radiation, investigations of low-dose hypersensitivity with targeted radiopharmaceuticals are moving forward [23] and could lead to novel strategies for cancer treatment. However, at present, both the radiobiology and the dosimetry of this field are topics of intense debate, and the implications for healthy tissues are unclear.

Thus far, we have highlighted how the points from a) to d) raised in the previous paragraph are at the centre of the radiobiological interest concerning the action of charged particles on normal

cells and how interrelated these aspects are. Furthermore, the fact that most such aspects are shared between internal irradiation regimes and external beams and the need to translate pre-clinical studies to therapeutic scenarios, hence supporting the case for in vivo studies and adequate modellization at both the physical and biophysical level, justify the choice of the Work Packages (WPs) in this proposal as detailed later (see paragraph 8).

Concerning biophysical modelling, a need for a switch from phenomenological to mechanistic models has recently been highlighted [11]. Mechanistic approaches allow testing of different assumptions on key open questions, thus leading to a deeper and deeper understanding of the intermediate steps leading from the initial energy deposition by radiation in biological targets to critical DNA damage and to subsequent endpoints (e.g., chromosome aberrations) that determine the "fate" of the cell, i.e. cell death or survival of a cell progeny with altered genome. Examples of the aforementioned open questions are the following: i) characterization of the critical DNA damage; ii) relationship between such critical damage and subsequent endpoints at sub-cellular level, typically chromosome aberrations; iii) link between chromosome aberrations and cell death or cell conversion to malignancy. Recently, Schipler and Iliakis [24] concluded that DSB clusters undermine local chromatin stability generating small DNA fragments, whose loss is likely to impair the function of all DSB repair pathways and to cause cell death and other endpoints including chromosome aberrations. However, it is still not clear how small these critical DNA fragments should be, because at least three different sizes have been considered up to now: the base-pair scale, related to the double helix, the kbp scale, related to nucleosome organization in the chromatin fibre, and the Mbp scale, related to chromatin organization in giant loops. The distance-dependence of the interaction probability between two DSBs (no matter whether complex or not) is another open question: while some authors hypothesise a rapid decrease with increasing distance, others describe it by a step function with a cut-off distance at the micrometre scale, in line with recent findings suggesting the existence, on the nuclear matrix, of specific repair complexes to which breaks migrate and accumulate for repair [25]. Mis-rejoining of chromosome fragments leads to various types of chromosome aberrations. Although it has been shown that Giemsa-stained asymmetric exchanges are strictly correlated with cell death in X-irradiated human fibroblasts [26], such correlation needs to be investigated for other radiation types (typically, charged particles) and other cell types; especially for high LET radiation, the role of complex exchanges needs to be elucidated. By contrast, symmetric exchanges like some specific reciprocal translocations are known to be related to cell conversion to malignancy [27]. These open questions will be addressed by the Unit of Pavia, applying and further developing a mechanistic biophysical model linking radiation-induced DNA damage, chromosome aberrations and cell death which will be measured by other units (NA, LNL, ISS). Furthermore, the model can provide useful information on specific scenarios that are less accessible by experiments, such as the effectiveness of a single charged particle traversing the

cell nucleus and the exposure of the cell to alpha particles starting from the cell surface, as it happens in targeted therapy (see below).

In the remaining part of this paragraph, we shall motivate the choice of concentrating our studies on three main "disease units", i.e reference organ models, which constitute the WP-1 subdivision. These are: Breast, Pancreas and Bone. The other two WPs will, in turn, focus on one or two such organ models based on the most up-to-date clinical panorama a well as to optimize the experimental plan. Hence, WP-2, which explores the basic action mechanisms of charged particle exposure from incorporated sources mimicking their energy and dose rate regimes, will focus mainly on the Bone while the WP-3, devoted to the set-up and carrying out of in vivo irradiations, will dissect the Breast model following exposure to external charged particle beams. We shall therefore now briefly examine the current state of research for these three organ models in relation to the issue of normal tissue damage following charged particle exposure. The mounting body of evidence in favour of an increased risk of coronary disease induced by exposure of healthy tissue to ionising radiation and the uncertainties linked with the RBE for the cardiac tissue [28, 29] was indeed initially supported by findings from epidemiological studies among the cohort of the Japanese atomic bomb survivors [30]. In particular, the relevance of the endothelium in the adverse effects of radiotherapy is demonstrated by recent evidence [31, 32] linking an increased risk of developing coronary and heart diseases in women treated for breast cancer when the left breast was irradiated versus those who received treatment on the right breast (table.1). These findings have led, among others, to the provocative suggestion to use proton therapy in the treatment of breast cancer [33]. Even so, a portion of the endothelium would be irradiated, the consequences of which need to be addressed. In fact, to testify the growing awareness of cardiotoxicity at the international level, an important European concerted action is in place (http://www.procardio.eu/), with whose of the WPs (RBE and ion exposure effects, led by prof. M. Durante at GSI, Germany) we plan to work in close collaboration on some common cell lines and looking at complementary endpoints .

Results of the patient group with >20 years follow-up			
Time after diagnosis years	Cardiac deaths		Mortality ratio left vs. right
	Left breast	Right breast	
<5	230	180	1.19
5–9	189	145	1.21
10–14	157	106	1.42
>15	234	145	1.58

Table 1: The risk for occurrence of cardiovascular disease after post-operative breast cancer radiotherapy [31]

Radiotherapy represents a major treatment option for patients with pancreatic cancer, but several studies demonstrated that ionising radiation might promote migration and invasion of tumor cells by intricate implications in the microenvironment, cell-cell junctions, extracellular matrix junctions, proteases secretion, and induction of epithelial-mesenchymal transition [34]. In particular, it has been reported that after photon irradiation interactions between pancreatic cancer cells and surrounding fibroblasts play an important role in aggressive tumor progression [35]. Several authors hypothesized that carbon ions may have a different effect on radiation-induced migration in comparison with low-LET radiations, although in vitro and ex vivo studies provided controversial results (Table 2), none evaluated the role of normal surrounding cells like fibroblasts.

Carbon-ion radiation and cell invasion in literature.

Article	organ	Cell line	In vitro or ex vivo	Dose	Migration	Invasion/metastasis
Akino et al. [9]	Lung	A549, EBC-1	In vitro	0.25 Gy in 1 fr, 1 Gy in 1 fr, 5 Gy in 1 fr	Decreased in both cell lines at each dose ($p<0.05$)	Decreased in both cell lines at 1 Gy and 5 Gy ($p<0.05$)
Fujita et al. [54]	Pancreas	MIAPaCa-2, BxPC-3, AsPC-1, Panc-1	In vitro	0.5 Gy in 1fr, 1 Gy in 1 fr, 2 Gy in 1 fr, 4 Gy in 1 fr	At 2 Gy: decreased in MIAPaCa-2, BxPC-3, AsPC-1 ($p<0.05$) and constant in Panc-1.	At 2 Gy: decreased in MIAPaCa-2 ($p<0.05$), constant in AsPC-1 and BxPC-3, enhanced in Panc-1 ($p<0.05$).
Goetze et al. [19]	CNS, Colon	U87, HCT116	In vitro	1 Gy in 1 fr, 3 Gy in 1 fr, 10 Gy in 1 fr	Decreased at each dose for HCT116 ($p<0.05$). Decreased at 3 and 10 Gy for U87 ($p<0.05$)	
Goetze et al. [20]	Colon	HCT116	In vitro	1 Gy in 1 fr, 3 Gy in 1 fr, 10 Gy in 1 fr	Decreased at each dose ($p<0.05$)	
Ogata et al. [26]	Human fibrosarcoma, Mouse osteosarcoma	HT1080, LM8	In vitro and ex vivo	0.2 Gy in 1 fr, 1 Gy in 1fr, 4 Gy in 1 fr	Decreased in HT1080 at each dose ($p<0.01$)	Decreased in HT1080 at each dose ($p<0.01$) Metastasis decreased when LM8 irradiated at 5 Gy
Ogata et al. [27]	Lung	A549	In vitro	1 Gy in 1fr, 5 Gy in1 fr	Decreased at each dose ($p<0.05$)	Decreased at each dose ($p<0.05$)
Riekenet al. [32]	CNS	U87-MG	In vitro	0.5 Gy in 1 fr, 3 Gy in 1 fr		Decreased at each dose ($p<0.05$)
Tamaki et al. [38]	Buccal mouse	NR-S1	Ex vivo	5 Gy in 1fr, 10 Gy in 1 fr, 30 Gy in 1 fr, 50 Gy in 1 fr		Metastases decreased at each dose ($p<0.05$ Gy)

fr, fraction; CNS, central nervous system.

Table 2: Carbon-ion radiation and cell invasion as reviewed in [34]

In addition to this, in light of a generalised radioresistance of this type of cancer to conventional radiotherapy there exists a strong interest in treating pancreatic cancer with charged particles either from external irradiation schedules [36], as also shown by the scheduled treatment of this disease at CNAO [37], or with internal irradiation. Hence, the issue of the surrounding healthy cells is of interest also in targeted radionuclide therapy of pancreatic cancer [2].

Another interesting model to study the possible detrimental consequences of healthy tissue irradiation by both external and internal irradiations is represented by the bone. The most common type of bone cancer is osteosarcoma while a more rare form is represented by

chondrosarcomas, which develop along vertebrae. For the latter, indications for the use of charged particle therapy come from the observation that chondrosarcomas are relatively radioresistant tumours and lie in the proximity of sensitive organs (e.g the spinal cord), hence benefitting from hadrontherapy treatment [38-39]. However, much more common and worrisome are osteosarcomas since they tend to develop in growing bones, thereby impacting lives of youngsters and increasing the risk for metastasis. An important paper published by Hall in 2006 [40] highlighted how, especially for pediatric tumors, IMRT would significantly increase the risk of secondary tumours due to the higher dose received by yet a smaller portion of the healthy tissue if compared with conventional photon therapy, advocating proton treatment (even better when dose is delivered actively by means of pencil scanning beams systems). A very recent paper, on the other hand, compares several labelled radionuclides pointing to a radiopharmaceutical employment of a drug conjugated with alpha-emitting Radium-223 (Xofigo) as the most promising approach in terms of internal irradiation [41]. However, the level of toxicity to normal tissue from alpha-emitters as well as from other radionuclides remains to be ascertained. Finally, to make the Bone "disease unit" even more attractive for modern radiobiology and implications on ameliorating patient radioprotection, are recent studies that have also demonstrated the close association between mesenchymal stem cells, which can differentiate, among others, in osteoblasts, and osteogenesis [42]. Furthermore, previous studies comparing the effects of X-rays and heavy ions (^{56}Fe) showed LET-dependent mechanistically different effects on mesenchymal cells [43]. Since mesenchymal stem cells are well-known for their ability to repair damaged normal tissues, these data raise the possibility that irradiation may perturb the autocrine/paracrine signalling by which they elicit their homeostatic action. In particular, no exhaustive data are present on the effect of lower Z ion types (such as alpha or protons) on mesenchymal stem cells secretome, whose study would be therefore of great importance for the side effects of charged particle radiotherapy [44]. There are several indications suggesting that the altered secretome of senescent cells of several types (including endothelial and fibroblasts) may either promote or block cancer cell proliferation, being one the most intriguing sublethal effects on normal cells with implications in cancer therapy [17]. Therefore, the Bone model could provide the ideal system linking several aspects listed in paragraph 1 and investigated as described in paragraph 8 and following. We shall also initiate a collaboration with prof. U. Galderisi (Experimental Medicine Department, Second University of Naples) who has recently reported on prematurely senescing mesenchymal stem cells [45].

