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Université Paris-Saclay

Cancer treatment: innovative therapeutic strategies based on the use of tumor targeted radiation modalities and nanoagents

Acronym: NanoTheRad

Strategy axis: Health challenge and wellness

Action: "From bench to bedside"

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Outline

Abbreviations

a.m.u	atomic mass unit
CAPP	Cold Atmospheric Pressure Plasma
CEA	Commissariat à l'énergie atomique et aux énergies alternatives
CNRS	Centre national de la recherche scientifique
CD	Cyclodextrin
CPO	Center of Protontherapy of Institut Curie, Orsay
DBD	Dielectric barrier discharge
EPR	Enhanced Permeability and Retention
IC	Institut Curie
IGR	Insititut Gustave Roussy, Villejuif
INSERM	Institut national de la santé et de la recherche médicale
IRMT	Intensity-modulated radiation therapy
ISMO	Institut of Molecular Sciences
Gy	Gray
kV	kiloVolts
LET	Linear energy transfer
LIDYL	Laboratory hosting UHI100 laser facility at CEA Saclay
LOA	Laboratoire d'Optique Appliquée at ENSTA hosting SAPHIR
NA	Nano-Agent
NE	Nano-Enhancer
MOFs	Metal Organic Frameworks
MRI	Magnetic Resonance Imaging
PARP	poly (ADP-ribose) polymerase
PARPi	PARP Inhibitors
PEG	Poly ethylen glycol
PET	Positron Emission Tomography
RadExp	Plateforme de Radiothérapie Expérimentale
ROS	Reactive Oxygen Species
SIRIC	Sites for Integrated Research on Cancer
UPSay	Université Paris-Saclay
UPSud	Université Paris-Sud
UHD	Ultra-high dose rate

I- PRESENTATION

Cancer is the main cause of death in the occidental population. The improvement and personalization of treatments to increase cure rates and reduce side effects of cancer treatments and to reduce the costs of these treatments is a major societal issue[1]. Nearly two-thirds of all cancer patients will receive radiation therapy. Actually, radiotherapy has long been shown to be effective for the treatment of localized tumors and is relatively well tolerated by patients. Over the last 20 years, radiotherapy techniques have changed considerably through the development of imaging, dosimetry, software and instrumentation. These changes have led to the development of conformational treatments adapted to the specific features of the tumor, thereby decreasing the secondary effects. In addition, the combination of radiotherapy and antitumor drugs (chemo-radiotherapy) has facilitated major improvements in efficacy and safety, and has led to a re-evaluation of radiobiological equivalence models. However these techniques do not allow the treatment of tumors located in sensitive tissues (brain, digestive system…) or radiosensitive patients (children, aged, with specific constitutive mutations…). Beyond acute adverse effects, the use of radiation is also limited by the risk of developing secondary tumors due to radiation-induced mutations. More importantly, despite all progress, it remains a subset of cancers such as triple negative Breast cancer, Glioblastoma, Head and Neck carcinomas, Lung cancer which cannot be cured by conventional treatments and for which improving radiation efficacy is a priority. Thus the improvement of radiotherapy protocols and the decrease of early and late adverse effects of radiation is a major challenge of cancer treatment. Hereby, we propose an ambitious project based on the development and the proof of concept of new irradiation sources together with tumor targeted nanoagents and drugs to improve the performances of radiation-based therapies. New radiation modalities such as IMRT, cyberknife and protons have been proposed as alternatives to spare tissues. However none of these techniques is able to fully spare healthy tissues. Very recently ultra-high dose rate radiation has been proposed as a new strategy able to damage tumor tissues selectively. But little is known about this revolutionary strategy. In parallel, the addition of nanoparticles has also shown powerful ability to amplify the effects of conventional x-rays. However this modality is still limited by the pain caused by injection of the colloids in the tumors and the poor knowledge of their physical and biological impact.

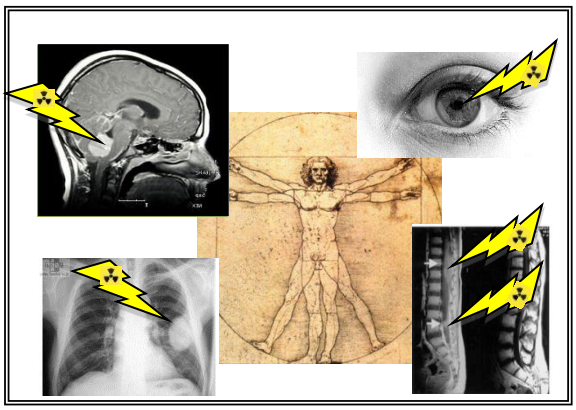

***Figure 1** : Clinical chalenges in radiotherapy*

Thus this project aims at developing strategies based on **new tumor targeted radiation modalities and nanoagents for radiation therapies**. The characterization and understanding of damage and biological consequences from the cellular scale up to the animal models will be essential to design the best treatments. Major outcomes including innovative treatment strategies, instrumentation, products and new knowledge are expected. This field is a niche of research that is growing in Europe and in the World. UPSay has unique forces to reach these objectives and answer this major societal issue.

I.a Specific strengths of Université Paris-Saclay

Unique expertise in radiobiology, oncology, cancer research, radiotherapy and dosimetry

UPSay hosts the leading community in cancer research and radiotherapy in France: Institut Curie (IC) and Institut Gustave Roussy (IGR, Villejuif), two comprehensive centers with hospitals and research laboratories. With fully equipped radiotherapy platforms they perform more than 10,000 treatments annually thanks to medical oncologists and radiotherapists, physicists. The center of protontherapy in Orsay (CPO) of the IC is the first protontherapy center in France and a worldwide leader in this domain. IC and IGR host two sites for Integrated Research on Cancer (SIRIC-Curie and SIRIC-SOCRATE) under the aegis of the National Cancer

[1] *Plan cancer 2014-2019 : social-sante.gouv.fr/IMG/pdf/2014-02-03_Plan_cancer.pdf*

Institute (out of the 8 sites in France). In this project, this community will give access to a platform (RadExp) and models (including 2D, 3D, PDX models*)* fully dedicated to cancer research.

