BIOphysical characterization of Helium and Oxygen ion beams for hadronTherapy

Acronym: BIOHOT

Proposed duration: Three years

Research field: Interdisciplinary (experimental and theoretical radiation biology; microdosimetry; Monte-Carlo modelization)

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Participating Units: NA, PV, RM3

ABSTRACT (max 3000 caratteri, spazi inclusi)

Abstract strutturato in: Contesto della ricerca; Descrizione degli obiettivi della proposta; Metodologia; Principale risultato atteso

Hadrontherapy (HT) presently uses protons and ^{12}C ions to treat deep-seated and radioresistant tumors due to their favorable inverse dose-depth profile and, in the case of ^{12}C ions, their higher relative biological effectiveness (RBE). However, particles of intermediate and higher charge, ^{4}He and ^{16}O ions, may improve dose localization and tumor control. A knowledge gap exists between predictions and available data. BIOHOT will study the biophysical properties of these ions through an integrated approach by in vitro measurements of clinically useful endpoints, cellular radioresponse predictive models, and microdosimetry. ^{4}He ions at clinically relevant energies (≅ 250 MeV/n) present reduced lateral scattering and range straggling, with a higher linear energy transfer (LET) compared to protons, hence better spatial selectivity and increased RBE, with still negligible fragmentation beyond the Spread-Out Bragg Peak (SOBP). This is attractive for pediatric patients, where lowering the risk of radiotherapy-induced secondary cancers is mandatory. Heavier ions such as ^{16}O, on the other hand, offer an even smaller later-scattering-generated penumbra and greater LET than ^{12}C ions across the entire target volume, making them in principle more effective at counteracting hypoxia-induced radioresistance. In addition, at the entrance (plateau), the physical dose to normal tissue could be further decreased thanks to the higher RBE, partly offsetting the bigger fragmentation tail. As the choice of the “optimal” ion depends on several factors, e.g. type of tumor and healthy tissue, target position and depth, and beam ballistics, biologically and LET-optimized SOBPs require a thorough biophysical characterization.Therefore, we shall measure normoxic and hypoxic cancer cell death and migration, DNA damage induction and repair proficiency in cells of tumors prospectively benefitting from ^{4}He and ^{16}O ions, i.e., osteosarcoma and pancreatic cancer. The former is most common among children, hence a candidate for ^{4}He-based HT; the latter is already treated with ^{12}C ions due to its radioresistance. Normal-tissue toxicity, a limiting factor for curative dose, will be evaluated using endpoints related to late sequelae, e.g. senescence and inflammation. An innovative 3D model will be also used for a better mimicry of the in vivo environment. Monte Carlo simulations coupled with microdosimetric measurements will complete the biophysical characterization of ^{4}He and ^{16}O beams in terms of LET profile, secondary productions and model-verified biological and physical parameters. Activities will be carried out at Heidelberg Ion-

Therapy center (HIT), where ^{4}He-based HT is starting, and at CNAO, where a new source is planned for 2023 as use of both ions is considered. Clinical SOBPs will be thus available at both facilities during the project. Obtained data will help evidence-based, disease-specific ion type selection and serve as facility intercomparison between HIT and CNAO.

Scientific proposal: (max 26000 characters, spaces included)

State of the art (max 4000 characters, space included)

Review critica della letteratura e degli eventuali esperimenti (in particolare se finanziati dalla CSN5 o, in generale, dall'INFN) sullo stesso tema

Radiotherapy is a cornerstone in cancer multimodal management. In particular, hailed as a promising strategy in the era of precision oncology, ion beam therapy (hadrontherapy, HT) with protons and ^{12}C ions, grows fast: 280,000 patients have been treated worldwide with protons and 42,000 with ^{12}C ions at the end of 2021 (1).Despite cost-effectiveness issues, debated clinical superiority from lack of evidence-based trials and radiobiological uncertainties, HT is deemed to expande (2) and broadening the spectrum of therapeutic ions is a new frontier (3-5). Specifically, ^{4}He and ^{16}O ions are seen as alternatives to protons and ^{12}C ions, respectively. Therapeutic quality beams with cutting-edge raster scanning technology for such ions are now available at the Heidelberg Ion-Beam therapy Center (HIT). Moreover, the addition of a new source built by INFN-LNS, AISHa (Advanced Ion Source for Hadron therapy), will soon allow pre-clinical research with ^{4}He and ^{16}O ions at CNAO (6). ^{4}He ions exhibit reduced lateral scattering, with half the distal dose fall-off, hence smaller range straggling compared to protons and a greater LET, thus a higher RBE along the SOBP (Fig.1), but smaller-than-^{12}C ion fragmentation tail. By maintaining the ballistic precision of protons, with improved dose conformation to the target and enhanced biological effectiveness void of the complications entailed by ^{12}C,

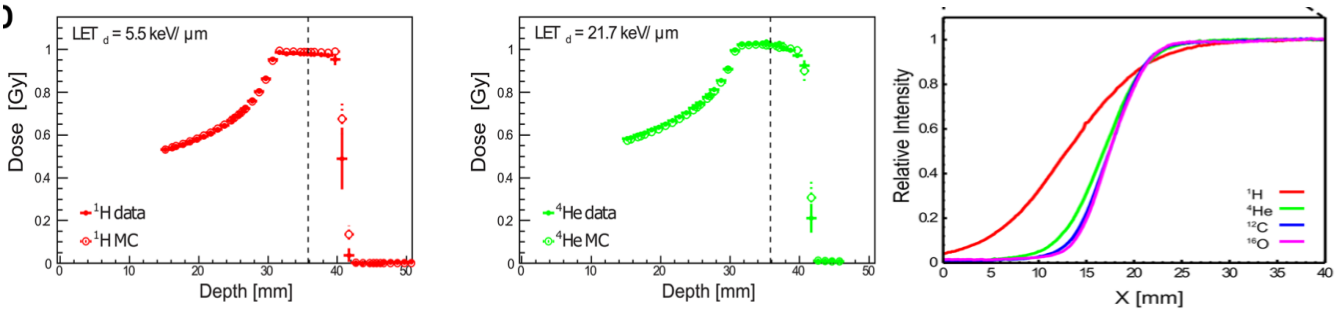


Figure 1: Comparison of depth-dose profiles for clinical proton (left panel) and ^{4}He beams (middle panel) at HIT showing the higher LET at mid SOBP and lack of significant fragmentation for the latter. The panel on the right shows a normalized intensity distribution for the 4 different beams available at HIT that reflects the much pronounced lateral scattering for protons compared to heavier ions. From ref. (15).