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3. Relevance of the proposed research activity with regard to the INFN mission and the research in the field

It has to be noted that for the first time a radiobiology proposal is presented to INFN that bring together the two main therapeutic irradiation scenarios (external beams and radionuclide therapy) focussing on the consequences for the healthy tissue, which involve also dose distribution calculations and possible bioimaging applications in addition to more basic mechanistic radiobiological research.

One of the INFN missions is the promotion of interdisciplinary research as to bring together a diversity of expertise and competences. ETHICS envisages a close collaboration between several aspects of applied physics (radiobiology, dosimetry, medical physics) as well as theoretical approaches (modellization of radiation interaction with biological structures and dose distribution). As a matter of fact, in the last "white paper" delineating the research lines of INFN for the period 2012-14 (page 29) could be read: « Application of fundamental physics to human health and to the environment is becoming a primary need and, as such, is acknowledged by modern research...In the field of hadrontherapy...radiobiology and modelling studies will grow." It thus appears evident that role that radiobiology has to play and the necessity that it relates organically with the medical physicists and the clinicians. Further broadening of contacts with researchers who are not usually involved in INFN activities will come from the collaborations that will be established as discussed in paragraph 5. In this context, in the ETHICS collaboration, Institutions such as CNAO and the participation of researchers of the IBFM-CNR based at the Polo Oncologico in Cefalù will provide the much needed feedback with real treatment scenarios. Finally, the close connection between nuclear physics (the "core business" of the INFN) and its applications is clearly highlighted in one of the statement from a meeting held in Paris at the end of last year of the Nuclear Physics European Collaboration Committee (one of the bodies of the European Science Foundation:" *Nuclear physics is a coin that has two sides: basic research and applications. Without basic research there would be little to be applied; applications resulting from basic research contribute to the wealth and health of society".* Radiation protection issues in medical applications are of increasing importance: A key question in radiation protection is how the radiation risk can be assessed

for: a) exposures at low dose rates and b) partial/inhomogeneous exposure of the body. These kind of exposures are typical of Radioisotope Therapy. That a growing interest exists is shown by several reports and platform
at the European level (HLEG or High Level and Expert Group Report on European Low Dose Risk Research – Radiation Protection, European Commission EUR 23884, 2009; http://hleg.de; http://www.melodi-online.eu/). In particular, the interest of INFN in such research topics has been recently strengthened by its official participation to the MELODI platform. The scientific outcomes of our proposal will certainly go in the direction of improving the quality of life of patients by reducing the risk of late-occurring complications and, more in general, towards a deeper understanding of charged particle exposure effects, also at the environmental level where chronic exposures dominate, thereby making it safer for the general public.

4. Aims and organizational structure of the project

ETHICS is structured in three Work Packages (WPs). The first two WPs reflect the exposure scenarios to charged particles herein studied, i.e. irradiation by external beams, and internal irradiation (radionuclide targeted radiotherapy), while the third is dedicated to the planning, setting-up and performing of the in-vivo experiments. In detail, the WPs are as follows: WP-1 External beam therapy (hadrontherapy); WP-2 Internal irradiation therapy (radioisotope or targeted therapy); WP-3 Pre-clinical studies (in vivo studies). To optimize the achievement of the proposal aims, each WP is organised in tasks (sub-WPs), to which one or more of the participating units will contribute, based on their expertise. In particular, WP-1 subdivision can be seen as divided in “disease units”, each task being devoted to a specific model system (tissue/organ) of interest in hadrontherapy for the repercussions on healthy tissue they may have in foreseen or current treatment; these are Breast, Pancreas and Bone. Each of such tasks will be divided in subtasks focussing on different aspects peculiar to the system of choice and appropriate endpoints. WP-2 will deal with basic questions inherent to current internal irradiation strategies and their effects on healthy cells, focussing on two of the above disease units, that is Bone and Pancreas, thereby allowing comparisons with results from WP-1. A small task here will be also devoted to chronic exposure of environmental relevance whose exposure regime is similar to that of nuclide therapy using biological systems of interest for cardiac complications but also of the respiratory tract, target of inhaled alpha-emitting radon descendants. Finally, WP-3 will be structured in three main tasks, i.e. Monte Carlo-based simulations of animal irradiation, the engineering of the irradiation set-up and the actual animal irradiation experiments. This WP will also include in vitro experiments conducted in parallel with the animal studies for feedbacks on techniques and results found in using the two approaches.

5. Expertise of the participant groups, infrastructures and external collaborations

One essential aspect of interdisciplinary and polyfunctional research projects, with multi-facet themes is the good integration and complementarity of the shared expertise. In ETHICS groups that have long contributed to the development of the current radiobiology knowledge at the national and international level take part, with a specific track record in the field of charged particles. In addition, groups not traditionally involved in INFN-funded interdisciplinary projects have been involved, whose contribution is expected to enrich the collaboration and establish links with clinicians and medical physicists, as necessary for a research to be operatively translational. In each of the involved laboratories adequate instrumentation is present as below detailed.

The Radiation Biophysics group of Naples has a long-standing research record in the biological effects of ionizing radiation and has focussed particularly on the radiobiological properties of charged particle beams, for studies in space radiation protection (biodosimetry, biological effects of shielding materials) and hadrotherapy applications (radiosensitization of cancer cells., effects of ion track structure, RBE determination of several ion beams, etc). It has been the laboratory of choice for data collection and analysis for several INFN-funded projects. In addition to standard cell culture equipment, the laboratories have state-of-the art instrumentation for measurement of radiation-induced cytogenetic damage: fluorescence microscopy and semi-automated computerized systems for scoring of chromosome aberrations either visualised by hybridization with whole chromosome fluorescent probes or by solid staining as in the case of dicentrics and micronuclei. It has also a sophisticated imaging and analysis software by which cell karyotyping can be carried out. In the last years it has acquired experience in the analysis by immunochemical methods of high LET radiation-induced prematurely senescent cells. Routinely used by the laboratory are a departmental 3-MV TTT-3 Tandem accelerator and a 250 V_p x-ray machine. Dosimetry by means of solid-state nuclear track detectors as well as Si-based detectors is also routinely performed together with spectra acquisition at multichannel analysers during ion beam irradiations.

The Institute of Bioimaging and Molecular Physiology (IBFM) of CNR encompasses laboratories for *in vitro,* preclinical *in vivo* and *ex-vivo* studies and for biomolecular analysis. IBFM-CNR includes the Cefalù support unit (UOS) which carries out its activities at the laboratories of the public-private company LATO (LAboratory of Oncologic Technologies), based at the Foundation Institute San Raffaele-Giglio in Cefalù. The Cell and Genomic Methodologies Laboratory is equipped with proteogenomics advanced molecular tools such as Microarray technology. One of the main target of Cell and Genomic Methodologies Laboratory is to study molecular mechanisms that regulate proliferation and survival of tumour cells for leading to the development of novel therapeutic approaches. The laboratory employs the use of cutting-edge tools in their field applied to genomic and cell cultures. Therefore, a main role is played by the development and integration of multidisciplinary skills. The Laboratory of Medical Physics and

Bioimaging processing deals with the study and optimization of the physical characteristics of the radiation used with experimental techniques and Monte Carlo simulations. It uses the Monte Carlo method with toolkit GEANT4 for radiotherapy applications with electron beams (conventional and high dose per pulse), photons and protons. The laboratory has experience in the use of instruments for carrying out accurate dosimetry (relative, absolute, and "in vivo") on beams of electrons and protons, carrying out radioprotection studies in systems involving the use of particle accelerators and unsealed sources. The equipe has also expertise regarding the processing, segmentation and quantification of RM, CT and PET images. IBFM-CNR includes highly experienced personnel in the fields of molecular biology and PET imaging and it is characterized by a multidisciplinary group of researchers (physicists, biologists, engineers, radiochemists, pharmacologist), working together ensuring the convergence of multidisciplinary expertise on research activities. All the activities are strongly translational, with the overall objective of cancer studying and care, using advanced biomedical technologies involved in pathology, preclinical and biomedical imaging.

The Unit of Pavia has a wide experience in modelling the effects of ionizing radiation on biological targets at different levels, with focus on chromosome aberrations and cell death. The activity is based on a model and a Monte Carlo code called BIANCA (BIophysical ANalysis of Cell death and chromosome Aberrations), and is characterized by a mechanistic approach that provides simulated dose-response curves for chromosome aberrations and cell death directly comparable with experimental data. This Unit has also experience in performing experimental activity, thanks to a solid experience on cellular biology and radiobiology making use of a wide array of techniques (cell culture, histochemistry, immunocytochemistry, Enzyme Linked Immunosorbent Assay). In particular, the experimental research topics include aspects of cellular biology that are known to play a fundamental role in tissue response to ionizing radiation, i.e. tumor-stroma interactions and extracellular matrix remodelling . The Centro Nazionale di Adroterapia Oncologica (CNAO), Pavia, is a nationwide facility whose mission is to provide hadron-therapy treatments (both proton and carbon ion scanned beams) and to perform research in the related fields. The CNAO synchrotron is able to accelerate proton and carbon ions in the energy range of 60-250 MeV and 120-400 MeV/u, respectively, with a beam penetration depth approximately ranging from 3 g/cm^2 to 27 g/cm^2 and steps of 0.1 g/cm^2. Dose is delivered with active raster scanning. Different spot sizes will be available, with a radial size adjustable from 4 mm to 10 mm (FWHM), in steps of 1 mm. The maximum available field size is 20 x 20 cm^2. Many in vitro (and in vivo) irradiations have been performed in the last 3 years with CNAO beams by several of the participant groups so that the irradiation set up and the dosimetry has been already tested and optimized for this sort of experiments. By participating to proposal as the one here illustrated, CNAO intends to confirm to be a centre of excellence for advanced research in the field of hadrontherapy not only for tumour eradication but, given the theme of this proposal, also as far as patient risks are concerned. Its participation to European networks

and collaboration with many other research institutions employing this challenging technology, (i.e. CERN, NIRS, GSI, HIT) makes CNAO a key player on the international scene. The recently set-up radiobiology laboratory in CNAO is well-equipped for the large scale experiments typically needed for ion beam radiobiological studies. It has 2 double-cabinets vertical flow, 2 CO2 HERACELL incubators, 1 inverted microscope Olympus IX71, water baths, laboratory refrigerators, and a -80°C freezer. The Department of Biology and Biotechnology "Lazzaro Spallanzani" (Prof. Rosanna Nano, UNIPV) will provide the equipments and appropriate structures for the part of the research that will not be not available in CNAO. In particular, it will provide centrifuges, ELISA readers, cameras and software to acquire and elaborate the imagines (Image-Pro-plus software is used in a wide range of applications including: Fluorescence Microscopy, Confocal Microscopy, Cytometry, Pathology).