Expertise in the development of innovative instruments for radiotherapy

One of the most important discoveries in radiotherapy has been published recently by the team of V. Favaudon (IC, UPSay) and his collaborators (CEA and IGR). By using a modified electron pulse, they found that an irradiation treatment with **ultra-high dose rate (UHDR**, <1Gy/s, approx. 100 times higher than conventional treatment), called **FLASH irradiation**, has the therapeutic effect similar than conventional irradiation but spares the healthy tissues[2]. An important work on dosimetry of the FLASH irradiation is still required.

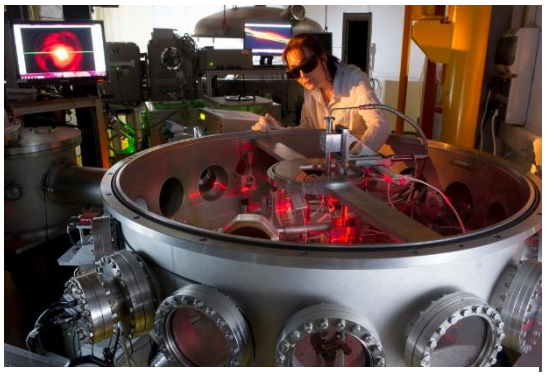

Figure 2: *laser-matter interaction area on UHI (LIDYL) installation*

UHDR mode with proton beams is also promising and can beneficiate of very recent installations, CPO for treatment and RadExp for research[3]. In this perspective a full knowledge of the mechanisms (physical and biological) involved in the differential answer of tissues to radiation is required. Exploring the **biological effects induced by UHDR protons** should rapidly validate the first treatment for human.

Femtosecond bunches of energetic electrons or protons are also delivered thanks to high intensity lasers. Thanks to the unique expertise in high-energy physics, UPSay hosts currently the most powerful laser in the world, APOLLON[4]. Fortunately beams with tenths of Tera-Watt (10^{12} W) peak-power can provide **ultimate dose rates of 10^{10} Gy/s**, in human-sized installations[5]. Exploiting their extra-ordinary properties of intensity, quality, stability and liability in pre-clinical models is now a major challenge.

Cold Plasma for therapy - Plasma is an ionized gas in which ions and electrons coexist. The development in the mid-1990s of cold atmospheric pressure plasma (CAPP) led to the formation of a new field for medical applications such as wound healing, skin treatment, biofilm inactivation, teeth disinfection, blood coagulation, sterilization of various surfaces and fine surgeries in which UPsay is fully involved in terms of development through LPGP and IC[6]. CAPP can also destroy cancer cells[7]. CAPP can also propagate within capillaries which is potential new approach *in vivo* by developing endoscopic devices allowing CAPP use in deep tissues or precise locations to spare sensitive organs.

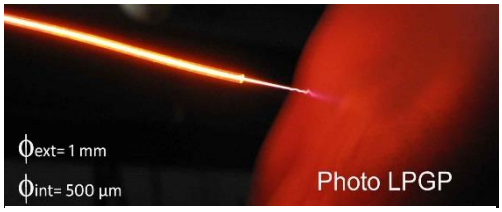

Figure 3*: plasma jet propagating ina capilar and impacting a biological target.*

Unique expertise in the development of nanoagents for medical applications

UPSay is leader in France and Europe in the development of NEs for medicine. It hosts major institutes known for their expertise in the development of a variety of innovative organic, inorganic and hybrid organic-inorganic nanomaterials[8]. UPSay also hosts research groups, which already have a worldwide recognized expertise in the association of NEs with radiation-based treatments to improve cancer treatments by conventional radiotherapy or particle therapy[9]. Some of the groups are also highly active in patenting and developing SMEs.

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[2] *Favaudon V. et al. Sci Transl Med.* ***2014****. 6: 245ra93.*

[3] *http://www.institut-curie.org/research/experimental-radiotherapy-platform-radexp*

[4] *http://cilexsaclay.fr/Phocea/Vie_des_labos/Ast/ast_technique.php?id_ast=9*

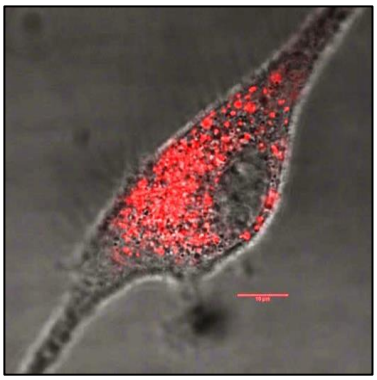
[5] *Fuchs et al. Phys. Med. Biol.* ***2009****. 54, 3315.*

[6] *Sousa JS. et al. Journal of Applied Physics.* ***2011****. 109(12):8.*

[7] *Irani S. et al. Arch Med Sci.* ***2015****. 11(6):1286-95.*

[8] *Agostoni V. et al. Scientific Reports,* ***2015****. Jan 21; 5: 7925*

[9] *Porcel E. et al. Nanomedicine.* ***2014****. 10(8):1601-8.*



The overarching goal of NanoTheRad is to combine the expertise of the above cited groups to develop novel, innovative NEs which in combination with radiation-based treatments will improve cancer treatments by radiation (photons and particles). These new NEs will be synthesized through different chemical methods. Diverse nano-objects will be engineered (nano-MOFs, metallic biocompsites…). They will all be composed of high-Z elements that are able to amplify electron emission when activated by radiation. In addition they will be coated with biocompatible molecules (i.e. PEG) and specific ligands (i.e. antibodies, folate…) with the aim of to improving tumor targeting.

Proximity of multi-disciplinary communities ready to function from fundamental approach to (pre)clinical evaluation

NanoTheRad project is a unique opportunity to establish a strong network among these groups, some of which having already collaborated. The existing expertise will enable the use of innovative sources and the compounds/nanoparticles as soon as the project will start and thus we will have time to optimise the best systems, to test them both *in vitro* and *in vivo* and to have a whole picture of their possible toxic side effects. With this project, UPSay will become the best strategic place to develop future innovative strategies for radiotherapy.