^{4}He ions may close the gap in achievable dose conformality and RBE between protons and ^{12}C ions. Hence, their eligibility for radioresistant pediatric tumors, where steep dose gradients close to organs at risk (OAR) may be required and secondary cancer risk must be abated (7). In fact, their therapeutic potential has been partly explored in pioneering work at the Lawrence Berkeley National Laboratory Bevatron before its discontinuation: about 2,000 patients had received ^{4}He HT, mainly for meningiomas and uveal melanoma, with satisfactory results (8,9). By rekindling the case for ^{4}He, HIT has sparked a surge of studies: Monte Carlo (MC) simulations (4,10) and analytical models (11) compared physical dosimetric characteristics with clinical outcome when coupled with biological models. Treatment planning system (TPS) optimization using dose calculation

algorithms and modeling of physical properties such as lateral scattering have also been performed (7,12-14). Along with multi-scale approaches incorporating radiobiology results in MC predictive models using the HIT therapy settings (15), this prompted a "roadmap" for ^{4}He HT (16). While no clinical experience with ^{16}O ions exists to date, in silico and experimental data on dose-depth distribution support their use (17-20). For heavier-than-^{12}C ions, the main attractiveness stems from an even higher LET distribution in tumors, hence a higher RBE, and further reduction of its dependence on the tumor oxygenation status, as measured by the oxygen enhancement ratio (OER). Hence, ^{16}O ions promise greater efficiency than ^{12}C ions at circumventing hypoxia-associated radioresistance. However, due to their greater Z, ^{16}O ions will release a comparably high LET also in the plateau, increasing normal tissue damage. Moreover, damage to OAR past the target might also increase due to a larger fragmentation tail. Indeed, the CSN5-funded experiment FOOT (FragmentatiOn Of Target) has studied projectile fragmentation of ^{16}O and ^{12}C beams (21). Yet, models concur that actual clinical advantage against hypoxia is reached with LET higher than 100 keV/µm, attained by ^{12}C ions only at the SOBP distal end (22,23). In fact, preliminary in vitro data obtained to implement prospective TPS for ^{16}O HT (17) support predictions (24) in favor of ^{16}O over ^{12}C ions for larger highly hypoxic tumor regions (Fig.2). Exhaustive radiobiological studies and improved dosimetric data with these ions are needed inputs for TPSs and biological models (13,15,25) to validate the beam quality in and across the facilities that are going to use them, e.g., HIT and CNAO.

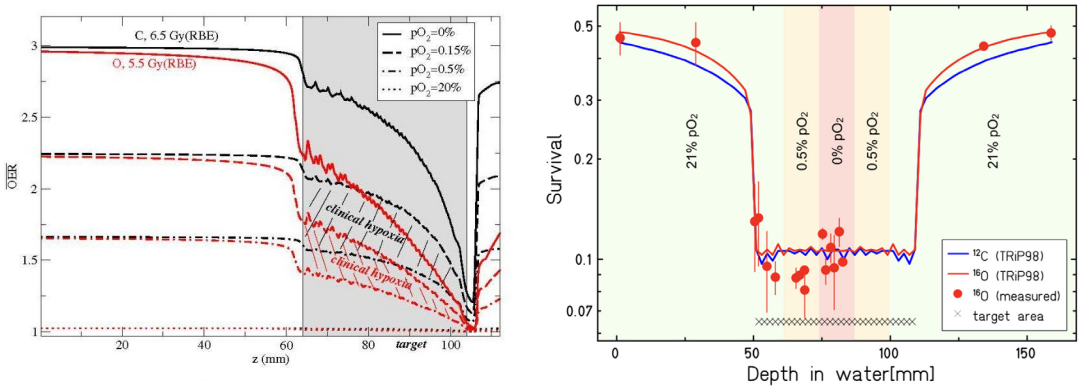


Figure 2: ^{16}O is predicted to exhibit a much lower OER than ^{12}C ions for most of the clinically relevant hypoxic region of a tumor (left panel, ref. 24).Clonogenic survival data obtained for rodent cells showing an increased effectiveness of ^{16}O vs. ^{12}C for differently oxygenated areas (ref.17)

Obiettivi (max 2000 caratteri, spazi inclusi)

Descrivere gli obiettivi e indicare chiaramente la rilevanza e l'attualità del progetto in relazione alle tematiche di interesse della CSN5

The main objective of BIOHOT is the radiobiological and physical characterization of ^{4}He and ^{16}O beams by experimental radiobiology, modeling and microdosimetry. CNAO will be the first Italian facility to introduce ^{4}He, and prospectively ^{16}O, in cancer HT thanks to a new INFN-built source. As these novel beams are suspected to exhibit intermediate radiobiological properties between protons and ^{12}C ions, understanding their actual biological effectiveness and physical properties is necessary before clinical implementation. A paucity of data on cellular response to ^{4}He and ^{16}O ion beams exist. In addition, recently developed biophysical models predictive of damage imparted by these ions need experimental validation. Finally, simulations and measurements of physical quantities (dose distribution, scattering, LET (including the contributions of the produced secondaries), are necessary to fully assess the clinical potential of these ions. Specifically:

- In vitro radiobiological measurements of osteosarcoma and pancreatic cancer cell death and repair kinetics will allow to experimentally evaluate the RBE across clinical-quality ^{4}He and ^{16}O SOBPs ; irradiation of endothelial and fibroblast cells will provide insights into normal-tissue damage for adverse reactions;
- Theoretical predictions of survival curve parameters and DNA damage by BIANCA biophysical model will be performed and validated by comparison with the experimental. Together with MC simulations of dose- and track-averaged LET, and equivalent quantities derived from experimental microdosimetric spectra (including projectile and target fragmentation), this will assist biologically- and LET-optimized treatment planning for ^{4}He and for similar multi-ion strategies with ^{16}O
- Our data will also serve as a facility intercomparison between HIT and CNAO. Our results may thus pave the way for future cancer and normal tissue type response-driven choices of the best-suited ion type for specific malignancies at CNAO.

Metodologia della ricerca (max 6000 caratteri, spazi inclusi)

Descrivere le metodologie da adottare nella ricerca, mettendone in luce l'originalità, aspetti innovativi del progetto, la fattibilità e sostenibilità del progetto, le risorse umane e strumentali disponibili, esplicitate per tutta la durata del progetto.

1. Experimental radiobiology

An array of methodologies, including state-of-the art immunochemistry and immunolabeling techniques, will allow to quantify ^{4}He and ^{16}O ion effectiveness at causing cancer cell death as well as normal cell damage: the former relates to tumor local control, the latter to healthy tissue complications. An innovative 3D model will mimic the in vivo scenario.