The RM1-GrcolSanità Unit (ISS) has a long-standing experience in charged particle radiobiology and research for the assessment of low-dose risk. The activity of the Unit is based on a multi- and inter-disciplinary approach, with biologists and physicists working together for designing and performing experiments as well as developing models on the mechanisms of radiation action. The major interests of the Unit concern radiation biophysics issues related to densely ionizing radiation (investigation of the relationship among energy deposition patterns, DNA damage induction, distribution and repair, cell differentiation, cell lethality and mutagenicity) and implications for radiation protection and therapy. The ISS vast expertise on interdisciplinary research in charged particle radiobiology will be complemented by the collaboration with ISS colleagues from contiguous laboratories that posses a solid experience in hematopoietic and mesenchymal stem cell studies and will coadjuvate the Unit for the Mesenchymal Stem Cell (MSC) isolation and characterization collaborating closely in the radiobiological measurements. The ISS group has designed and developed facilities for low dose/dose rate exposure to alpha particles and □-rays of biological samples in physiological conditions. These facilities will complement the charged particles accelerators in the ETHICS research activities focused on the dose –rate dependence of biological effects.

The INFN-LNL Group is characterized by a multi-disciplinary expertise (radiation physics, dosimetry, radiation protection, biology, radiobiology) and has been active for more than 25 years in the field of radiobiology of ionizing radiations, within national and international scientific collaborations. In the framework of research programmes mainly funded by INFN, the INFN-LNL Group has developed light- and heavy-ion irradiation facilities especially dedicated to radiobiology investigations, installed at the INFN-LNL 7MV Van de Graff CN accelerator and Tandem-ALPI accelerator complex. By means of these facilities monolayers of cell cultures, in sterile and controlled-temperature conditions, can be irradiated in "track segment condition" by light and heavy ion beams extracted in air as a function of accelerated ion species, energy, dose and dose-rate as well as in different irradiation geometries (including whole or partial cell population irradiation). In addition to the accelerator-based irradiation facilities, an alpha-source irradiator

has also been developed to allow continuous/protracted cell irradiations whilst cells are incubated, i.e. in physiological conditions. The INFN-LNL Unit has a Co-60 gamma-beam irradiation facility (mod. GB-150, "panoramic" source; Nordion, Canada - dose-rate : 3-4 Gy/min down to 0.05 Gy/min, depending on the distance source-sample) and a fully equipped cell biology laboratory for cell culturing and processing after irradiation (n.4 CO2 incubators; n.2 vertical and n. 1 horizontal biohazard flow hoods; inverted phase-contrast microscope and fluorescence microscope, both equipped with CCD cameras for image analysis; -20°C freezer; -86°C ultra-low temperature freezer; dewar for cell cryo-preservation; microcentrifuge and refrigerated centrifuge; cytospin; coulter counter; scepter cell counter; spectrophotometer; autoclave for steam sterilization; oven for dry-heat sterilization; thermostatic water baths; horizontal and vertical gel electrophoresis apparatuses).

The main activity of the LNL Group is related to the study of the targeted and non-targeted effects (low-dose hyper-radio-sensitivity and induced-radio-resistence; bystander effects; genomic instability; adaptive response) of ionizing radiations (gamma rays and accelerated charged particles) in cultured mammalian cells, in terms of cell inactivation, micronuclei induction, mutation induction at hprt locus, protein expression, DNA damage, as a function of dose and radiation quality (radiation type and energy).

The PLASMA-X Laboratory, Department of Life, Health and Environmental Sciences, Aquila University, is equipped with a plasma-laser source producing ultra-soft x-ray, whose modulation allows to generate photoelectron with an energy range comprised between 10 and 500 eV which well mimics the delta-rays structure radially departing from the particle tracks and responsible, ultimately, for the energy depositions at the end of the range. The laboratory is also provided with a biological sample-holder, whose motion can be opportunely controlled, for cell irradiation under sterility conditions. A hardware/software system allows the control of both the x-ray beam and of the images.

As detailed in the experiment database with supporting documents stating the interest in our proposal and shown in paragraph 1, there are a number of external collaborating groups, of scientifically renowned reputation, with which we hope to strengthen in time links: In view of the rapidly changing ways research is being funded, it is essential that connections based on a common scientific agenda be in place as a prerequisite for future grant applications at the European level.

6. Expected impact of the research and its relationship with the Horizon2020 themes

It can be reasonably expected that our research programme may shed light on the basic action mechanisms underlying the action of charged particles on normal cells and on the interplay between these and the tumour microenvironment from two complementary perspectives, i.e.

external beam and internal irradiations. The envisaged distribution of tasks among groups and based on selected "disease units", together with the concerted contribution of experimental and theoretical approaches and both in vitro and in vivo studies, will help focus the specificity of the final findings, hence of pragmatic usefulness for patients and clinicians. However, not only clinics may benefit from the research we intend to carry out since the exposure regimes and model systems we propose to investigate have some degree of overlap with risk estimates for the general public, therefore the low-dose and chronic exposure scenarios which most people experience may gain in knowledge for specific biological mechanisms which in turn may lead to a greater accuracy in defining radiation risk estimates. Our proposal well fits with the Horizon 2020-Priority: "Scientific Excellence" because of the qualified participation of national groups and international partners and can be related to the "Societal Challenges" theme, Specific objectives: "Health" since one of the main objectives of our proposal lies in the reduction of the risks for human health deriving from common or expanding radiation modalities used in disease treatment. As for the Horizon 2020-EURATOM Radiation Protection theme, the topics we shall investigate have been clearly identified as prioritary by the strategic research agenda by the most important European scientific platform in the field of the protection from low or very low ionising radiation doses (MELODI).

7. Risk assessment of the proposed activities and contingency plan

Inherent to each research programme, especially those that heavily rely on irradiation facility, the risks of failure or under-achievement or partial completion of milestones cannot be ruled out. This may in turn affect also the theoretical part of the collaboration calculations/simulations that may need experimental data as input or mean of comparison for their predictions. One possibility to minimise such risks may be to diversify the beam time requests between the foreseen facilities (LNS, LNL and CNAO). At the ISS two alpha irradiators exist so no criticalities are expected with respect to protracted alpha exposures.With the alpha particle irradiator also present at LNL laboratories, the risk of not being able to complete WP-2, for instance, become minimal. For contingency plans to be effectively and rapidly enforced it is important that the each of the involved group of the collaboration is constantly aware of the progress made by its counterpart(s) of the same WP or sub-WPs. This can be easily achieved by scheduling periodical on-line meetings where possible criticalities are immediately made of public domain. Another typical unknown, specific of radiobiology experiments, is represented by the handling of biological samples: Cell lines may not grow as fast as expected, experiments may be partially or entirely lost due to loss of sterile conditions and/or post-irradiation contaminations. The use of one than more group of the same cell line due to common tasks may serve as an effective contingency plan, although part of the information may not be recovered since each group tend to carry out complementary measurements of different endpoints. The use of the Metafer Metasystem platform (Units of NA and ISS) for automatic analysis of the genomic and cytogenetic damage will allow measurements on the large number of samples, needed for

statistical significance of the responses expected at low doses. This may reduce dead times between experiments and maximise data throughput. Finally, one important source of indetermination, especially regarding the WP-3 is represented by the set up of the in vivo irradiations and the handling of small animals, which requires specific regulations and protocols. While all will be done to guarantee that the official paperwork will be in place and that the engineering design will be checked, in case of unexpected delays, alternative facilities for small animal irradiations (with photons) are available at one of the prospective collaborators, the laboratory of prof. Kevin Prise (QUB, Belfast) where we have actually envisaged to carry out irradiations in 2016 as to be prepared for comparisons with high LET data from LNS. Also, collaborating groups that work in close association with the laboratories in Cefalù as well as another foreign collaborator, dr. Marie Boyd, have animal expertise and experience in the field of radiopharmaceutics. An eventual reshuffling of milestones (previously agreed upon with the proposal assigned referees) and of the expected temporal sequenceof the experimental plan could allow us to minimize disruption in the achievement of the final goals.

8. Experimental working plan

As introduced in paragraph 4, the experimental working plan will follow a Work Package (WP) structure in the attempt to optimize the contribution each Unit will give based on their expertise and infrastructures. Especially for the WP-1 (and to a lesser extent for the other two) the criteria for sub-task repartition will be to focus on a single “organ/tissue” system, for which appropriate cell lines will be chosen for the in vitro studies or mice model for the in vivo experiments. the rationale is to keep the translational character of our research well defined.

8.1 Detailed activity of WP-1: External beam therapy

This WP will examine in vitro cellular response to external charged particle beams (proton, helium-4, carbon and oxygen ions) using three “disease units”: breast, prostate and bone. The effects of end track high-LET electrons mimicking the delta rays associated with ion tracks will be studied by irradiating cells with ultra-soft x-rays. For the breast system endothelial and normal breast epithelial cells will be used. Fibroblasts as representative of widespread normal connective tissue will also be used. To represent the normal tissue associated with pancreas irradiation, human fibroblasts will be used. The bone system will be the only one in this proposal to envisage also the associated tumour cell line to study the interplay between osteosarcoma sublethally damaged normal cell, the mesenchymal stem cell niche and the tumour microenvironment, which is profoundly influenced by the first two cell types.