A unique platform of experimental radiotherapy RadExp

A unique platform (RadExp) dedicated to experimental radiotherapy and radiobiology has been developed on UPSay with the financial support of INCA, "Région Ile de France" and IC. RadExp facility is run by expert radiobiologists and physicists (4 ETP) who give access and supervise the use of all the different irradiation systems available at UPSay. It guarantees the dose delivered to any sample and takes care of new users training. According to the needs of the users, the platform helps the users to irradiate their samples (e.g. cells, mice) but can also assume complete experiments when specific expertises are required. The RadExp platform has a dedicated animal facility and can conduct preclinical studies. Part of this grant will be dedicated to complete the imagery equipment (scanner X, IVIS photo imagery, radio detector Typhoon) with the imagery Vevo LAZR system that can be used to visualize internal tumors, total hemoglobin and identify leakage and blood accumulation. Moreover, NEs or molecules that absorb near infrared light can be identified. Proximity of the imagery instrument and irradiation settings will allow the monitoring of early responses after irradiation. In the frame of NanoTheRad, all members of the consortium using RadExp will benefit of the reduced cost of IC internal users for the duration of the project.

European and international positioning

The specificities of the FLASH irradiation have been reproduced in several institutes in the world (CHUV, Lausanne - Switzerland; Stanford Canc Inst. CA USA; RMIT University, Melbourne - Australia) and render the competition extremely active. However, the site of UPSay being the first to have published such breakthrough discovery is so far considered as the leader in the field.

The combination of NEs and radiation is a subject of study that emerged in several excellences research sites in the World. For example, the combination of NEs and radiation is a subject of study emerges at MIT (Boston, US) and Australia (Prof A. Rosenfeld, Univ. Wollongong). Currently, the French community has the leadership in the production and clinical evaluation of NEs for radiotherapy. In particular it is worth mentioning that this is at UPSay that these major clinical trials are performed in the two hospitals Gustave Roussy and IC associated to UPSay. The trials focuses on the effects of hafnium dioxide-based NEs developed by Nanobiotix (Paris) and gadolinium-based NEs developed by Nano-H (Lyon). Hence the position of UPSay in this field is central. In addition the development of NEs for radiotherapy motivated the development of SMEs (DNA Therapeutics, Bioalliance/Onxeo) from UPSay laboratories. Several European projects of the excellence panel are coordinated by researchers of this community including Marie Curie ITN projects (Cyclon-HIT, ARGENT, RADIATE), ERC grants (XMED, *V. Malka (ENSTA)*; TERNANOMED and SQUALAC, *P Couvreur (Inst. Galien, UPSud)*.

With two of the most important French comprehensive centers (SIRIC SOCRATE and SIRIC CURIE) within UPSay and the two largest radiotherapy departments in France, the UPSay is worldwide recognized as a

leading player in cancer treatment and Radiotherapy. However, the number of clinical trials with innovative radiotherapy protocols or new associations remains limited and would be strongly stimulated by increasing the number of programs dedicated to this field.
In the LabeX NanoSaclay, there is a flagship dedicated to Nanomedicine.
With this project, UPSay will become the best place to develop future Innovation in radiotherapy.

I.b General objectives

NanoTheRad aims at:

- Developing new and more efficient protocols of radiotherapy
- Bringing together the forces of UPSay to reinforce and make visible the leadership of the university in the field of Innovative radiotherapy
- Answering European calls as a group and be "force de proposition" in Europe

In addition to

- Transferring the knowledge and know-how to a new generation
- Participating to industrial development of UPSay
- Disseminating the outcomes to the socio-economic stakeholders including hospitals, patient associations as well as policy makers and the general public.

II- THE SCIENTIFIC PROJECT

Among innovative protocols in radiotherapy, FLASH radiotherapy (a clinical dose administered at very high rate: >1 Gy/s) is a discovery made in UPSay. It has the potential over conventional radiotherapy to spare healthy tissue while keeping same anti-tumor efficiency[10]. This property was demonstrated in lung in which the minimal dose of FLASH irradiation with electrons inducing fibrosis was twice higher than with conventional irradiation while both irradiation protocols have the same ability to control tumor progression. This has been demonstrated with FLASH electron irradiation. The effect on other organs including bone marrow, brain, skin, and eye will be investigated within the frame of NanoTheRad. This study will also be performed with proton FLASH irradiation. Clinical trials are planned for the next three years. This studies will be performed in the WorkPackage (WP1.1).

Beside the well advanced development of FLASH therapy, new approaches are prospected such as laser induced ultimate doses or cold plasmas. These last 20 years have actually revealed new energetic particle sources driven by high intensity laser. The progress is very fast and particle energy can now reach 100 MeV in reasonable size installations[11]. With the great performances of the femtosecond duration of electron and proton bunches, one can expect that pushing the limit of UHDR radiation with these machine should be a revolution in terms of development of new type of accelerator for medicine. NanoTheRad should allow the decisive progress to adapt these machines to biology. NanoTheRad will contribute to decisive progress by adapting ultimate dose rate machines to biology (WP1.2). In addition, the outcomes of UHDR sources will be compared to another innovative modality namely cold plasma treatment (WP1.3).

In parallel, the added value of using nano-agents to amplify the effect of medical radiation has been proven by Hainfeld and co-workers who demonstrated that gold nanoparticles prolong the life of mice treated with 160 kV x-rays[12]. Nanoparticles were administered in xenograft directly, which is not applicable in clinic. Since then more sophisticated nanoparticles composed of amplifying agents and tumor targeted molecules have

[10] *Favaudon V. et al. Sci Transl Med,* ***2014****. 6: 245ra93.*
[11] *Malka V. Laser plasma accelerators. Physics of Plasmas.* ***2012****. 19(5):11.*
[12] *Hainfeld JF. et al. Physics in Medicine and Biology* ***2004****. 49:N309-N315 ; Hainfeld JF. et al. British Journal of Radiology* ***2006****. 79:248-253 ; Hainfeld JF. et al. Journal of Pharmacy and Pharmacology* ***2008****. 60:977-985.*

been developed to allow concentration of nanoagents[13]. In tumors, a major challenge is to develop intravenous injection. Nanoagents composed of high-Z elements such as hafnium and gadolinium (developed respectively by Nanobiotix and Nano-H SMEs) have been designed with the aim to not only amplify the effects of conventional x-rays but also improve tumor diagnostic using the same compound[14]. These innovative nano-agents are currently tested in clinic at UPSay (Institut Gustave Roussy, UPSud). However the administration of the drugs proceeds via intratumoral injection, which is painful for the patients, badly reproducible, and not extendable to different cancer cases. At this stage it is proven that the performances of radiation-based therapies may be improved by using specific nanoagents (or nano-enhancers). However, it is now necessary to develop more sophisticated nano-platforms that are able to be injected intravenously, to target tumors, to pass the blood brain barrier, and able to be adapted to different cancer cases with minimum toxicity (WP2.1) and best efficiency (WP2.1 and WP2.2).