1.1 Effects of ^{4}He and ^{16}O ions on tumor cells

1.1.1 RBE

To determine RBE for cell killing, clonogenic dose-response curves will be obtained for osteosarcoma Saos-2 and pancreatic PANC-1 cells. Survival fractions (SFs) are best fitted by the linear-quadratic model SF = exp (-αD-βD^2): the ratio α/β predicts in vivo cancer radioresponse (26). Interestingly, the few available data point to a higher ^{4}He RBE for radioresistant cancers (α/β<10) than for radiosensitive ones (α/β>10) at 20-30 keV/µm (16), the LET values expected along a ^{4}He SOBP (Fig.1). Knowledge of ^{16}O RBE is also poor (27,28). Cells will be irradiated along a typical 6-cm ^{4}He and ^{16}O SOBP (maximum energies 230 Mev/n and 400 MeV/n, respectively; doses 0-6 Gy) and assessed for colony-forming ability (29). For ^{16}O ions, RBE will be measured also under hypoxia using an original portable chamber (30), where cells are gassed for ~4 hr and then irradiated, maintaining hypoxic status. Therapeutic 6 MV X-rays from a Clinac 2100 linear accelerator (Varian Medical Systems, USA) will act as reference radiation.

1.1.2 Apoptosis

Charged particles effectively induce apoptosis (31,32), with a propensity of light ions to induce this death pathway (33). Apoptosis will be quantified by Annexin V assay (Figs 3 and 4), using a state-of-the art high-sensitivity and throughput AttuneTM NxT acoustic-focusing flow cytometer (ThermoFisher Scientific).

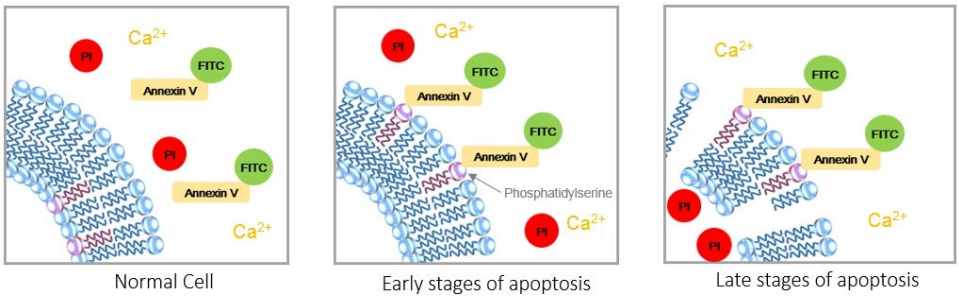


Figure 3: Annexin V/Propidium iodine (PI) assay for detection of apoptosis, a genetically controlled death programme by which multicellular organisms eliminate potentially harmful cells. In radiotherapy, it eradicates cancer cells not undergoing clonogenic death. Annexin V is a protein with high specificity for phosphatidylserine. Externalization of cell membrane phosphatidylserine occurs upstream of the cascade of biomolecular execution of the apoptotic program, of which it is an early and reliable marker.

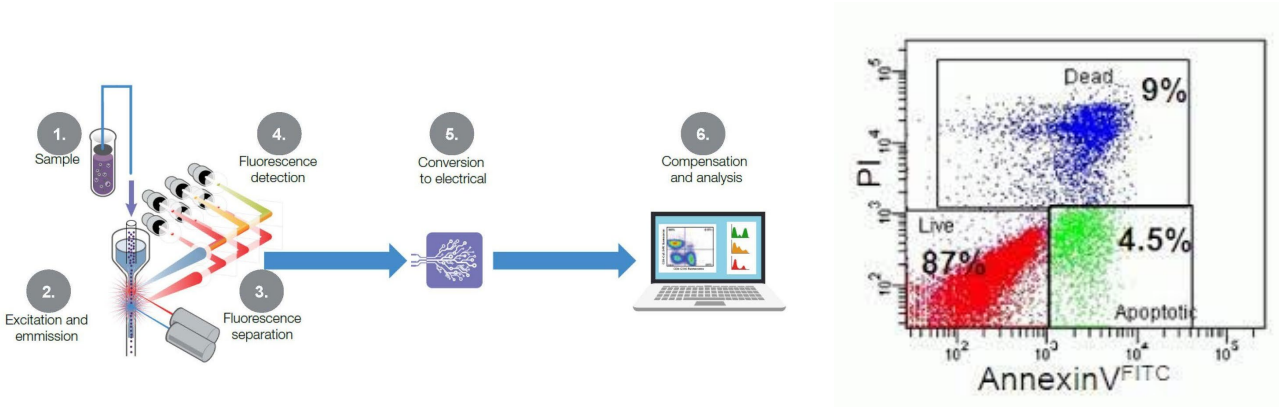


Figure 4*:* Principle of flow cytometric analysis of fluorescence-labeled cells (left); representative quantification of normal (live), apoptotic and necrotic (dead) cell subpopulations (right). In the latter panel, apoptotic cells are positive only to Annexin V conjugated with fluorescein isothiocyanate (FITC), contrary to necrotic ones that also fail to exclude PI (right).

1.1.3 Repair

Diminished cancer cell repair capacity is expected from the higher LET of ^{4}He compared to protons and of ^{16}O compared to ^{12}C (34). The immunofluorescence foci assay detects proteins, such as γ-H2AX and 53BP1, recruited to the sites of radiation-induced DNA Double-Strand Breaks (DSBs) and allows repair kinetics study by fluorescence microscopy (35-37). An automated workstation consisting of a fluorescence microscope remotely controlled by dedicated software (MetaSystems, Germany) will be used (Fig.5).

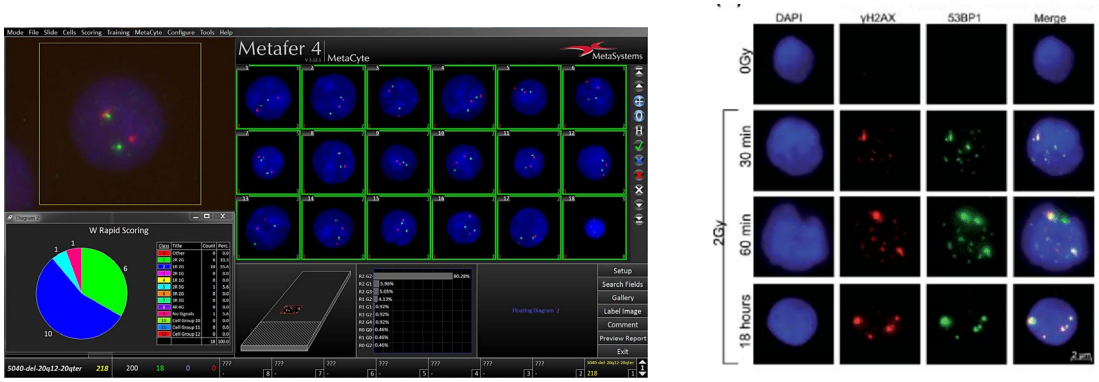


Figure 5: Representative images of the processing software used for automated foci analysis and counting (left) and of radiation-induced foci co-localization in cell nuclei (right). Image stacks will be taken at 0.5 μm-interval to minimize errors due to foci focal plane distribution. Co-localized γ-H2AX and 53BP1 foci will be automatically counted for each cell.