8.1.1 WP-1.1 Breast

8.1.1.1 *DNA damage modelling (Pavia)*

Within the framework of WP-1 Pavia will carry out both simulations and experimental work. In

WP-1.1 the activity will be modelling activity and will consist of simulations of DNA/chromosome damage and cell death. Whenever possible, the model will be applied to the same cells used by the experimental partners: for WP-1.1 these will be represented by endothelial cells and/or normal breast epithelial cells by modifying the code ad hoc to represent realistically the cell type according to its shape and dimensions. The starting point will be a biophysical model, implemented as a Monte Carlo code called BIANCA (BIophysical ANalysis of Cell death and chromosome Aberrations), which at the moment can simulate chromosome aberrations and cell death following exposure to protons, He ions and C ions (as well as photons for comparison) at doses of the order of a few Gy . The current version of the model assumes that DNA cluster damage plays a fundamental role for subsequent endpoints and that some chromosome aberration types lead to cell death. The model and the code will be specifically refined and implemented to reproduce as close as possible scenarios of interest for the present project. The simulations will concentrate on particles and energies of interest for hadrontherapy, basically low-energy protons and carbon ions, plus possibly oxygen ions, for which the interest in the hadrontherapy community is on the rise due to their possible better counteracting tumour re-oxygenation compared to carbon ions, for example, to the extent that these ions are under consideration for patient treatment at Heidelberg, Germany. Low-energy particles are always present in the distal region of therapeutic Bragg peaks and thus represent a potential risk for normal tissues beyond the tumor region. Particular attention will be devoted to protons of energy around 1 MeV or even below, which can be significantly more effective than photons although a RBE of 1.1 is assumed in clinical practice . The focus will be on doses below about 1 Gy, which are typically present in the descending part of therapeutic Bragg peaks and, more generally, in normal tissues also at the entrance of the beam. Besides, at doses of such magnitude, most of the damage inflicted to normal cells will be sub-lethal in nature, hence of importance for late effects such as transformation, genomic instability premature senescence and/or differentiation, etc. In this framework, the code will be purposely extended to deal with the case of cells traversed by exactly one particle, which is the lowest possible dose. This is also of interest for WP-2 (targeted radionuclide therapy) and for exposures of environmental interest (e.g. alpha particles from inhaled radon indoor). However, the effects of higher doses will also be characterized, considering their relevance for new modalities such as hypofractionation. Concerning the considered endpoints, the model will be applied both to cell killing, because many (early) effects in normal tissues are related to cell death, and to non-lethal effects such as transmissible chromosome aberrations, in particular translocations. The latter in general allow the cell to proliferate, but give rise to a cell progeny with an altered genome, which can lead to late effects including (second) cancer. To characterize the mechanisms underlying the induction of critical damage in normal cells, the relationship between different endpoints will be investigated qualitatively and quantitatively. More specifically, the yields of DNA "Cluster Lesions", which in the current version of the model is a parameter to be adjusted *a posteriori* by comparison with experimental data on chromosome aberrations and/or cell survival, will be compared with data on DNA fragmentation, with the aim of characterizing this

critical damage. The focus will be on DNA fragments with different scale size (from bp to the Mbp), which have received particular attention by the radiobiology community .The data for comparison will be taken both from the literature, and from the activity of the ISS Unit, which has a wide experience on DNA fragmentation down to the kbp scale, which seems to be the most promising candidate as a critical initial damage for subsequent endpoints including chromosome aberrations and cell death. In addition to the role of DNA cluster lesions, the model will also be applied to clarify the relationship between chromosome aberrations and cell death, which is another open question in charged particle radiobiology, although there is a general consensus that some chromosome aberrations, basically those involving (large) acentric fragments, lead to cell death . On this basis, the current version of the model assumes that dicentrics, rings and deletions visible in Giemsa lead to cell death. This hypothesis will be tested also for the cell lines and the radiation types used by the experimental partners; furthermore, since also some types of complex exchanges are likely to play a role, the model/code will be modified refining the scoring of complex exchanges (which will be divided on the basis of their transmissibility to the cell progeny, basing on the work by *Savage, 1995*) and assuming that also non-transmissible complexes lead to cell death. These data may be compared with those collected by Naples unit by means of m-FISH karyotyping of epithelial normal breast cells.

8.1.1.2 Molecular mechanisms and gene expression profile (Naples/CNR-IBFM)

Breast cancer (BC) represents a highly heterogeneous group of tumours at both the clinical and molecular level. Radiation to the breast is often given after breast-conserving surgery to help lower the chance of recurrence in the breast or nearby lymph nodes. Radiation may also be recommended after mastectomy in patients having either cancer larger than 5 cm, or cancer in the lymph nodes. External beam radiation is the most common modality of dose delivery for women with BC. Irradiation carries a risk of skin burns and long-term cardiovascular and pulmonary toxicity. It also increases the risk of persistent post-surgical pain. The success of radiotherapy mainly depends on the total administered dose. This must be homogeneously delivered onto the tumour and must preserve the surrounding healthy tissue. However, several patients are hypersensitive to ionizing radiations and may develop important radiation-induced early and late side effects. The prediction of these side effects remains currently not possible and involve the decision to limit the given dose with the risk to decrease the therapeutic benefit. Therefore, one of the major challenges in radiobiology is to accurately predict normal tissue radiosensitivity. Gene expression profiling studies have provided for BC a molecular classification into clinically relevant subtypes, new tools to predict disease recurrence, response to different treatments, metastatic progression, and new insights into related oncogenic pathways. Microarray-based expression studies have demonstrated that BC is a clinically diverse and molecularly heterogeneous disease comprising subtypes with distinct gene expression patterns, associated with different prognoses and outcomes. In order to highlight molecular mechanisms induced by radiation treatment in healthy tissue surrounding cancer cells, we will adopt an Omics-based approach using high throughput technologies.

In particular, we will perform gene expression profile by cDNA Microarray to study gene and networks activated following charged particle beams. In addition, an increasing amount of data suggests that there is a reciprocal relationship by which radiations stimulate the immune system, which in turn contributes to cell death. Ionizing radiations (IR) activate both pro-and anti-proliferative signal pathways producing an imbalance in cell fate decision, regulated by several genes and factors involved in cell cycle progression, survival and/or cell death, DNA repair and inflammation. IR leads to the activation of several immunological proteins modulating the expression of numerous immune mediators which could promote cell proliferation. Thus, targeting the IR induced inflammatory signaling pathways offers the opportunity to identify potential biomarkers that could predict normal cell outcome. High-throughput methodologies, such as DNA microarray, allow to analyze mRNA expression of thousands of genes simultaneously in order to discover new genes and pathways as targets of response to charged particle beams. In order to identify and understand the molecular mechanisms involved in the response to different radiation treatment, this task will be centered on the study of radiation effects at different doses and energies, by an *in vitro* approach, using the non tumourigenic epithelial mammary MCF10A cell line as a model of healthy tissue. Therefore, we intend to carry out an experimental plan articulated as follows:

1. Non tumourigenic epithelial mammary MCF10A cells will be treated with electrons and hadron radiation beam. Vitality and morphological assays will be performed in treated and untreated cells at different time after radiation exposure;
2. Two-color microarray based gene expression analysis (Agilent Technologies) using Whole-genome cDNA microarray containing all known genes and transcripts of an entire human genome will be performed in cells treated and untreated;
3. Microarray validation experiments by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) analysis will be conducted in order to identify new candidate genes responsible of radiobiologically relevant responses.
4. Integrated metabolic pathway enrichment analysis will be performed in order to highlight similarities and differences in cell response to electrons or hadron
5. Cell network of specific biomarkers selected will be designed in order to test their role to improve radiation treatment plan based on molecular predictive models.

8.1.1.3 Inflammatory profile and role of cytokines in normal cell response(Naples/CNR-IBFM)

Radiation therapy has a significant effect on the immune system modulation through the activation of cytokine cascades. Cytokines are produced by normal, tumour cells and tumour-infiltrating lymphocytes (TIL). These molecules can greatly influence cellular radio-sensitivity and tissue complications. Thus, the analysis of cytokine signature is a topic of interest in radiobiological studies to understand the complex relationship between radiation, inflammation and immune response. the inflammatory response is believed to play a role in radiation-induced cardiovascular complications and in the perpetuation of genomic instability,

also examined by other units in the collaboration. This field opens new avenues for radiation research and therapy in order to optimize and personalize treatment. Therefore, in order to identify and understand the inflammatory response induced by radiation treatment, this task will be centered on the study of radiation effects at different doses and energies performing:

1. Analysis of cytokines and chemokines in culture mediums of treated and untreated cells with electron and hadron radiation beams by Luminex technologies (IL- 1 β , IL- 2 , IL- 4, IL- 5 , IL- 6, IL -7 , IL-8 , IL- 10 , IL-12 (p70) , IL-13 , IL-17 , G-CSF , GM-CSF , IFN- γ , MCP- 1, MIP- 1, TNF-α);
2. ELISA test of immunological related molecules such as cytokines, chemokines and growth factors in culture mediums will be conducted ;
3. Immunoblotting validation experiments will be assayed on selected protein to search for useful biomarkers in defining proper radiation treatment plan.

8.1.1.4 Genomic and sub-lethal cellular damage (Naples, RM1.Gr.coll.Sanità)

Sublethally damaged proliferating normal cells are those that pose the major threat to the genomic stability of the normal cells and the homeostatic balance of tissue and organs. Chromosome aberration induction and cellular premature senescence will be investigated in normal epithelial breast cells MCF-10 and human cardiac microvascular endothelial cells or HCMEC (as well HUVEC cells from umbilical vein as a reference) respectively, by Naples by means of beta-galactosidase activity (and possibly analysis of secreted factors) and FISH techniques for the visualization of a wide range of structural chromosome aberrations the yield of complex-type and transmissible aberrations will be compared with the simulation by Pavia. Senescence data will be compared to those collected by Naples for different ion beams along the Bragg curve in close collaboration with the PROCARDIO European initiative which focuses on heavier ions. In parallel, RM1-Gr.coll.Sanità will study acute genetic damage (and possibly delayed responses in the progeny of irradiated cells) by means of th cythocalasin B (CB)-induced block assay for detection of micronuclei, i.e. genomic fragments lagging behind at cell divisions, and delayed reproductive death. Protons, carbon and oxygen ion beams will be used at LNS, LNL and CNAO.