Paris-Saclay has the best scientific environment, platforms (see the Annex gathering available accelerators for the project) and expertise to characterize the mechanisms of action of these different agents and irradiation on living systems and to overcome the technological obstacle of these developments. The strategy that consists of developing in parallel or in combination alternative radiation protocols and nano-enhancers is unique and highly promising.

In this context NanoTherad will target the following scientific objectives (SOx):

SO1: assessing best strategies to enhance radiation effects in tumor tissues and to decrease adverse effects on normal tissues
SO2: assessing best strategies to decrease tumor radioresistance
SO4: improving knowledge of physical, chemical and biological mechanisms that underlie efficient radiotherapy
SO4: evaluating the protocols, predict and control their biological impact

The NanoTheRad program has selected the projects that are sufficiently advanced to expect to be able to perform studies on living systems during the first years. All the set-up and nanoagents will be studied in 2D cell cultures, eventually 3D spheroids (which recapitulate some features of tumors as hypoxia and heterogeneity) and if possible in animal models. Studied models vary according to the teams and objectives and cover brain tumors, head and Neck carcinoma, skin cancer, pancreas and lung carcinoma, hematologic cancers. Toxicity on healthy tissues will be extensively studied in parallel. Expertise in preclinical studies is a long-standing asset of the teams involved in NanoTheRad project. **The ultimate aim is to perform preclinical assays in view of preparing future clinical trials.**

1. PERFORMANCES OF INNOVATIVE RADIATION STRATEGIES FOR CANCER THERAPY

WP 1.1. Ultra High Dose Rate (UHDR) with Electrons

Task 1.1.1. FLASH irradiation

***Purpose**: To demonstrate that tumor cells respond differently to UHDR irradiation and Conventional irradiation.*

Sub-task 1.1.1a. Dosimetry detectors for FLASH radiotherapy

Ultra-High Dose (UHD) rate dosimetry is a real challenge since no established methodology for absolute dosimetry exists. We thus propose a cross-disciplinary action aiming at a dual goal:
(i) Setting innovative monitoring detectors optimized in terms of time resolution, dose-rate independence and energy spectrum response and performances.
(ii) Setting a detailed calorimetric study that will be used and parameterized for all calculations in various experimental environments including, first the relativistic electrons used in FLASH studies, then photons and proton beamlines in the prospect of ultrahigh dose-rates with these particles in a near future. Moreover new simulation codes, incorporating new models to take into account temporal and spatial distributions at

[13] *Kong T. et al. Small **2008**. 4:1537-1543 ; Salvati A. et al. Nanomedicine-Nanotechnology Biology and Medicine **2011.** 7:818-826.*
[14] *Rima W. et al. Biomaterials **2013**. 34:181-195 ; Sancey L. et al. Br J Radiol. **2014**. 87(1041):20140134.*

different scales, are required to help the understanding of the physical, chemical and biological response of normal and tumor tissues. In the NanoTheRad project, dosimetric methods will be developed. New Monte Carlo models will be derived from experimental observations, and could be then considered to evaluate new treatment option strategies.

Sub-task 1.1.1b. Protocol FLASH and hematopoiesis

Sparing normal tissues at the expense of tumors using FLASH technology evokes the possibility of differential DNA damage formation and repair in normal, quiescent cells and appears to stem from the differential susceptibility of normal progenitor cells to the duration of the beam-on time. Hematopoietic, gastrointestinal and neurovascular syndromes are deadly acute radiation syndromes (ARS)[15]. Indeed, after total body irradiation (TBI) at doses above 10 Gy, mice die from aplasia and hematopoietic stem cells (HSC) are lost or rendered incapable of reconstituting hematopoiesis.

We will be the first to investigate the beneficial potential of total body FLASH-irradiation (TBI-FLASH) by **monitoring the survival[16], hematopoietic stem cell (HSC) recovery after dose escalation and compare the effects of conventional and FLASH-irradiation on HSC.** The hematopoietic toxicity studies are essential steps to address the association of FLASH with immunotherapy that will be discussed in task 2.2.3. In parallel, we will use total body FLASH-irradiation in mouse and human models of leukemia and study leukemia progression in mice receiving TBI-FLASH.

Sub-task 1.1.1c. Protocol FLASH for pediatric cancer

Toxicity of radiotherapy is particularly at concern in treatment of pediatric tumors as the **quality of life of cured children** might be impaired by abnormal growth, behavioral or cognitive defects and risk of radio-induced cancers. To recapitulate the effect of irradiation in young brain, we will perform FLASH and conventional irradiation at various dose (1-6 Gy) on 10 days old mice and compare survival and adverse effects by analyzing histology of young and adult irradiated brains.

In parallel we will compare the efficacy of both treatments on medulloblastoma cell line cultures and xenograft on mice animals. These tumors will also be grafted orthotopically in the brain cerebellum of young mice to be treated with conventional or FLASH irradiation. Tumor growth and tolerance will be monitored. Long term effects will be analyzed three month after treatment by monitoring recurrence metastasis formation and brain irreversible damage. The risk of developing radio-induced tumors will be explored in WP2, task 2.2.2a.

Task 1.1.2. Ultimate dose rate effect

Purpose: *regarding the promising results already obtained with UHD Resources (see above) it is of major interest to go further and study the biological impact of even higher "ultimate" dose rate (Giga Gray/second). This will be performed by using unique instrument developed at UPSay. In this perspective, these advanced instruments and interface with radiobiological platform will be developed.*

Sub-task 1.1.2a. Improving electron transport for cell targeting

In the community of ultra-high and ultimate dose rate instrumentation, laser-accelerated electron beams commonly deliver femtosecond bunches with enough charges and energies (100-MeV) suitable for *in-vivo* irradiation. Nevertheless they are still suffering from the small size of the beam (~ mm^2) and its spatial instability. In this project, we will develop magnetic lenses and filters to effectively spread the emission to a larger surface (~ cm^2) while keeping other characteristics. Translation to *in vitro* and *in vivo* irradiation would be immediate, as the remaining part of the infrastructure (biological target positioning, real-time dosimetry, biology facility) would be shared with protons.