1.1.4 Migration

Low-LET radiation may promote tumor invasion but high-LET data are elusive (38). Hence, the study of the influence of ^{4}He and ^{16}O ions on the modulation of migration is important, especially in pancreatic cancers (39). Migratory capability of surviving irradiated cells will be assessed by two methods: the scratch assay or the wound healing assay to evaluate single and collective cell migration (Fig.6). In parallel, migration capability will be evaluated by the Boyden chamber assay for quantification of single cell migration (Fig.7).

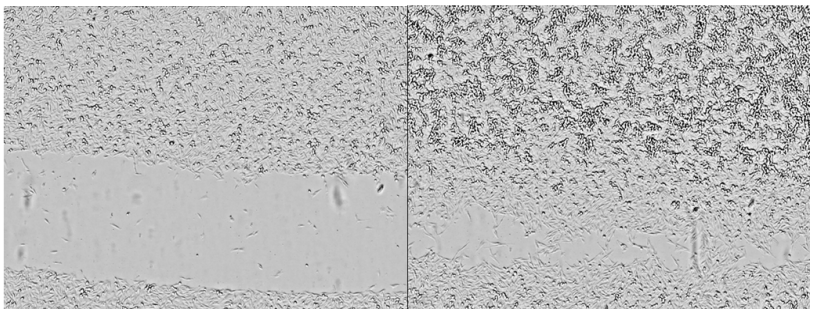


Figure 6: Scratch Assay of mucosal melanoma cells: on the left a time 0 and on the right 48 hours after the formation of the wound (400X).Briefly, a scratch is performed on a cell monolayer and the cell free area measured at different time points until wound closure

Figure 7*:* Schematic representation of the Boyden assay principle.Two medium-filled compartments are separated by a microporous membrane. Irradiated cells are placed in the upper compartment and allowed to migrate through the membrane pores into the serum-rich lower compartment. After 48 h, the membrane is fixed and stained, and the number of cells that have migrated is determined

1.2 Effects of ^{4}He and ^{16}O ions on normal cells

Healthy tissue damage, the main limiting factor for curative dose, will be measured in MRC-5 fibroblasts and HUVEC endothelial cells. Heritable chromosome aberrations (CA), which enhance secondary cancer risk (40), will be studied by mFISH karyotyping. Repair capacity will be assessed by the foci assay (1.1.3) measuring residual damage as it correlates with normal tissue radiotoxicity (41). Premature senescence (PS), oxidative stress and inflammatory responses, on which no data exist for clinical ^{4}He and ^{16}O beams, reflect endothelium disruption, hence development of cardiovascular pathologies and tumor aggressivity (42-44).

1.2.1 mFISH

mFISH (45,46) techniques allow to identify high-LET biomarkers and to analyze CA dose- and radiation quality-dependence. The “C-ratio” (complex/simple interchanges) and the “F-ratio” (interchromosomal/intrachromosomal exchanges) are radiation signatures. Complex exchanges (CA involving at least three breaks and two chromosomes (47)), and the F-ratio are expected to increase after high-LET radiation (48,49). Since tumor cells have more than 46 chromosomes, mFISH will be carried out in MRC-5 normal cells, providing a necessary input for modeling. Cells will be seeded 24-48h before exposure to 2 and 4 Gy of ^{4}He and ^{16}O at the SOBP entrance. Calyculin-A G_2-condensed chromosome spreads will be processed and at least 100

spreads per sample analyzed.

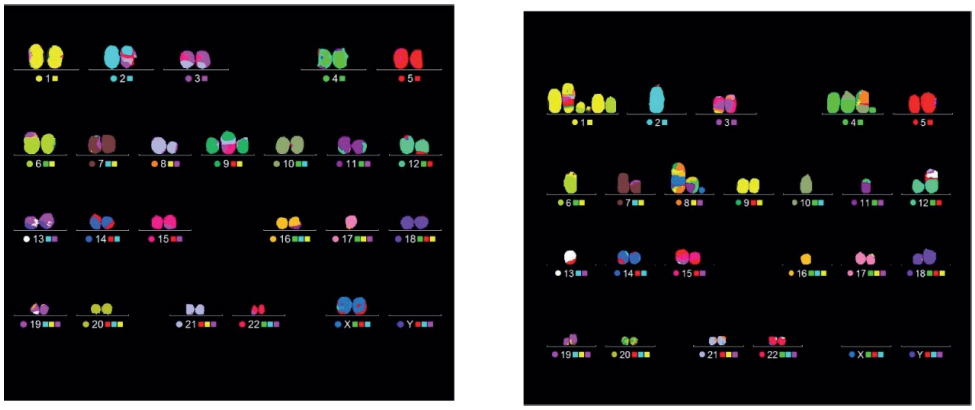


Figure 8: Images depict karyotypes from samples irradiated at the CNAO beamline with 4 Gy of protons and show typical simple exchange between chromosomes 2 and 17 from a cell exposed at the entrance (left panel); the right panel shows several complex rearrangements due to high-LET alpha-particles (from ref. 50).

1.2.2 Senescence, oxidative stress and inflammation

PS will be studied by histochemical β-Gal assay (51) in HUVEC cells exposed at the ion beam entrance (0.5, 2 and 4 Gy). A peculiar array of secreted factors, collectively known as SASP (Senescence-Associated Secretory Phenotype) accompanies PS, affecting both normal and cancer cells (52). HUVEC secretome will be studied by flow cytometric analysis of soluble cytokines (interleukin-6) and the novel senescence-specific surface marker SCAMP-4, a key SASP modulator (53). Persistence of reactive oxygen species (ROS)-related oxidative stress and inflammatory responses will be studied by flow cytometry-based MitoSox assay (directed to mitochondrial ROS) and detection of inflammation markers (PD-L1 and HLA-G1). Generalized oxidative stress will be evaluated by fluorescence microscopy analysis of fluorogenic probes activated upon oxidation by ROS.

1.3 3D system

Biologic scaffold are attractive organ engineering strategies (54): by whole organ decellularization, all cellular components are removed from porcine liver, generating an acellular 3D unit that maintains native organ-specific structures in terms of hierarchical anatomical geometry and bioactive molecules (55). Such scaffolds (Fig.9) retain the 3D tissue architecture. At CNAO, this system was used for the first time with photons and ^{12}C ions (56) and will allow examination of morphological alterations induced by different types of radiation. Apoptosis will be measured by TUNEL assay: slices from scaffolds formalin-fixed and formalin-embedded at different times post irradiation (up to 4 weeks) will be processed, the number of apoptotic cells in each section counted by microscope observation and correlated to radiation type and dose.

Figure 9: Hepatic scaffold. The right image is obtained by the observation of the scaffold under the phase-contrast microscope at a 100X magnification.Compared to 2D cultures, bioscaffolds facilitate cell adhesion, tissue integration, remodeling and differentiation. They also promote cell viability, proliferation, recreating an environment suitable for the diffusion of oxygen and cell growth factors. Co-cultures (tumor + endothelial + fibroblasts) can also be prepared.