8.1.2 WP-1.2 Pancreas (Pavia)

The experimental activity of the Pavia Unit within the WP-1 will be dedicated to the evaluation of the effects of sublethal doses of different radiation qualities on the stroma mechanisms regulating cell adhesion and migration (risk metastasis). Increasing our knowledge of the tumour microenvironment mechanisms regulating cell migration and invasion is central to understanding tumour progression and metastasis. Indeed, the local tumour microenvironment contributes to the transformed phenotype in cancer by providing specific signals that alter the cell behaviour and

promote metastasis. The tumour stroma includes a variety of non-epithelial cells, for example inflammatory cells (lymphocytes, macrophages and mast cells) and fibroblasts, along with a series of extracellular matrix (ECM) proteins and extracellular molecules. In particular, fibroblasts have a strong association with cancer since they release growth factors, chemokines and many of the proteins laid down in the extracellular matrix that promote angiogenesis, inflammation and tumour progression/metastasis. Besides factors of the microenvironment surrounding the tumor, also the tumor treatment itself may affect the migratory behavior of tumor cells. Sublethal photon irradiation was recently suspected to increase tumor cell motility and promote locoregional recurrence of disease. Increasing cellular movement in malignant neoplasms would undermine the therapeutic intent and possibly impose a greater risk of deep locoregional tumor infiltration and metastasization. Furthermore, photon irradiation is known to modulate the expression of extracellular matrix proteins, and thus alter the motility-determining environment of tumours . In this framework, the activity of the Pavia Unit will focus on three main issues, as detailed below.

8.1.2.1 Effects of paracrine diffusible factors secreted by fibroblasts irradiated with varying radiation quality on the adhesion, proliferation, migration and invasion of pancreatic cancer cells (Pavia, Naples)

Pancreatic tumor cells will be incubated with irradiated fibroblast-conditioned media, and several parameters important for the establishment of the risk of metastases will be evaluated, such as cell proliferation and cell migration. To test whether the medium collected from irradiated fibroblasts is able to increase pancreatic cell motility Boyden chamber assays will be performed. Pancreatic tumour cells will be plated on the upper compartment and incubated with conditioned media from differently irradiated fibroblasts. Three days after the pancreatic cells that will reached the undersurface of the transwell membrane will be fixed and MGG stained, and counted using a light microscope. Comparison with media collected from sham irradiated tumour cells will allow to determine whether irradiated fibroblasts secrete factors that increase cell motility of tumour cells. Pilot tests are also foreseen to evaluate whether conditioned media from irradiated tumour cells have any influences on normal fibroblasts motility. Cell proliferation of tumour cells incubated with conditioned media will be evaluated at different times after irradiations by means of MTT assay. If possible, directly induced senescence or secretome-mediated senescence will be evaluated by beta-galactosidase assay and/or soluble factor analysis in conjunction with Naples and the effect of premature radiation-induced senescence will be assessed onpancreatic tumour cells to investigate the Senescence-associated Secretory Phenotype (SAPS)

8.1.2.2 Analysis by ELISA of the concentrations of soluble MMPs in supernatants from pancreatic tumour cells irradiated with varying radiation quality (Pavia, LNL)

Matrix metalloproteinases (MMPs) are a family of extracellular endopeptidases that selectively

degrade components of the extracellular matrix. MMPs are implicated in tumor cell invasion because they mediate the breakdown of the basal membrane. In addition, they seem to be important for the creation and maintenance of a microenvironment that facilitates tumor cell survival. The concentrations of MMPs, tissue inhibitor of metalloproteinases (TIMPs) and other soluble factors candidate to be responsible for the observed effects will be determined by ELISA in the conditioned media (also in collaboration with LNL).

8.1.2.3 Evaluation of the migration capability of pancreatic tumour cells following irradiation with varying radiation quality (Pavia, LNL)

To test whether different radiation qualities (p, C and photons) have different influences on the migration capability of pancreatic tumour cells Boyden chamber assays will be performed. Irradiated pancreatic tumour cells will be plated on the upper compartment and incubated for 3 days. Pancreatic cells that will have reached the undersurface of the transwell membrane will be fixed, MGG stained, and counted using a light microscope. Comparison with sham-irradiated tumour cells will allow to determine whether irradiation has any influences on cell motility of tumour cells. Analysis of the the concentrations of MMPs tissue inhibitor of metalloproteinases (TIMPs) and other soluble factors candidate to be responsible for the observed effects will continue (also in collaboration with LNL).

8.1.2.4 Non-targeted low dose and low dose-rate effects (LNL)

Within the WP-1 the INFN-LNL Group plans to study the response of human normal fibroblasts and endothelial cells to ^{12}C-ions at the accelerators of INFN-LNL, INFN-LNS and CNAO, in a wide energy, dose and dose-rate range close to the clinical hadrontherapy regime and to exploit the action of ^{16}O-ion beams given their possible use in cancer treatments.

In addition, to mimic the lighter fragments produced by the high energy carbon and oxygen ions, monoenergetic lighter ions, mainly low-energy protons and helium-4 ions, will be studied at the INFN-LNL CN accelerator (up to 6 MeV and 12 MeV, respectively). To investigate the action of low-energy, low-fluence light ions, present in the Bragg curve tails, in human normal cells (tissues), various irradiation geometries (including the partially-shielded one) and fluence-rate will be adopted to mimic the inhomogeneous irradiation typical of real clinical treatments. In particular, the non-targeted effects at the low-doses and low-dose-rates will be faced. Emphasis will be paid to low-dose hypersensitivity, bystander effects and genomic instability phenomena, in terms of early and late cell survival, variation of non-lethal hprt mutation frequency (in progenies of low-dose irradiated cells) and DNA damage. Cell survival will be tested by the well-established cell colony forming assay; the hprt mutation frequency, widely considered as biomarker of a variety of DNA alterations linked with an increase in the risk for (secondary) radiation-induced cancer, will be determined in the irradiated cells and in their progenies by the 6-thioguanine test (by counting the 6-thioguanine resistant colonies formed in the early and

late surviving cells after radiation exposures); the DNA damage will be evaluated by the Comet assay. Irradiation experiments will be also performed by using Co-60 gamma-rays for comparison purposes.

8.1.2.5 *Mimicking end-of-range secondary electrons by soft x-ray irradiations (Aquila)*

The experimental study of the biological effects produced by low doses of ionizing radiation is important in understanding the mechanisms that link the radiation physical absorption dose events to the damage inflicted upon biological structures. Energy deposition events by charged particles are ultimately reconcilable with highly inhomogeneous and localized energy deposition events dose with energy ranging between 10 and 100 eV. These events have been correlated with their effectiveness in giving rise to biological effects by formation of ionizations clusters the order nanometers. That are believed to be co-responsible for lesions in the DNA or other cellular structures. This mechanism is closely related to the release of energy by the ions or protons through the so-called δ rays. The goal is to study these effects using soft X-rays (photons with energy between 50 eV - 1.5 keV) produced by the laser-plasma source at the University of L'Aquila. The use of soft X-ray is functional to the aim of the proposal, i.e. unveiling the biological effects due to irradiation of the normal tissue by charged particles because they can give rise to photoelectrons in an energy range from 10 to 500 eV, which will give rise to clusters of ionization typical of the high-LET radiation off-track secondary electrons. Moreover, since the predominant physical process by which energy will be absorbed by the biological samples is by the photoelectric effect, mainly from the peripheral structures of the cell (membrane and cytoplasm) and not directly from the DNA, it will be possible to study the biological damage when the biological effect does not originate in the DNA itself, hence it well integrate with cell signalling and non-targeted studies conducted by the other unit on normal human fibroblasts

8.1.3 WP-1.3 Bone

8.1.3.1 *Interplay between normal, tumour and mesenchymal stem cell following acute exposure to charged particles (RM1.Gr.coll.Sanità, Naples)*

Experiments will be carried out after acute irradiation of osteosarcoma cells (Saos-2) with charged particles at doses of clinical relevance with the aim of investigating the activation of the TGF-β/Smad2/3 pathway, responsible for inhibition of the Mesenchymal Stem cells (MSCs) osteogenic differentiation. MSCs are non-hematopoietic multi-potent stem-like cells that are capable of differentiating into both mesenchymal and non-mesenchymal lineages. In fact, in addition to bone, cartilage, fat, and myoblasts, it has been demonstrated that MSCs are capable of differentiating into neurons and astrocytes. MSC-related researches and clinical trials have evoked exciting promise in a variety of disorders and tissue regeneration. Recently, it has been demonstrated that circulating MSCs from bone marrow or adipose tissue migrate and persist in the tumor stroma, and that the interactions between MSCs and tumor cells induce tumor

growth promotion. Studies will be performed on the radiation sensitivity of MSCs to low doses of charged particles in terms of cell growth, cell death, DNA damage/repair, chromosome damage and premature senescence (in collaboration with NA), modulation of growth-factor release and metabolic pathways. The interaction between charged particles irradiated MSCs and Hematopoietic Stem and Progenitor Cells (HSPCs) will be also investigated. The clonogenic capability of granulocyte, erythrocyte and megakaryocyte precursors will be analyzed after incubation with medium from MSCs irradiated with different doses.

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8.2 Detailed activity of WP-2: Internal irradiation therapy

Radiation protection issues in medical applications of charged particles are increasingly attracting attention and becoming of societal importance due to the widespread diffusion of both radiodiagnostic and radiotherapeutic practices. A key question in radiation protection is how the radiation risk can be assessed for: a) exposures at low dose rates and b) partial/inhomogeneous exposure of the body: These exposure modalities are often encountered in Radioisotope Therapy. The experience with radioisotope radiotherapy (where the dose is delivered to the tumor cells by continuous exposure at declining low dose-rates) shows a cytoreductive effect at doses lower than those needed with external therapy, therefore involving remarkably lower normal tissue toxicity. In particular, alpha emitters are the most potent and promising vehicles in the so-called Targeted Alpha Therapy (TAT) or in alpha radioimmunotherapy, (α-RIT), when antibodies are used as targeting vectors.

The alpha emitters presently used for therapy in nuclear medicine are listed in Table 3. The high LET value of the alpha particles is responsible for the severe damage that these particles produce in both healthy and malignant cells. However the short path of the alpha particles also means that toxic effects on adjacent healthy tissue may be minimized. The advantage of alpha emitters over gamma and beta emitters has been demonstrated in specially designed preclinical trials, suggesting it may be a treatment of choice for minimum residual diseases. Understanding the effects of alpha emitters requires consideration of spatio-temporal properties of the energy deposited by the radiation as a consequence of that exposure. In general, these spatio-temporal properties are the key for interpreting a variety of radiobiological observations, many of them being relevant to internal emitters, such as the dose-rate dependence, the effect of radiation quality and the non-linear responses (e.g., non-DNA targeted effects and adaptive responses).

At the ISS are available two facilities that allow continuous exposures to gamma rays and alpha particles at variable low dose-rates. The former will be used to mimic, in highly controlled experimental conditions, the scenarios encountered in Radioisotope Therapy with alpha emitters. Low dose/dose rate gamma rays will be used as reference. These facilities are both placed inside CO_2 cell culture incubators in order to perform radiobiological studies after exposures lasting days/weeks under physiological conditions.