Sub-task 1.1.2b. Therapeutic dosimetry challenge for fs-bunches

The delivery of electrons in few femtoseconds duration bunches allows the study of interaction of ultimate dose rate ionizing radiation *in vitro* and *in vivo*. The dosimetry currently performed is achieved by comparing detector responses calibrated with conventional electron accelerators, which is not applicable in the

[15] *MacVittie TJ. et al Health Phys.* ***2015****. 109(5):342-66.*

[16] *Lewandowski D. et al. Blood.* ***2010****. 115(3):443-52.*

present case. In this project a new calibration method will be optimized. The deposited dose will be determined via the quantification of radicals using fluorescence spectroscopy [17].

Sub-task 1.1.2c. Effects on biological systems with fs-bunches

We will characterize the effects of ultimate dose rate of electrons *in vitro* by irradiating the biological models described in this project. Cells will be exposed to ultimate dose rate electrons at 2 Gy to 10 Gy. For characterizing the biological effects of y high dose rate, cell survival and proliferation, cell death, DNA strand breaks and repair, genome instability and cell cycle checkpoints will be studied 3D models of human cancer cells as well as xenografted tumors.

WP 1.2. UHDR Proton Beam

Task 1.2.1. FLASH Protontherapy and Proton minibeam radiation therapy (pMBRT).

***Purpose**: The response of tumor cells treated by protons FLASH and ultimate dose rate irradiation will be compared to the effects obtained with the other sources.*

Sub-task 1.2.1a. Evaluation of FLASH protontherapy effects

Proton accelerators used in clinic can deliver UHDR protons. So far, they are the only instruments that could rapidly be used for clinical trial with FLASH irradiation. The main interest of **FLASH radiotherapy** over conventional treatment is to spare healthy tissues[18]. A preliminary experiment has demonstrated the feasibility of treating animals with FLASH protontherapy. To determine if we observe the same beneficial effect with FLASH protons than with electrons, we propose to investigate the biological effects of FLASH protons on three relevant organs:

1. Lung: The first FLASH study performed with an electron beam evokes a sparing effect on the healthy lung in terms of DNA damage signalization. We thus propose to evaluate the consequences of FLASH proton irradiation during the acute (DNA damage response) and late (lung fibrosis) phases.
2. Eye: Patients suffering from uveal melanoma are classically treated by protontherapy. This therapeutic strategy is limited by the damages done to the surrounding healthy tissue (*i.e.*, the vascular plexus) and FLASH protontherapy could thus be of great interest to treat this pathology. We propose to compare the treatment by conventional *vs.* FLASH protontherapy of two xenografted tumor models derived from uveal melanoma patients.
3. Brain: Treatment of brain tumors is a challenge as adverse effects are highly deleterious (in particular in children see task1.1.c). The quality of the treatment could be improve with a better targeting with protons and a lower effect on healthy tissues. We will test this hypothesis in glioblastoma grafted models.

Sub-task 1.2.1b. Evaluation of pMBRT effects

The pMBRT [19] combines the prominent advantages of protons for RT and the remarkable tissue preservation provided by the use of submillimetric field sizes and a spatial fractionation of the dose, delivered as UHDR as in MBRT. The technique has been implemented at the CPO in clinical setup (conventional dose-rate, mechanical collimation) [20]. This technique would certainly benefit from the use of high dose-rates.

We thus propose a cross-disciplinary project aiming at a dual goal:

1. Define a first setup where high dose-rates can be used in combination with pMBRT.
2. Perform dosimetric studies with a single crystal diamond dosimeter developed for protons beams by the CEA-LIST team. This device aims to give accurate dose measurement due to the specific properties of diamond as tissue equivalence and small detection volume,
3. An experiment in which we will irradiate healthy rat brains will be performed and the data compared with those that we already obtained in order to establish a potential gain.

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[17] *Balcerzyk A. et al., Analyst. **2014**, 139, 1707-12; Foley et al, Angewandte Chemie-IE, **2005**, 44(1) 110-2.*

[18] *Favaudon V. et al. Sci Transl Med, **2014**. 6: 245ra93.*

[19] *Prezado Y. and G. Fois, Med. Phys. **2013**. 40, 031712.*

[20] *Peucelle C. et al. Med. Phys. **2015**, 42, 7108-7113.*

Task 1.2.2. Evaluation of ultimate dose rate proton irradiation

Purpose: *Combined effects of protons and ultimate dose rates are strongly expected. In this way the unique infrastructures at LOA and LIDYL must be equipped with advanced devices for real-time dosimetry and interfaces with radiobiology.*

Sub-task 1.2.2.a. Improve setup of p+ fs-bunches and biological validation

SAPHIR and UHI infrastructures have been constructed with the ambition of serving as proof of concept [21] for protontherapy. They can provide protons up to 20-MeV range. For application on single-layers or spheroid cell cultures, a specific collimation set-up based on a magnetic system is needed. The current irradiation protocol has been validated by absolute dosimetry (CPO/Orsay) and by survival rates of mono-layer cell cultures[22].

So far the use of this machine is limited as the delivered dose is 1 Gy/min due to a low repetition rate of 1 Hz. For increasing the available dose range an automated plasma source refreshing will be developed to reach a rate of 10 Gy/min.

The biological effects will be evaluated on glioblastoma and head-and-neck radio-resistant cell lines. Experiments will be performed on monolayer 2D but also on three-dimensional (3D) cell cultures.

Sub-task 1.2.2b. Dosimetry – a better characterization of proton-bunches (SAPHIR and UHI)

An **appropriate dosimetry for ultra-short sources** is a common issue for both electron and proton sources. This new dosimetry requires the development of a devoted diagnostic and will be done through a collaboration with the DOSEO platform and calculation performed with theoreticians.

On both installations, **the duration of the proton emission** is in the order of picoseconds, and very difficult to measure. We propose to design and construct a device to probe transient spectrum of specific material with sub-picosecond time resolution by using time-resolved spectroscopy in quartz or in liquid solution. The availability of such a device for routine operation on a laser-produced radiation source would be mandatory for experiments where the effect of UHDR on biological target would be studied for different durations of bunches. Moreover the knowledge of the **proton spectrum** is also fundamental assumption for the correct calibration of **live dosimetry**. We propose the construction of a satellite, absolutely calibrated, Thomson parabola, to serve as reference for the irradiations. Furthermore detailed functional limits of a transmission ionization chamber through additional experimental campaigns on a conventional proton source (e.g. CPO) are required.