2. Methodology: modeling

Radiobiology simulations will be carried out by the BIANCA model/MC code developed in Pavia, which predicts cell death and CA.assuming that radiation induces DNA "Critical Lesions" (CLs) that produce CA, some of which lead to cell death. Following production of a database describing the survival of V79 cells (as a reference) by monochromatic ion beams, BIANCA predicts survival curves for any other cell type. An analogous database was produced for blood lymphocyte CA, which are indicators of late normal tissue damage including secondary tumors (40). Such databases can be read by a transport code or TPS. Recently, interfacing BIANCA with the FLUKA code lead to good agreement with in vitro and in vivo data for proton and C-ion therapeutic beams (57,58); RBE re-calculations for C-ion patients were in line with the two models used in clinics, LEM-I and MKM (59). As for LEM and MKM, BIANCA can evaluate normal tissue damage by CA; furthermore, BIANCA is suitable for the ion beams currently used in clinics. Additionally, comparisons against clinical (MKM,60) and translational (UNIVERSE,61) biological models will be performed in BIOHOT. Extension of the UNIVERSE model will be carried out to take into account hypoxia. MC simulations of track- and dose-averaged LET will be performed by algorithms already developed (62,63) and publicly released in the *Hadrontherapy* Geant4 application (64). The MC will adapt to simulate the detectors and the beams that will be used for the experimental yd and yf reconstruction (see microdosimetry). Biological damage evaluation will be then performed taking into account the LET estimation and applying the MC algorithms already implemented inside *Hadrontherapy* application.

3.Methodology: Microdosimetry

Biological and clinical properties of positively charged particles derive from the physical properties of their interaction with the target volume. When ions penetrate the biological matter, they lose part of their energy, they slow down and the ionization density along the particle track increases. Because of the increased ionization density, also the amount and complexity of damage to critical cellular structures increases. The RBE depends on several variables, which include radiation type, charge and velocity of the charged particles, dose, fractionation, cell type, biologic endpoint, etc. The RBE of different radiation qualities is strictly related to the amount of energy deposited along a track (the so-called LET). In this context microdosimetry, measuring the random processes of energy deposition in micrometric sites, offers valuable tools to evaluate the LET and consequently the RBE. In the framework of BIOHOT project MicroPlus probe microdosimeter will be adopted to this scope. The MicroPlus probe is an array of 3D right parallelepiped shape sensitive volumes (diodes) with area 30 um x 30 um,

fabricated using silicon on insulator wafers with an active layer of 10 um thickness. The LET distribution at different depths along the Bragg peak adopted for the radiobiological irradiation will be estimated. The detector will be put at different positions varying the depths by inserting calibrated PMMA layers in front of the microdosimeter. The corresponding mean chord length of the active region will be determined with specific MC simulation. Experimental LET will be then compared with the specific algorithms implemented in the Hadrontherapy Geant4 application.

Organizzazione del Progetto (max 6000 caratteri, spazi inclusi)

Eventuale divisione in workpackage, con indicazione dei responsabili e sedi coinvolte

- Breve descrizione delle attività previste (per sede o WP)

Cronoprogramma del progetto nel suo insieme

- Milestone per l'intero progetto. Il numero e la distribuzione temporale delle milestone deve essere tale da consentire ai revisori di verificare lo stato di avanzamento del progetto

BIOHOT is organized in the following Activities: Experimental Radiobiology, Modeling , Experimental Microdosimetry.

NA UNIT

The activities of the NA unit will predominantly focus on Experimental Radiobiology. Specifically, NA unit will perform measurements with therapeutic quality ^{4}He and ^{16}O ion beams at HIT and CNAO on the determination of the RBE for cancer cell killing by clonogenic assay (see Research Methodology, section 1.1.1) in collaboration with RM3 Unit, on apoptotic cancer cell death (1.1.2), on repair proficiency of cancer and normal cell lines using the foci assay (1.1.3), and responses related to senescence, oxidative stress and inflammation in normal cells (1.2.2). For RBE determination clonogenic dose-response curves will be obtained also with the 6 MV clinical photon at Pavia, Foandazione Maugeri. Both RBE determination and DNA repair activities will be carried out in close connection with RM3 Unit as to provide more reliable measurements and statistically robust data. This is necessary since the clonogenic assay requires several replicate experiments to accurately characterize the radioresponse and inter-laboratory validation serves as data quality assurance. A similar rationale applies to the study of radiation-induced repair since the foci assay methodology may be sensitive to immunolabeling quality and intercomparisons will strengthen data reliability. As regards the normal cell response, all analyses will be performed on live cells at the Radiation Biophysics Laboratory (NA) following irradiation at the various facilities. In fact, the endpoints indicated in 1.2.2 (senescence, oxidative stress and inflammation) are relevant in terms of their onset, persistence and perpetuation among the descendants of the exposed cell populations. In addition, thanks to the participation of INFN-LNS researchers, MC modeling and microdosimetry experiments that are closely related to the experimental radiobiology tasks will also be carried out.