Alpha emitters	Functional feature	Validated and "under study" Treatments
^{223}Ra	Calcium-like chemistry (tendency to concentrate in the bones). Metabolic targeting	Bone metastases of various cancers such as castration-resistant prostate cancer, breast or lung cancers
^{213}Bi	Cancer-specific biomolecules (targeting vectors) that will carry an attached radioisotope as payload to its destination. Useful for treatments of malignancies characterized by isolated cells or microscopic cell clusters	Leukaemia, melanoma, metastatic breast, ovarian and gastric cancer
^{211}At	Cancer-specific biomolecules (targeting vectors) that will carry an attached radioisotope as payload to its destination. Useful for treatments of malignancies characterized by isolated cells or microscopic cell clusters	Brain cancer, prostate cancer micrometastases, neuroblastoma, acute myeloid leukaemia, cancers of the ovary and intestine
^{225}Ac	Cancer-specific biomolecules (targeting vectors) that will carry an attached radioisotope as payload to its destination. Useful for treatments of malignancies characterized by isolated cells or microscopic cell clusters	Leukaemia
^{227}Th	Cancer-specific biomolecules (targeting vectors) that will carry an attached radioisotope as payload to its destination. Useful for treatments of malignancies characterized by isolated cells or microscopic cell clusters	Breast and ovarian cancer
^{224}Ra	Intra-tumoral implantation of specially prepared radioactive sources continually releasing short -lived α-emitting atoms from their surface. High-LET brachytherapy (called diffusing alpha-emitters radiation therapy (DART) useful for treatments against solid tumours	Approach validated in a series of preclinical experiments on mice. Ready to its first-in-man clinical trial

Table 3: Alpha emitters used for therapy in nuclear medicine

The ISS alpha irradiators (there are 2 available, for parallel use) can house a Cm-244 source or an Am-241 source. With the present configuration, i.e. source-sample distance of 59 mm, the average incident LETs are ~122 keV/µm and ~125 keV/µm respectively and the corresponding dose rates about 2.3 mGy/min and 84 mGy/min. These dose rates can be reduced by using a grid to decrease fluence or modifying the source-sample distance of the present configuration (although with some change in energy). Stainless steel Petri dishes with a 3-µm Mylar base have been especially designed allowing irradiation of cell monolayers on different areas up to about 25 cm2. The uniformity of the alpha-particle dose is better than ±7% with respect to the average dose. The Petri dishes can house companion dishes (of the same size of the commercial for experiments with inserts focused to cell-cell communication (Fig. 2).

The gamma irradiator can house 3 different Cs-137 sources to cover a dose-rate range from 3 µGy/h (0.05 µGy/min) to 24 mGy/h (0.4 mGy/min) A sketch of the irradiator is shown in Fig. 2. Higher dose rates (up to 0.8 Gy/min) can be obtained on site using the Gammacell Extractor (Nordion) or at the LNL, where a gamma source is also available (Co-60 gamma-beam irradiation facility; mod. GB-150, "panoramic" source; Nordion, Canada - dose-rate : 3-4 Gy/min down to 0.05 Gy/min, depending on the distance source-sample).

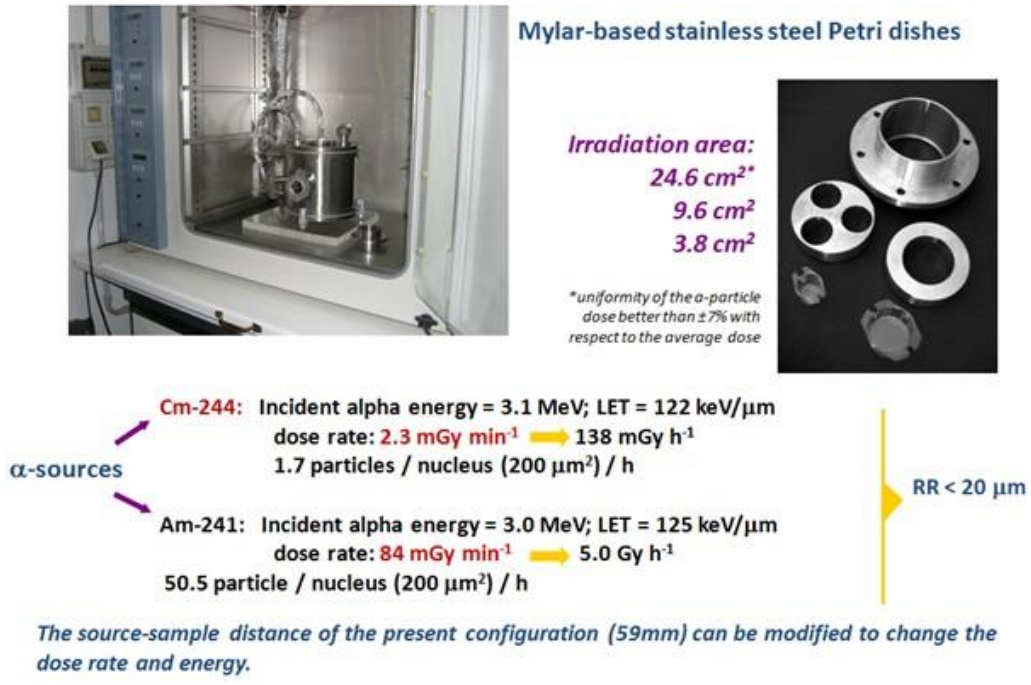

Fig.2: Characteristics of the alpha particle irradiator at the ISS

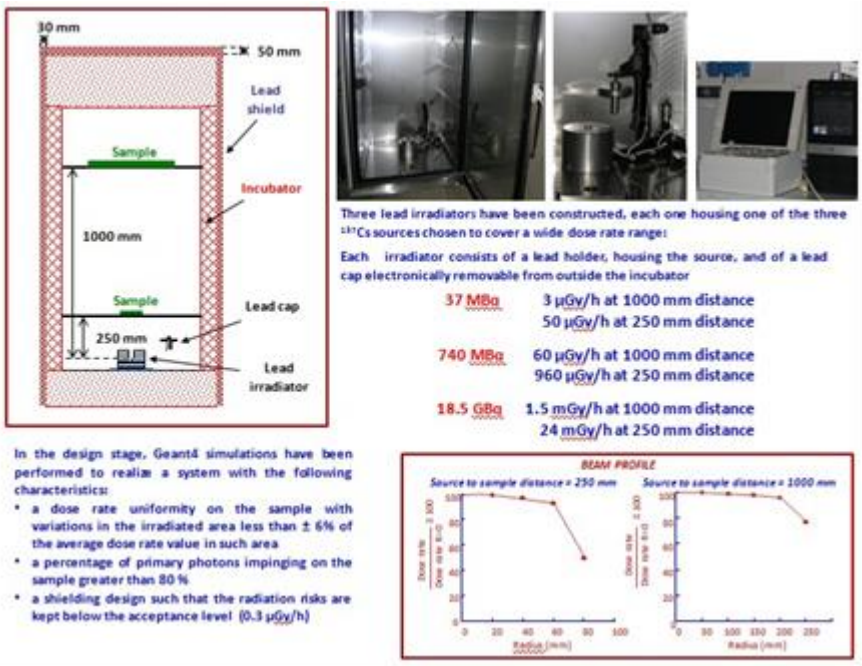

Fig.3: Characteristics of the gamma irradiation facility for low dose/dose rate in vitro biological studies at the ISS (research infrastructure funded in the framework of the EU NoE DoReMi)

8.2.1 WP-2.1: Pancreas

8.2.1.1 Modelling (Pavia)

In the framework of WP2, the Unit of Pavia will investigate the action of particles of interest for tumor targeted-therapy, basically alpha particles having energies of some MeVs. Up to now, the BIANCA model/code (see above) has been applied and validated for alpha-irradiated V79 cells, which are Chinese hamster normal fibroblasts. As a consequence, the first step of the work will consist of extending the model/code to simulate the induction of DNA cluster damage, chromosome aberrations and cell death in normal human cells (typically AG01522 human fibroblasts, which will be used also by the experimental partners, plus possibly other cells of interest, such as endothelial cells); the simulation results will be compared with experimental data taken from the experimental partners and/or from the literature. The second step will consist in modifying the code to make the particles start from the cell surface, rather than from an external source. This way, the simulations will reproduce a typical scenario for targeted therapy, in which alpha-emitting radionuclides linked to antibodies are attached to specific antigens located on the tumor cell surface; to reproduce an isotropic distribution, the particles will start from random positions with random directions. The simulation results obtained under these conditions will be compared with those obtained with external (parallel) beams from WP-1. As a final step, the model/code will be further modified to take into account the

low-dose rate conditions that characterize targeted therapy.

8.2.2 WP-2.2: Bone

8.2.2.1 Interplay between normal, tumour and mesenchymal stem cell following protracted exposure to charged particles (RM1.Gr.coll.Sanità, NA)

In the framework of WP2 activities, ISS will evaluate the radiation sensitivity of Mesenchymal Stem Cells (MSCs) in a scenario reproducing protracted radionuclide treatment. MSCs represent an important component of the bone marrow (BM) microenvironment. Treatment of cancer patients with ionizing radiation (IR) can result in adverse side effects on non-target tissue affecting the bone marrow and hematopoietic systems. Ionizing irradiation effects on the bone marrow are complex and long-lasting. An understanding of these effects remains a challenge for scientists seeking to develop safe therapies to treat tumorigenic cells without causing damage on the hematopoietic systems. Some lines of evidence indicate that MSCs, within the BM, can survive doses of IR, detrimental to the hematopoietic system. Previous study carried out at ISS on the amplification of the HSPCs purified from cord blood demonstrated the effectiveness of the Wharton's jelly-MSCs (WJ-MSCs) not only to preserve the hematopoietic stem/progenitor cells (HSPCs) but also to improve the repopulating efficacy of the amplified HSPCs, also in the absence of added cytokines and growth factors. The advantage of using WJ-MSC obtained from umbilical cord fragments is determined by their high expansion potential and by the fact that the stemness of WJ-MSC lasts longer than that of MSC from adult tissues.

MSCs from Wharton Jelly of umbilical cord are already available and characterized according to the criteria defined by the International Society of Cellular Therapy. Methods are also well established for Isolation and purification of HPSCs (CD34+); hematopoietic clonogenic assays and HPC unilineage culture and ematopoietic migration assay. Co-cultures techniques of MSCs with hematopoietic stem and progenitor cells (HSPCs) or other cell types is an approach routinely used at ISS. Osteosarcoma cells (U2OS or Saos-2) will be purchased from ATCC (Rockville, MD) for the specific use in this proposal.