Then we will estimate the potential treatment improvements brought by new treatment solutions combining metallic nanoparticles and non-conventional beams (UHDR, protons,…), by using Monte Carlo simulations available at the DOSEO platform to estimate nanodosimetry at the cell level and to determine dose enhancement factor implied by nanoparticle injection.

The interaction of energetic particles with biological media leads to highly ionized spurs **leading to complex molecular damages**. The complete characterization of these molecular events is a **prerequisite to a possible nanodosimetry when using new sources in radiotherapy protocol**. We can provide investigation of the chemical reactivity and dynamics that occur in the early stages of the dissociation of a multiply ionized medium (biomolecule immersed in an aqueous environment) at the femtosecond time-scale, using state-of-the-art time-dependent ab initio molecular dynamics (MD) simulations **at the nanoscale level**.

WP 1.3. Cold Atmospheric Pressure Plasma (CAPP)

The plasma source used in this study is a nanosecond pulsed atmospheric pressure cold plasma micro-jet created by a dielectric barrier discharge (DBD). Changing the gaz composition and the electric pulses allow to generate a large variety of plasmas. For the first time, these plasmas will be developed in close collaboration with radiobiologists who will characterize the intracellular compounds (ions, free radicals, oxidative products…) and their biological consequences in the perspective to identify the most efficient plasma treatment.

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[21] *ERC: POC «Medical Phase Contrast X-ray Imaging with Laser Plasma Accelerator ». V Malka (LOA).*

[22] *Bayart et al., in preparation*

Task 1.3.1. Setup optimization for CAPP propagation in capillaries

***Purpose**: To optimize a new setup for cell irradiation by plasma*

CAPP can propagate on relatively long distance (few tens of centimeters) within capillaries. This is of particularly importance regarding the treatment of tumors in deep tissues by endoscopy. We propose to optimize our plasma device (shielding gas, input and output voltage, frequency, geometry of the device…) so that the plasma generated at the exit of the capillary retains its properties towards cancer cells.

Task 1.3.2. Dose-equivalent molecular-events activated by CAPPs treatment

***Purpose**: To perform the dosimetry of plasma irradiation*

In order to improve the efficiency of CAPPs, it is necessary to investigate the cellular and molecular pathways that contribute to the cell sensitivity/cell resistance to CAPPs treatment. This can be achieved by focusing on the ROS/RNS intracellular defenses, the activation of signal transduction pathways (*oxidative stress, DNA damage response, cell death)* and detection of intracellular ROS/RNS production by using selected fluorescent probes, as a relevant local equivalence of dose. This approach is encouraged by newly published time resolved detection[23]. These information will be useful for further development of standard dosimetry (to be discussed with NanoTheRad partners involved in dosimetry monitoring and simulation development).

Task 1.3.3. Proof of concept of cold plasma treatment of "head and neck" mouse model

***Purpose**:* to study the anti-tumor activity of CAPP *in vivo*

This will be performed on a mouse model of "head and neck" tumor cells (tumors in the upper aerodigestive tracks, UADT). This model, already used at Curie Institute, is based on the intra-cheek orthotopic transplantation of TC-1 tumor cells expressing oncoproteins type 16 papillomavirus and mimicking virus-induced tumors in the UADT. The closeness of fragile organs (salivary glands, eyes) make it a good model to assess efficacy and safety. In parallel, we will investigate the efficiency of CAPPs *in vitro* by looking at the ROS/RNS production, the viability and the mechanism of cell death (apoptosis versus necrosis) and look for biomarkers that could be associated to sensitivity to the Plasma treatment. This markers will be confirmed on animal models.

2. COMBINATION OF RADIOTHERAPY AND NANOMEDICINE: TOWARDS PERSONALIZED MEDICINE

Radiotherapy is limited by the need to minimize the harmful effects of exposure due to the dose delivered to the healthy tissue surrounding the tumors. Strategies based on tumor-targeted drugs together with radiation treatment are rapidly developing worldwide. Due to the inherent variability of tissues to radiation, multiple factors impact on therapeutic outcome including early stage damage and then biological impact. Thus two approaches are currently proposed to improve radiotherapy outcomes. The first consists of combining the radiation treatment with NEs able to amplify the dose deposition in tumors; they are called **nano-enhancers** (NEs). Clinical trials are already in process (for instance products of Nanobiotix, Paris, France and Nano-H, Lyon, France). However, in spite of encouraging results, this protocol is still limited by a lack of tumor specific localization of the drug (especially in the brain). The second approach consists of using agents to enhance the biological impact of radiation (they are called **radiosensitizers**. The two approaches can be combined. In this project we propose (i) to develop new NEs that are able to target tumors and amplify the effects of radiation (WP 2.1) and (ii) to test existing drugs to increase the biological impact of radiation (WP 2.2).

[23] *Rumbach P. et al. Nature Comm.* ***2015****. 6:7248.*

WP 2.1. Design and test of new NEs

It is known that small (<5 nm) metallic nanoparticles such as Au [24], Pt [25] and Gd [26] are good candidates to amplify medical radiation effects, called NEs. However these agents lack tumor specificity and further development are needed to improve their delivery in tumors.

Task 2.1.1. Engineering and characterization of diverse nano-enhancers

***Purpose**: This task aims at producing various NEs composed of high-Z elements, which are able to amplify local dose deposition in tumors when activated by ionizing radiation.*

Experts in nanosciences will develop new NEs that contain metals, tumor targeted molecules and biocompatible coatings (see Figure 4). These NEs will (i) have sizes less than 200 nm to be administered by intravenous route; (ii) be prepared by "green" methods devoid of any toxic solvents or other substances; (iii) possess a protecting shell (i.e. PEG) to confer them long blood-circulating properties; (iv) bear tumor targeting ligands to accumulate at the tumor site (such as as folate, RGD and transferrin), and (v) possibly be functionalized with elements used for medical imaging (fluorescent tag, F18 for TEP, Gd for MRI). Different types of NEs satisfying all the requirements will be produced by researchers of the chemistry department. Thanks to the local expertise, these agents will be rapidly available in gram-scale quantities to perform the biological evaluation.