PV UNIT

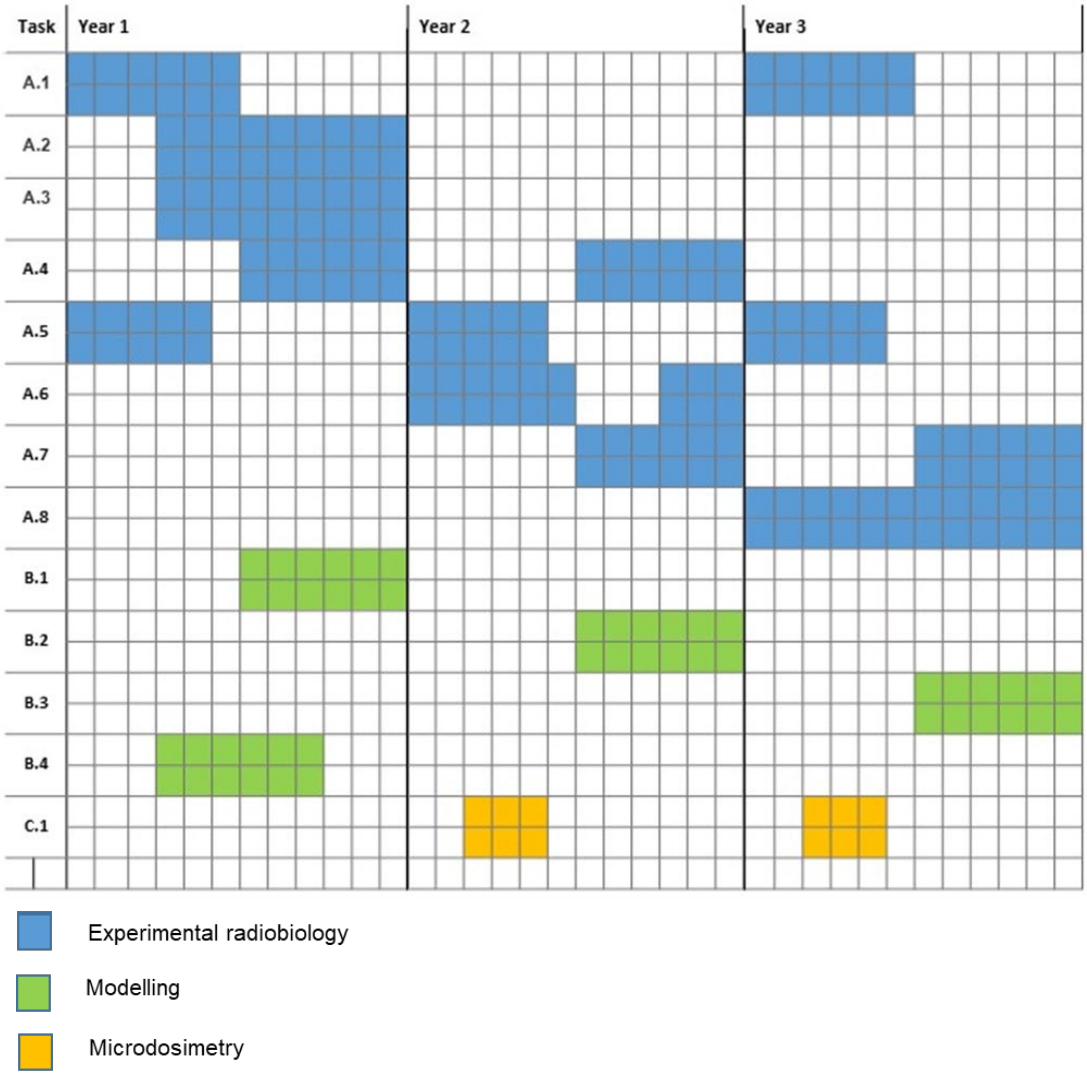
The activities of PV Unit will span both Modeling and Experimental Radiobiology, the latter being carried out by the CNAO research group. The PV modeling group will extend the BIANCA model to predict cell death by ^{4}He and ^{16}O beams for the tumor cell line used in the experiments. This will require constructing an *ad hoc* radiobiological database as follows: the photon experimental survival curve for tumour cells will be used to derive the model parameters to simulate survival curves for different monochromatic ion beams; linear-quadratic fitting of each curve will then allow constructing a database that will be read by a transport code like FLUKA. This will make it possible to predict tumor cell survival and RBE along the considered ion beams. Such predictions will be compared with the survival data produced by the NA and RM3 units, to validate BIANCA for pancreas tumor cell killing by the ion beams. Concerning normal tissue damage, BIANCA already allows predicting ion RBE values for lymphocyte CA; these predictions will be compared with the data produced by RM3 Unit using mFISH (Research Methodology Section, 1.2.1). Furthermore, an *ad hoc*

database will be constructed to predict aberration yields in the used normal cell lines: the experimental photon dose-response curve will be used to derive the model parameters to simulate the aberration dose-response by monochromatic ion beams, and linear-quadratic fitting of these curves will allow constructing an aberration database that will be read by a transport code like FLUKA. Finally, the yields of the model “critical lesions” will be compared with those of DNA damage foci data obtained by NA and RM3 units, to evaluate whether such foci can be identified as critical lesions. The PV Unit will use the innovative 3D model as described in the Research methodology section 1.3. Bioscaffolds prepared by UNIPV/San Matteo colleagues will be overnight incubated in fresh growth medium in order to recondition scaffolds, and then they will be populated through the seeding of the normal and/or tumor cells. Three days after repopulation, scaffolds will then be irradiated 6 MV photons or ^{4}He and ^{16}O ions (2Gy and 4Gy); afterwards, they will be fixed one per dose every 7 days for 4 weeks. Histological sections will be stained using different techniques including hematoxylin and eosin, periodic acid-Schiff, masson, alcian blue and picrosirius red. Apoptosis induction will be assessed by means of TUNEL assay.

RM3 UNIT

The role of the RM3 Unit will be mainly devoted to the radiobiological characterization of the novel beams in the selected normal and tumor cell lines related to methodologies described in Sections 1.1.1, 1.1.3 and 1.2.1. In particular, its main focus will be on the measurements of CA using the 24-color mFISH on which it has a consolidated track record (65-68).Studies on these cells will provide information on the effects of the tested ions on normal tissue damage, in terms of the induction and type of chromosome aberrations to be compared with senescence endpoints by NA Unit and provide data for the modelization needed by PV Unit for modeling work. Induction and kinetics of DNA DSB of repair will be assessed through the quantification of radiation-induced foci following immunostaining with both γ-H2AX and 53BP1 antibodies. Concerning Saos-2 osteosarcoma and PANC-1 cells, the radioresponse will be evaluated through clonogenic dose-response curves (in collaboration with NA Unit). Experiments with clinical photons will be carried out in both normal and tumor cells for RBE assessment.

Below is the project GANTT and relative activities (tasks) explained:



A.1: Irradiation of cancer cell lines and MRC5 cells by clinical 6 MV photons
A.2: Optimization of protocol for mFISH karyotyping of MRC-5 normal cells
A.3: Optimization of protocol for flow cytometry/fluorescence microscopy analysis of senescence/ROS/inflammation in HUVEc normal cells
A.4: Irradiations of cancer cells with therapeutic ^{4}He beams
A.5: Irradiation of 3D bioscaffolds with clinical photons, therapeutic ^{4}He and ^{16}O ion beams
A.6: Irradiation of normal cells with therapeutic ^{4}He ions
A.7: Irradiation of cancer cells with therapeutic ^{16}O ions
A.8: Irradiation of normal cells with therapeutic ^{16}O ions
B.1: Prediction of tumor cell death and chromosome aberrations in healthy cells following photon irradiation (same cells used in the experiments)
B.2: Prediction of tumor cell death and chromosome aberrations in healthy cells following ^{4}He ion-irradiation (same cells used in the experiments)
B.3: Prediction of tumor cell death and chromosome aberrations in healthy cells following ^{16}O ion-irradiation (same cell types used in the experiments)
B.4: Estimation of the LET-d, LET-t and RBE for the healthy and tumor cells for ^{4}He and ^{16}O irradiations
C.1 Microdosimetry measurements at CNAO and HIT

Milestones:

First year
M1.1 Clonogenic data for cancer cell lines Saos-2 and PANC-1 from photon irradiation (30/06/2023)
M1.2 Preliminary clonogenic data from ^{4}He irradiation of cancer cell lines Saos-2 and PANC-1: initial estimation of RBE for cell killing (31/12/2023)
M1.3 Preliminary assessment of DNA damage by foci assay and apoptosis in cancer cell lines following ^{4}He irradiations (31/12/2023)
M2.1 prediction of tumor cell death and chromosome aberrations in healthy cells following photon irradiation (same cells used in the experiments)
M2.2 LET-d, LET-t and RBE estimations for the two cancer cell lines considered and for the two beams