At ISS solid experience exists on: methods for cytokines detection in the conditioned media by ELISA (Enzyme-Linked ImmunoSorbent Assay); surface markers analysis by flow cytometry, assays for osteogenic differentiation, cell viability and proliferation, cell cycle and apoptosis rate, early and delayed survival (representative of cell killing and lethal mutation respectively), DNA damage/repair and micronuclei induction. Genomic and chromosome damage (in collaboration with Naples) will be analyzed using the Metafer Slide Scanning Platform that allows an automatic analysis on a large number of samples (fundamental for low dose studies).
Identification of mRNAs and miRNAs that are differentially expressed after irradiation in mesenchymal stem cells will be performed by mRNA and miRNA microarray analyses (Agilent Technologies), possibly in collaboration with Naples Unit (specifically CNR-IBFM laboratories)

Experiments will be carried out after continuous irradiation of osteosarcoma cells (Saos-2) with low dose rates of alpha particles with the aim of investigating the activation of the TGF-β/Smad2/3 pathway, responsible for inhibition of the MSCs osteogenic differentiation. Studies will be performed on the radiation sensitivity of MSCs to low dose rates of alpha particles in terms of cell growth, cell death, DNA damage/repair, modulation of growth/signalling factors release and metabolic pathways. Experiments will be also carried out after gamma irradiation for RBE evaluation at low dose rate.

8.2.2.2 Non-targeted low dose and low dose-rate effects (LNL)

Within the WP2 the INFN-LNL Group plans to investigate the detrimental and beneficial action of alpha particles in normal human fibroblasts and osteosarcoma cells by using accelerated helium-4 ions with energy in interest for the targeted radionuclide therapy, at low dose (fluence) and low-dose rate. To mimic targeted radionuclide therapy cell/tissue inhomogeneous irradiation conditions, whole and partially-shielded beam irradiation will be performed. Emphasis will be paid to the non-targeted effects (bystander, genomic instability;..) and their possible effects in the risk increase of second cancer risk in healthy tissues surrounding the treated malignant tissue. Early and late cell survival (in particular in the progenies of low-dose irradiated cells) and non-lethal hprt mutation frequency in normal human fibroblasts will be determined as a function of alpha-particle dose (fluence) and energy.

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8.3 Detailed activity of WP-3: Preclinical studies (NA/CNR-IBFM)

The pathological processes of radiation injury begin immediately after radiation exposure, but the clinical and histological features may not become apparent for weeks, months, or even years after treatment according to the type of tissue. Radiation injury is commonly classified as acute, consequential, or late occurring, according to the time before appearance of symptoms. Acute (early) effects are observed during the course of treatment or within a few weeks after treatment. Late effects, sometimes called "consequential late effects", are caused by persistent acute damage. Late effects emerge months to years after radiation exposure. Carcinogenesis is

an important late effects, consequence of radiation exposure. In addition to the contribution of radiation itself, the presence of the tumour may predispose the surrounding normal tissue to injury. Tumours change their surroundings in several ways: physically distort normal tissue architecture resulting in defects that can add to damage produced by therapy; release proteolytic enzymes and factors that facilitate invasion and metastasis and cause fibrosis due to collagen deposition.

Today, molecular and cellular processes involved in radiation response are very complex and still not fully understood and need therefore to be further clarified.
Preclinical models are a crucial component of research in radiation therapy and in nuclear medicine. Small-animal irradiation systems must mimic the clinical application of radiation therapy as closely as possible. The aim is to achieve a highly conformal dose to the target volume, while sparing the surrounding healthy tissues. To support the demands on complexity of radiation delivery, one treatment planning systems, also with different requirements regarding the conventional one, has to be developed also for preclinical irradiation. Moreover, there is the need for improved small animal radiotherapy research to develop both irradiation and imaging techniques, and to advance in radiobiology research.

For many years, animal radiation studies were mostly performed using fairly crude experimental setups with radiation fields that did not conform to the target solely. The preclinical models were never thoroughly validated on animal models. The majority of animal data acquired from radiation research that are used in clinical radiotherapy were derived from broad field, single beam irradiation where the dose was roughly estimated. Similarly, little attention is commonly paid to the beam exit region where leaking backscatter can significantly influence the dose. In the animal research platforms, human radiotherapy conditions can be faithfully mimicked if radiation beams is downscaled in geometry, but also in energy. The small size of mice demand a technical precision that exceeds those typically used for human therapy.

The same dose will entail much larger dose uncertainties in a small animal structure than in a human structure, at a certain depth and in conditions of sufficient dose build-up. Therefore, exposing small animals to such beams would cause dose build-up of the order of the animal size itself. Most beam delivery systems have precision of a few millimeters. A preclinical model has to take into account the physical dimensions of a small animal. For example, a mouse lung measures only one or two centimeters and tumors within the lung are even smaller. Therefore, a precision beam of a few millimeters does not suffice for mouse radiotherapy studies, where sub-millimeter precision is required, ideally of the order of 0.1 mm or less. This generates high demands on the mechanical precision and accuracy of the setup system and of dose distribution evaluation. It is needed to device new strategies and protocols to consider correct delivered dose values and the opportune field size .

8.3.1 Monte-Carlo simulations (NA/CNR-IBFM)

To consider all necessities that a preclinical model requires, Monte Carlo calculations must give a considerable contribution to characterize the beam's dosimetry simulating the physics characteristics of the beam, the beam line and the irradiation setup.

The simulations will be performed with the Geant4 toolkit. This package allows the simulation of the passage and interaction of particles with matter. It is written in C++ language code and based on Monte Carlo methods. It contains a large variety of physics models covering the interaction of electrons, muons, hadrons and ions with matter from 250 eV up to several PeV. It provides advanced functionality for all the domains typical of detector simulation: geometry and material modeling, description of particle properties, tracking, event and run management, user interface and visualization. Geant4 Monte Carlo simulations offer accurate dose calculations and can be used to reproduce accurately models for radiation source.

In particular, the Geant4 application, named *hadrontherapy*, will be used with the last version published in the advanced examples of the official Geant4 release (9.6 version). *Hadrontherapy* was implemented to address typical needs related to the proton/ion-therapy applications. The simulation starts from the scattering system up to the diagnostic monitor chambers and the final collimators, placed just before the target. The application uses the LowEnergy package, taking into account interactions radiation-matter (fluorescence, Auger effect, etc.) in order to perform a complete and very precise calculation; all these ones hadronic processes are activated in the GEANT4 toolkit.

The main features of this example are generic proton/ion transport beam line, 3D depth dose distribution curves reconstruction in any material and kind of beam, stopping powers calculation for simple geometries, calculation of the LET (Linear Energy Transfer) for any kind of particle and material, and the calculation of 3D RBE distributions for proton and generic ion beams.

Hadrontherapy application can be used to provide a good source of information about how to build and optimize the experimental setup for preclinical applications. For example, the geometrical dimensions of the system, position of small animal, source surface distance SSD etc., are variables that can be managed with Geant4 simulations.

With the aid of Geant4 toolkit it will be simulated a simple geometric reconstruction of the area to be irradiated of the small animal. Based on an X-ray images, the physical parts of the area around the tumor will be reproduced, like the bone, the tissue, etc. This will allow to derive 3D dose maps in order to prepare the treatment on the small animal. Afterwards, simulations will be performed with the implementation of DICOM images related on digital examinations of the small animal area to be treated. DICOM images will be given as input to *hadrontherapy* application to study in more detail the damage to healthy tissue surrounding the tumoral area.

Thus, Monte Carlo simulations will allow us to understand how well we can deliver dose to targets in a small animal subject, and to estimate accurately the dose released to the Organ at Risk (OAR), main goal of this project.

Treatment planning to target small structures within animals is far from routine, and much more work is needed before complex dose distributions can be processed with high confidence. Preclinical models research, that include dosimetric and RBE studies, has an immense potential and may open new avenues of radiobiological research.

8.3.2 WP-3.2 Realization of the experimental setup (NA/CNR-IBFM)

To perform preclinical studies, it is necessary to build an appropriate experimental setup. The CATANA (Center for Advanced Nuclear Applications and hadron therapy) facility will be used for the proton therapy experiments. The main goal of CATANA is the study and the application of hadrotherapy for the treatment of shallow tumours like uveal melanomas and subfoveal macular degenerations. By means of Geant4 simulations it will be possible to obtain the optimal configuration of the small animal housing during treatment. The appropriate size of the housing and the location in which it is inserted, will be obtained by simulations. The material of small animal housing will be Polymethyl methacrylate (PMMA). Moreover, it will be realized a 3D representation of the structure using a design software. The 3D representation of the small animal housing will be the basic scheme for its realization. This will be done in collaboration with the INFN - LNS workshop in Catania. This preliminary phase is essential in order to perform proper preclinical studies.

8.3.3 WP-3.3 In vivo irradiations (Naples/CNR-IBFM)

Aim of this task is to analyze molecular mechanisms induced by hadrons in preclinical models in order to study the behavior of healthy tissue exposed to radiation. In addition, we will compare *in vivo* cell response to treatment in mouse with or without BC cell inoculation in order to analyze the influence of tumors on healthy tissue in radiation treatment cell response. Therefore, we describe the following experimental plan:

1. Mice will be divided in three groups: 3 mice inoculated with MDA-MB 231 BC cell line will be used as BC *in vivo* model and named GROUP A; 3 mice without inoculation will be grouped in GROUP B; 3 untreated mice without inoculation will be used as controls and included in GROUP C;
2. During the study GROUP A, B and C mice will be monitored for body weight and clinical signs until sacrifice to obtain also survival curve;

3. Radiation treatment will start when GROUP A tumours will be visible at X-ray imaging; In addition, X-ray imaging studies will be conducted before and at the end of radiation treatments;
4. At the early time and at the end of radiation treatment GROUP A, B and C mice will be sacrificed and tissue removed for molecular analyses as follow: radiation treated healthy tissue surrounding tumour will be collected for GROUP A; radiation treated mammary healthy tissue will be collected for GROUP B; untreated mammary healthy tissue will be collected for GROUP C and used as control sample;
5. In order to establish deep molecular differences produced by hadron treatments, a whole-genome cDNA microarray expression analysis will be performed.
6. Microarray validation experiments will be conducted by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) analysis in order to identify new candidate genes responsible of radiobiological responses.
7. Integrated metabolic pathway enrichment analysis will be performed in order to highlight similarities and differences in cell response to hadron radiation treatments in GROUP A and B compared to the control GROUP C.
8. Measurement of cytogenetic damage (e.g. chromosome aberrations and/or cellular senescence) on paraffin samples or cells grown in vitro from explants

Cell network of specific biomarkers selected will be designed in order to test their role to improve radiation treatment plan based on molecular predictive models.