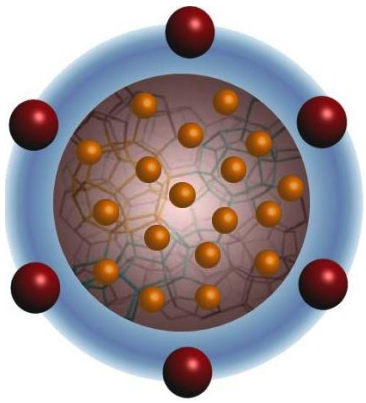

***Figure 4**: scheme of nanocarriers composed of high-Z elements (yellow), tumor targeted molecules (red), biocompatible coating (blue), assembled with various framework.*

Sub-task 2.1.1a. MOFs nanocages composed of Gd, Fe, Au, Pt,

Nanoparticles made of metal organic frameworks (nanoMOFs), a new class of biocompatible highly porous hybrid materials with versatile structures will be developed. NanoMOFs have been widely used for drug entrapment but never as tools to improve radiotherapy or particle therapy (hadrontherapy). In a first step, Fe trimesate and Gd trimesate will be studied. Their performance in radiation-based therapies will be optimized by doping these NEs with lanthanides or by changing the nature of the metal sites. In a second step, the surface of the nanoMOF will be functionalized with iron oxide in order to bring magnetic properties. Finally, the external surfaces will be functionalized with PEG and tumor targeting ligands following a newly developed "green" strategy [27]. Briefly, cyclodextrins (CDs) will be attached to the NanoMOF surface following a "green" technology, in aqueous media. Advantage will be taken of the presence of these CDs to further functionalize the surface with PEG-adamantane and/or adamantane-PEG-ligand. Indeed, it is well known that adamantine form very strong complexes with CDs. The efficacy of this strategy has been demonstrated in vitro with an adamantane-PEG-mannose [33]. It will be applied here to graft transferrin, folate and RGD. The versatility of the systems will allow grafting other ligands of interest chosen in the consortium, according to the in vivo data.

Sub-task 2.1.1b. Bimetallic coordination network-based NEs

The nanoparticles composed of bimetallic coordination networks such as Prussian blue analogues display advantages including (i) a very high proportion of Fe (III), Mn (II) or Gd (III) ions located on the surface which enhances longitudinal relaxivity; (ii) aqueous-based one-step "green" synthesis and post-coating with several biocompatible polymers; (iii) sizes between 3 and 50 nm and (iv) good chemical and colloidal stability over time, (v) adjustable surface chemistry to associate PEG and tumor specific molecules. A series of nanoparticles having shown no toxicity *in vitro* is already available for the project.

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[24] *Hainfeld et al, J Pharmacy and Pharmacology, **2008.** 60 :977-985.*

[25] *Porcel et al, Nanotechnology, **2010**. 21 : 085103-085110.*

[26] *Lux et al, Angewandte Chemie Int, **2011**. Ed 50 : 12299-12303.*

[27] *Agostoni V et al. Scientific Reports, 2015. Jan 21. 5:7925*

Sub-task 2.1.1c. Pt based bicomposites

It has been demonstrated that Pt-based NEs are able to improve hadrontherapy [28]. New Pt-based NEs of different sizes (from 1 nm to 100 nm) will be synthesized by radiolysis, in which a "green" one-step method is used to reduce the complexes and produce metallic species that aggregate to form small (< 5nm) nanoparticles. At the end, the solution is sterile (no mass loss) and ready-to-use for biological study. Bimetallic Pt-Fe nanoparticles coated will be synthesized for theranostic, taking advantage of their magnetism. Furthermore, porous Pt "nanoballs" of controlled porosity will be synthesized in mesophases (used as templates). Pt will be associated to luminescent C-dots for theranostic. All these nanoparticles will be coated with PEG and tumor targeted ligands.

Sub-task 2.1.1d. Macromolecular composite NEs

Macromolecular objects are based on cyclodextrin (CD) supramolecular aggregates and giant calixarenes forming multifunctional nano-objects of around 2 -10 nm and including noble metals. CD are cage molecules able to form stable inclusion complexes with Gd complexes bearing adamantine moieties [29] leading to high-relaxivity of interest for MRI. These nanoagents can be readily functionalized with PEG and targeting ligands.

All the nanoagents developed in this task will be thoroughly characterized by a set of modern techniques to gain insight on their size distribution, surface properties, stability and mechanisms of interaction with biological media. This includes dynamic light scattering (DLS), TEM, zeta potential and X-ray photoelectron spectroscopy measurements. In order to determine the effect of radiation on NEs structure and their spatial arrangement in biological media, Small Angle X-ray Scattering (SAXS) will be used. Thus important information will be gained on the structural modifications of the NEs induced by irradiation and on the influence of NEs self-assembly on their enhancing properties.
In the perspective of medical application, it will be absolutely needed to characterize the stability of the native (tag free) NEs in different biological buffers, as well as their interaction with surrounded proteins. Thus in the frame of this project, it is planned to acquire a powerful equipment (Microcalorimeter&viscosizer) able to (i) measure automatically ultra-low volumes and (ii) perform isothermal titration microcalorimeter (ITC) to characterize the nanoparticle/protein interaction. This instrument will be installed at UPSay, on the CLUPS platform. Its access will be free and available to the participants of NanoTheRad during the time of the project.

Task 2.1.2. Primary test on human cells

Purpose: *This task aims at demonstrating the proof of concept on human tumor cells, at quantifying the amplification efficiency of NEs combined with different ionizing sources (photons and fast particles). This should help determining the best agent and strategy to test in vivo.*

Sub-task 2.1.2a. Effects of NEs in cells with and without combined irradiation

Special attention will be given to the possible toxicity of the nanoparticles investigated in this project. The toxicity of NEs in cells will be quantified by performing proliferation and clonogenic assay of cells loaded with different NEs concentrations. Survival studies will be associated to analysis of cell morphology, attachment and intercellular adhesion (using xCELLigence), quantification of apoptosis/necrosis and cell cycle analysis (by Flow cytometry), quantification of the ROS intra-cellular level and cell cycle analysis.
Similar studies will be performed after irradiating NEs treated cells with the different devices described above starting by conventional photon irradiation. Genotoxic effect will be determined by immunostaining (γH2AX quantification). The proof of concept will be demonstrated using different human cancer cell line monolayers. In parallel, differential amplification effect of NEs will be assessed in subpopulations of cancer cells expressing or not the markers of cancer stem cells (i.e. $CD24^{low}CD44^{high}$ *vs* $CD24^{high}CD44^{low}$) [30]. Finally, it is planned to test the efficiency of NEs on 3D models (spheroids).