Second year
M1.1 Migration data in cancer cells on 2D system following ^{4}He irradiation (30/06/2024)
M1.2 Preliminary data on chromosome aberrations by mFISH, repair kinetics by foci and senescence/ROS inflammation in normal cells following ^{4}He irradiation (30/06/2024)
M1.3 Effectiveness of therapeutic ^{4}He ion beam irradiation at damaging cancer and normal cells (31/12/2024)
M1.4 Preliminary data on clonogenic survival, apoptosis and DNA damage in cancer cell lines following irradiation with therapeutic ^{16}O beams (31/12/2024)
M1.5 Histological analysis of bioscaffolds populated with cancer and normal cells irradiated with ^{4}He beams (31/12/2024)
M2.1 prediction of tumor cell death and chromosome aberrations in healthy cells following ^{4}He ions (same cells used in the experiments) (31/12/2024)
M3.1 Microdosimetric measurements at the HIT facility and comparison with Monte Carlo data (31/12/2024)

Third year:
M1.1 Preliminary data on chromosome aberrations by mFISH, repair kinetics by foci and senescence/ROS inflammation in normal cells following ^{16}O irradiation (30/06/2025)
M1.2 Effectiveness of therapeutic ^{4}O ion beam irradiation at damaging cancer and normal cells (31/12/2025)
M1.3 Histological analysis of bioscaffolds populated with cancer and normal cells irradiated with ^{16}O beams(31/12/2025)
M2.1 Prediction of tumor cell death and chromosome aberrations in healthy cells following ^{16}O ions (same cell types used in the experiments) (31/12/2025)
M3.1 Microdosimetric measurements at the CNAO facility and comparison with Monte Carlo data (31/12/2025)

Descrizione del gruppo di ricerca (max 3000 caratteri, spazi inclusi)

Descrivere ruoli e compiti delle unità partecipanti, le competenze di ogni gruppo, le infrastrutture da utilizzare, le collaborazioni internazionali

NA UNIT

The Radiation Biophysics group of NA unit has a long-standing tradition in the field of experimental radiobiology, which has unfolded over the years consistently within the interdisciplinary activities funded by INFN-CSN5, as well as within MUR-funded projects and at the international level. Indeed, it represents one of the very few groups with such expertise in a university physics department. Its main research interests have traditionally focussed on the radiobiology of charged particles, especially in the field of hadrontherapy. It was indeed among the groups participating in the preliminary radiobiological validation of CNAO prior to its clinical approval. Particle radiation effects on both cancer and normal cells have been examined with traditional assays, such as the aforementioned clonogenic test, but more extensively with cytogenetic analysis of DNA damage by the use of fluorescence hybridization techniques (e.g.,FISH) and endpoints including micronuclei and foci. It possesses all infrastructure needed to sustain the project and has recently acquired a state-of-the-art flow cytometry apparatus, which has allowed the unit to expand its expertise in more modern applications of biophysical techniques and which will be instrumental for BIOHOT activities. Associated for this project with NA unit are researchers from INFN-LNS, whose competence is on MC modeling by Geant4 of hadrontherapy beams for radiobiological validation of experimentally derived parameters and experimental microdosimetry. Their leading role in these fields are widely recognized at both the national and international level. Expected FTE: around 3.

PV Unit

The computational radiobiology group of Pavia has a long experience in modeling/simulating radiation-induced biological damage. Specifically, the group has developed a biophysical model called BIANCA, which predicts cell death and chromosome aberrations in different cells exposed to different radiation types. While up to now, the model has been validated for photons, protons and C-ions, in BIOHOT it will be extended/validated for He- and O-ions, for the same cell types studied by the experimental partners. associated to this unit is the CNAO Foundation, which is also a Center of Research and Development, whose activities have the objective of providing continual improvements in the capacity to cure. CNAO offers a rare unique possibility of clinical and pre-clinical research in a multidisciplinary environment with a broad range of competences. CNAO offers the opportunity to external researchers to take advantage of the biology laboratory for sample preparation and processing. Within the CNAO expansion plan, this will include rooms dedicated to microscopy, cell handling, cytology/histology and small animals preparation. Expected FTE are 1.6.

RM3 Unit

The expertise of the RM3 is mainly devoted to the implementation of molecular cytogenetics techniques for the analysis of ionizing radiation-induced DNA damage. Techniques such as immunofluorescent evaluation of radiation-induced foci by means of staining directed against gamma-H2AX and 53PB1 antibodies and clonogenic assay are routinely in use in our laboratory. All the equipment needed are available at the Lab of Genetics, Dept Science, University Roma Tre.

The Metafer platform by Metasystems (available at ISS TISP) allows the analysis of radioinduced molecular damage in terms of CA and DSBs. Specific modules allow the analysis of chromosomes in mFISH and the foci. Expected FTE are 1.6

All participating units have had and continue to have collaborations with multiple international institutions and research groups. As concerns BIOHOT, the relevant ones are listed in the next section.

Coinvolgimenti esterni alla CSN5 (max 1500 caratteri, spazi inclusi)

Indicare eventuale coinvolgimento di:
- altre Commissioni Scientifiche INFN;
- istituzioni esterne e laboratori di ricerca nazionali e/o internazionali;
- industrie, soggetti pubblici o privati che cofinanziano la ricerca;

Nel caso di ente pubblico o privato esterno coinvolto nella ricerca, indicare la tipologia con breve descrizione della "background experience" di ogni Ente partecipante, e esplicitando il ruolo all'interno del progetto

CNAO is one of the four centers in Europe, and six worldwide, offering treatment of tumors with both protons and ^{12}C ions. The role of CNAO is at the core of BIOHOT as it will provide the ^{4}He and ^{16}O ion beams (produced by an INFN-built new source) that the project aims at biophysically characterizing for future clinical applications. In addition, its researchers will provide an innovative 3D model that promises advances in the understanding of radiation-induced cellular effects at a level closer to the in vivo scenario.
Fondazione Maugeri (Radiotherapy Service) will make available a 6 MV linear accelerator for the RBE determination. People participating are M. Liotta (Medical Physicist), P. Tabarelli De Fatis (Medical Physicist), G.B. Ivaldi (Head of Radiotherapy Unit)
University of Pavia/S. Matteo Group will make available the expertise and the instrumentation at the Experimental Surgery Laboratories that is needed to obtain the decellularized porcine liver scaffolds. The participating team is highly multidisciplinary and comprises: L. Cobianchi, S. Croce, A. Peloso, F. Dal Mas , M.A. Avanzini

HIT is a synchrotron based-facility which has treated more than 7000 patients with raster-scanning protons and ^{12}C ions since 2009. Recently, patient treatment with ^{4}He ions beams started in 2021 while ^{16}O ions are available for biophysical investigations. The BioPT (BioPhysics in Particle Therapy groups, 15 members: scientists, postdocs and PhD students) will support the planning and the performing of biophysical experiments with these ions at HIT within the BIOHOT project.