8.3.4 WP.3 references

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9. Task repartition among the participants and milestones

The contribution of the participating units in the WP sand sub-WPs will be distributed as follows:

- **WP-1: NA, LNL, PV, RM-ISS, LNL, AQ**
  - **WP-1.1**: NA, PV, RM-ISS
  - **WP-1.2:** PV, NA, LNL, AQ
  - **WP-1.3**: RM-ISS, NA
- **WP-2: NA, RM-ISS, PV, LNL**
  - **WP-2.1**: PV
  - **WP-2.2**: LNL, NA, RM-ISS

- **WP-3 and sub-WPs: NA**

The milestones are schematically shown below for the foreseen duration of the research programme.

WP	*Milestones 2015: for a revised version after audit in October 2014 see INFN database*
All WPs	*Periodic internal reports in slides and/or text documents on the activity status*
WP-1 ***WP-1.1, WP-1.2, WP-1.3***	**WP-1.1** Theoretical characterization of DNA cluster damage following exposure charged particles **(June 2015)** **WP-1.1** Treatment with charged particles of non-tumorigenic breast epithelial MFC-10 cell line and Inflammatory profile and role of cytokines following exposure to charged particles **(December 2015)** **WP-1.1:** Genomic and sublethal cytogenetic damage in charged particle-irradiated (protons) cells of interest for cardiovascular complications **(December 2015)**

	WP-1.2 Evaluation of the effects of paracrine factors secreted by charged particle-irradiated normal human fibroblasts and possible evidence of SAPS **(December 2015)** **WP-1.2** Preliminary evaluation of early and late cytogenetic damage in human normal fibroblasts and endothelial cells following low-LET irradiation as reference for subsequent exposure to ion beams and thereof experimental set up **(June 2015)** **WP-1.2** Preliminary evaluation of the early and late cytogenetic damage in human normal fibroblasts and endothelial cells irradiated with charged particle broad-beams (protons and helium ions) **;** first results on the induction of Hprt mutations in human normal fibroblasts irradiated with low-LET radiation for reference against subsequent ion exposures **(December 2015)** **WP-1.2** Data on the effects of low-energy electrons generated by ultra-soft x-rays yo mimic the effects of radiation track delta rays on normal human cells **(December 2015)** **WP.1.3** : Evaluation of the interplay between healthy cells, stem cells and tumour microenvironment by analysis of the secretome, metabolic pathways and cytogenetic damage endpoints following acute exposures to charged particles. **(December 2015)**
WP-2 ***WP-2.1, WP-2.2***	**WP-2.1:** Characterization of DNA cluster damage following exposure to low-energy alpha particles **(June 2015)** **WP- 2.2** Early and late cell survival (in particular in the progenies of low-dose irradiated cells) and non-lethal hprt mutation frequency in normal human fibroblasts after chronic alpha particle exposure **(December 2015)** **W.P-2.2** Data on the signalling between normal, MSCs and tumour bone cells after protracted alpha-particle irradiation **(June 2015)**
WP3 ***WP-3.1, WP-3.2, WP-3.3***	**WP.-3.1** A special version of the hadrontherapy Geant 4 toolkit including the preclinical optimal setup with a geometric reconstruction of the area to be irradiated and small animal dose calculation (**June 2015)** **WP-3.2** Realization of the experimental setup with the construction of the small animal housing in PMMA material and ah hoc collimator on the CATANA beam line at LNS **(December 2015)** **WP-3.3:** Approval by the Governmental review committee on animal care of preclinical irradiations (**December 2015)**

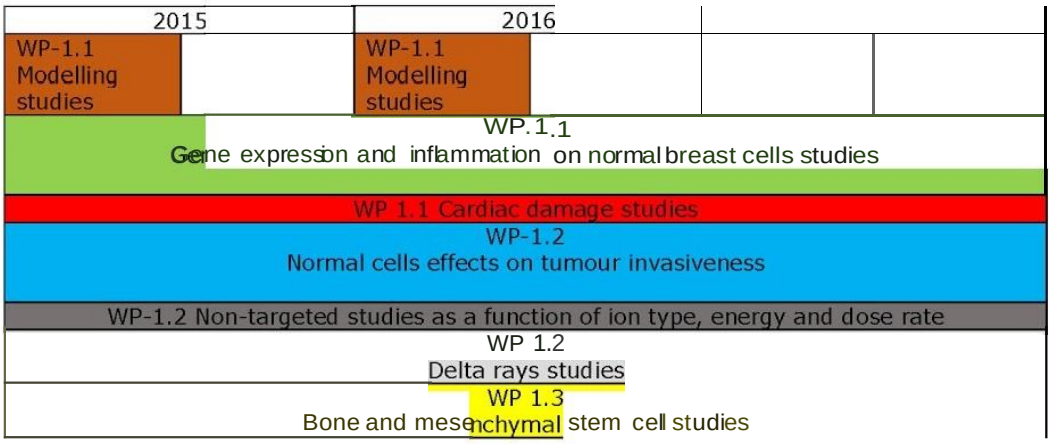
WP	*Provisional milestones 2016*
WP-1 ***WP-1.1, WP-1.2, WP-1.3***	**WP-1.1:** Modellistic implications of the higher biological effectiveness of low-energy protons (**June 2016)** **WP-1.1:** Genomic and sublethal cytogenetic damage in charged particle-irradiated (carbon ions) cells of interest for cardiovascular complications **(December 2016)** **WP-1.1** Gene expression profile of non-tumorigenic breast epithelial MFC-10 cell line following charged particle exposure and validation experiments by qRT-PCR **(December 2016)** **WP-1.2** Measurements of the release of proteins involved in extracellular matrix remodelling following p and C-ions (**December 2016**) **WP-1.2:** Preliminary data on DNA damage and the mutation rate at Hprt locus in human normal fibroblasts irradiated with protons and helium-4 ion beams **(December 2016)** **WP-1.2:** Early and late cytogenetic effects in human normal fibroblasts and endothelial cells irradiated with charged particle beams (protons, helium and carbon ions) of different energies and dose-rates at LNL, LNS (C12 ions) and CNAO (C12 ions) accelerators (**December 2016)** **WP**-1.2 Selective irradiation of cells to study the efefcts of delta rays **(December 2016)** **WP-1.3**: Study of the interaction between MSCs irradiated with charged particles (p/C-ions) and hematopoietic stem and progenitor cells (HSPCs) **(June 2016)** **WP.-1.3**: Data on cellular and molecular end point for MSCs irradiated with low doses of charged particles (p/C-ions) (**December 2016**)
WP-2 ***WP-2.1, WP-2.2***	**WP-2.1 :** First results on the early and late cell survival measurements in osteosarcoma cell line irradiated with gamma rays (at LNL Co-60 source) **(June 2016**) **WP-2.2 :** Evaluation of the early and late cell survival in human normal cells and bone cancer cells irradiated with both broad- and partially-shielded helium-4 ion beams **(December 2016)** **W.P.2.2**: Biological responses at molecular and cellular level after low dose/dose rate irradiation of MSCs with gamma rays and alpha particles and RBE evaluation for the relevant endpoints **(December 2016)**
WP-3	**WP. 3.3:** Finalizing the murine model and optimizing sub-cutaneous tumour growing **(June 2016)**

WP	*Provisional milestones 2017*
WP1 ***WP-1.1, WP-1.2, WP-1.3***	**WP-1.1:** Genomic and sublethal cytogenetic damage in charged particle-irradiated (oxygen ions) cells of interest for cardiovascular complications **(December 2017)** **WP.1.1** Integrated metabolic pathway analysis and cell network of specific selected biomarkers **(December 2017)** **WP-1.2** Acute and delayed cytogenetic response of fibroblasts and endothelial cells irradiated with oxygen ion beams of different energies and dose-rates at LNL and LNS accelerators: completion of data acquisition on evaluations of the DNA damage and mutation rate in fibroblasts irradiated with protons and helium-4 ion beams; Cell survival of human normal fibroblasts and endothelial cells irradiated with carbon ion beams at CNAO **(June 2017)** **WP-1.1** Evaluation of the early and late cell survival and the DNA damage in human normal fibroblasts and endothelial cells irradiated with charged particle beams (protons, helium, carbon and oxygen ion beams) of different energies and dose-rates at LNL and LNS accelerators (Completion) **(December 2017**) **WP-1.2** Evaluation of the migration ability of irradiated tumor cells **(December 2017)** **WP-1.3** Study of delta rays by irradiation with various modalities and energies of soft x-rays energies. **(December 2017)** **WP-1.3** RBE evaluation for carbon and oxygen ions of the relevant end points investigated in the bone system (**December 2017)**
WP2 ***WP-2.1, WP-2.2***	**WP-2.1** Implementation in the BIANCA model/code of conditions typical of targeted nuclide therapy (typically, alpha particles starting from the cell surface) **(June 2017)** **W.P. 2.2** Completion of studies on early and late cell survival in human normal fibroblasts and osteosarcoma cell lines irradiated with both broad- and partially-shielded helium-4 ion beams; completion of data collection on mutation rate in normal cells irradiated with protons and helium-4 ions **(December 2017)** **WP-2.2** Dose rate and radiation quality dependance data on the interaction between MSCs irradiated with low dose alpha particles and gamma radiation and hematopoietic stem and progenitor cells (HSPCs) **(December 2017)**

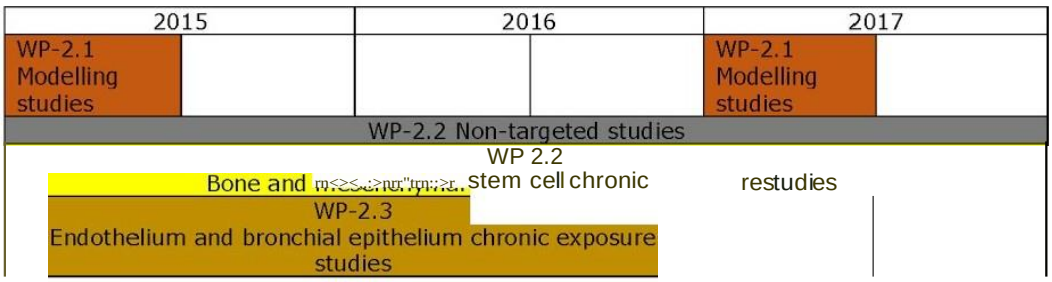
WP3 ***WP-3.1, WP-3.3***	**WP-3.1** Special version of hadrontherapy including the preclinical optimal setup and small animal dose calculation with the DICOM image implementation **(December 2017)** ***WP-3.3*** Hadrontherapy treatments and healthy tissues recovery **(June 2017)** ***WP-3.3*** Molecular and cell network analysis from in vivo experiments with ion beams **(December 2017)**

10. Temporal plan

Temporal plan for WP. : External beam therapy



Temporal plan for WP-2: Internal irradiation therapy



Temporal plan for WP-3: Pre-clinical studies