Queen's University Belfast (QUB, Belfast, UK) has long standing experience in the field of low- and high-LET radiobiology and has previously participated in several successful experimental campaigns at the INFN-LNS CATANA beam line, in collaboration with Dr. Cirrone. QUB will provide fundamental support for radiobiological measurements under hypoxic conditions with ^{16}O through the use of bespoke portable hypoxia chambers and customized stainless-steel dishes specifically made to fit these hypoxia chambers. Researchers involved in the experiment will be Dr. P. Chaudhary (biologist) and a PhD student (physicist) who will start in September.

Indicare progetti in corso o finanziati negli ultimi cinque anni

su tematiche analoghe (max 1500 caratteri, spazi inclusi)

Con coinvolgimento diretto dei proponenti. Indicare finanziamenti sia all'interno dell'INFN, sia a livello di progetti europei/nazionali/regionali

The NA Unit was leading the project ETHICS (pre-clinical Experimental and THeoretical studies to Improve treatment and protection by Charged particleS), which included eight Sections over the 2015-18 period. It focused on the integrity of healthy tissues for specific disease scenarios (breast, pancreas and bone) and their interplay with the irradiated tumor for the currently ions used in hadrontherapy, i.e., protons and ^{12}C ions. Work was carried out at INFN-LNL, INFN-LNS and CNAO. In the last four years it has led the radiobiology workpackage of the CSN5 CALL NEPTUNE (Nuclear process-driven Enhancement of Proton Therapy. UNravEled).

The development of the BIANCA model has been mainly carried out within the MC-INFN project, which is still ongoing in CNSV. Furthermore, BIANCA has been applied/validated for therapeutic beams of protons and C-ions within the ETHICS INFN project, funded by INFN-CNSV in the period 2015-2018.

The RM3 Unit has been involved in several INFN-funded projects during which it tested the combined radiobiological effects (clonogenic test; rejoining of DSBs) of hadrontherapy in vitro, through the irradiation with proton and ^{12}C ions at CNAO, and of magnetic fluid hyperthermia (MFH) on cultured pancreatic cancer cells (2016-2018 HADROCOMBI, 2018-2019 HADROMAG, 2020-2022 PROTHYP)

The CNAO experimental group involved in BIOHOT has actively participated in several INFN projects: ETHICS, NEPTUNE, HADROMAG, PROTHYP.

Descrizione dell'impatto e delle ricadute dei risultati della ricerca (max 2000 caratteri, spazi inclusi)

Impatto nella comunità INFN, possibili applicazioni dei risultati ad altri ambiti, segnalando eventuali problematiche di protezione della proprietà intellettuale. Menzionare le ricadute di trasferimento tecnologico, se potenzialmente presenti.

BIOHOT will address the prospective adoption by CNAO of novel ion beams (^{4}He and ^{16}O) to expand the clinical potentialities of hadrontherapy (HT). In fact, the new source able to provide the scientific community with such beams for preclinical research has been built by INFN-LNS. Working in close collaboration with the Heidelberg Ion-therapy center, where ^{4}He is de facto a clinical reality, the results of this research program will serve as a critical step for facility intercomparison to benchmark the therapeutic quality of the CNAO ^{4}He beam. In addition, building on HIT experience in testing also 16O, our data may pave the way for the implementation of this additional ion type, alone or in multi-ion TPSs with ^{12}C ions. more specific to the INFN community (e.g. the ongoing MC-INFN activity), possible useful results may come from the Monte Carlo simulations and microdosimetry activities envisaged by BIOHOT. In a broader scenario, the societal impact of this research may be relevant for improving the chances of treatment of pediatric cancers and of cancers refractory to treatment because of intrinsic/acquired radioresistance, for which ^{4}He and ^{16}O ions respectively hold great expectations.

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ULTERIORE DOCUMENTAZIONE

- Allegare eventuali dichiarazioni di endorsement da parte di Enti esterni **(non obbligatorio)**.
- Per il responsabile scientifico del progetto allegare il CV (massimo 10000 caratteri) e una lista di massimo 10 pubblicazioni scientifiche o brevetti.

Three letters of endorsement from CNAO, HIT and Queen's University Belfast (UK) are attached alongside the project file.

RICHIESTA FINANZIARIA

Richiesta finanziaria, dettagliata e per sede, per il primo anno di attività del progetto.

NA Unit

Typology	Description	Request (k€)
Travel	Two runs for two people for cellular photon irradiations at CNAO (0.75 k€/person/run); two runs for two people for cellular helium irradiation at CNAO (0.75 k€/person/run); two runs for two people for cellular irradiation with helium at HIT (1.0 k€/person/run)	10
Consumables	Cell cultures disposable plasticware, purchase of HUVEC cell line; media and supplements for cell growth; liquid nitrogen and CO_2 for cell culture maintenance; DNA damage antibodies (foci assay); reagents for flow cytometry measurements of apoptosis, senescence, oxidative stress and detection of inflammation markers	26

PV Unit

Typology	Description	Request (k€)
Travel	One run for two people for data acquisition with helium ions at HIT (1 k€/person/run)	2
Consumables	Cell cultures disposable plasticware; media and supplements for cell growth and 3D scaffold preparation; histology kits, TUNEL kit, Boyden chambers for migration assay	15
Inventory	One high-performance notebook for modeling work to be used during travel to HIT	1

RM3 Unit

Typology	Description	Request (k€)
Travel	Two runs for two people for cellular photon irradiations at CNAO (0.75 k€/person/run); two runs for two people for cellular helium irradiation at CNAO (0.75 k€/person/run); two runs for two people for cellular irradiation with helium at HIT (1.0 k€/person/run)	10
Consumables	Cell cultures disposable plasticware; media and supplements for cell growth; probes for molecular cytogenetics and karyotyping (mFISH); cell fixation reagents; DNA damage labeling; counterstaining; antibodies	24

Please note that detailed offers will accompany this proposal as separate .zip files for each Unit. They will also be uploaded to the database (form EC/EN2a). Some of the requested amounts for travel will be put under sub-judice pending actual beam tume assignment/availability at HIT and CNAO.

Stima di richiesta finanziaria per gli anni successivi

Breve cenno alle spese attese, con particolare riguardo a spese importanti per apparati o strumenti.

It is likely that the financial requests for the years following the first one will be grossly in line with those of the first year for consumables; however, as most of the ion beam irradiation both at CNAO will be in 2024 and 2025, the requests for travel may increase (pending beamtime availability).Please also note that microdosimetry measurements will occur in the second and third year, they require longer times, hence longer stay at the facilities (hotel costs, meals, etc) than those for radiobiological studies having to operate at lower currents/doses.