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[28] *Porcel et al, Nanotechnology* ***2010****. 21(8) : 085103.*

[29] *Bettistini et al, Chemistry : European Journal,* ***2008****;14(15):4551-61.*

[30] *Bensimon J et al., Mol Carcinog ,* ***2016****. 55 :245-54*

Sub-task 2.1.2b. Improvement of fast ion radiation (hadrontherapy)

Recent works have demonstrated that NEs could amplify the effects of ions radiation (as protons and carbon). Quantification of the effects of the addition of the NEs developed in NanoTheRad to protons and fast ions (200 MeV protons or 220-300 MeV/a.m.u carbon ions) will be performed *in vitro.* Some experiments performed in presence of a radical scavenger will allow the quantification of hydroxyl radicals and their role in the process. This experiment will be performed with beams of different linear energy transfers (LET) in order to mimic the effect at the entrance or at the end of the ion track.

For the most relevant NEs, the effect of the combined treatment on 3D cell culture like spheroids will be performed to assess the role of hypoxia and local necrosis.

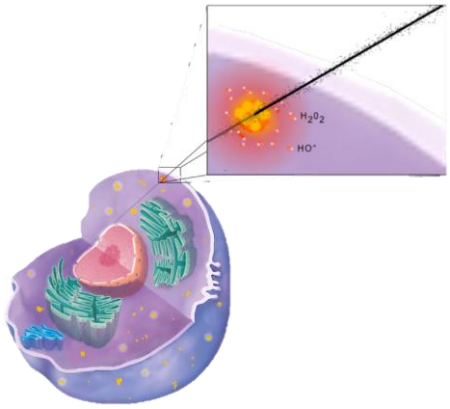
Task 2.1.3. Nanodosimetry

Purpose: *The nanodosimetry is required in view to predict the effect of the combined protocol. The effect of NEs and radiation is linked to the emission of electron and production of radicals in the environment of the NEs. This task will be dedicated to the full characterization of the electronic properties of NEs and to the theoretical simulation of the effects using models able to develop new treatment planning.*

Sub-task 2.1.3a. Electron emission by activated NEs

The electron emission induced in a few tenths of femtoseconds in the close vicinity of the NEs under irradiation will be determined using soft x-ray photoionization technique installed at SOLEIL synchrotron. In order to extract the properties of free standing NEs, a focused beam of NEs will be used to interact with the synchrotron radiation. The total electron yields will be recorded. In order to achieve the scientific goals, proposals will be submitted to SOLEIL program committees at the next open call (February 2017). In-house beamtime will allow the consortium to make preliminary tests.

Sub-task 2.1.3b. ROS production by activated NEs



It is known that ionizations produced by irradiation are responsible for the production of ROS such as $OH^{\bullet}$ or $O_2^{\bullet-}$ radicals and derivatives such as H_2O_2. The relative role of each ROS in the effect of NEs is poorly documented.

Understanding the effects of the local energy deposition and the chemistry of ROS close to NEs is crucial, essentially in the earliest steps after the ionization spurs formation or in the track-end of protons (Bragg peak). The determination of ROS concentrations is a great expertise of UPSay in several research groups that developed fluorescent specific molecules (e.g. coumarin[31]) or nanosensors (e.g. YVO_4: Eu-based nanoparticles [32,33]). The study will be assessed using unique installations of microscope platforms delivering incident x-rays (SOLEIL)) and incident protons (vertical beam of microsonde at CEA) as ionizing radiation. The local concentrations of ROS will be given as a function of the irradiation type and the presence of selected NE. This approach will be similar to the one used in WP1.2/task 1.2.2 with CAPP. The first experiments will be performed with agents that are already known as good NEs (2017) and later with newly developed NEs (2018-2019). The effect of the metal core size on the production of ROS will be investigated (using gold NEs from 10-70 nm).

As NEs can also affect the mechanisms of proteins, we will study the effect of NEs on NADPH oxidase, the sole enzyme whose function is to deliver $O_2^{\bullet-}$ for cell communications and non-specific immune defense. It is a key player in various cancers.

Sub-task 2.1.3c. Simulation of nanoscale effect and dosimetry

The potential treatment improvements brought by new treatment solutions combining metallic nanoparticles and non-conventional beams (UHDR, protons), will be evaluated using our new MC simulation platform thanks to advanced functionalities, such as powerful variance reduction techniques

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[31] *S. Foley et al. Angewandte Chemie-International Edition.* ***2005****;44(1):110-2.*

[32] *D. Casanova D. et al., Nat. Nanotech.* ***2009****. 4, 581-585*

[33] *Bouzigues C. I. et al. Chem. Biol.* ***2014****. 21, 647-656*

necessary to follow high-LET low energy electrons at a microscopic scale, and providing high resolution in the nanometer scale dose pattern.

Task 2.1.4. Biodistribution

***Purpose**: The action of NEs is strongly depending on their distribution in the body, tissues and cells. In particular the uptake in tumors is essentially depending on the blood vessel vasculature and the Enhanced Permeability and Retention (EPR) effect. However the diffusion of agents (i.e. NEs, Dbait) through tight junctions is poorly understood. The purpose of this task is to characterize, from the whole body down to the subcellular scale, the localization and distribution of the NEs with the perspective to develop products with tumor specific targeting. The first location to be studied will be brain tumors and subcutaneous HNSCC tumors (in the mouth).*

This task will be realized by using advanced techniques of multimodal imaging available at UPSay. Molecular imaging gathers a large spectrum of techniques to observe molecular events from the whole body down to the subcellular scale. We will take advantage of Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI) and ultrastructural chemical imaging by nanoscale secondary ion mass spectrometry (nano-SIMS) and analytical transmission electron microscopy (analytical TEM: EELS, EFTEM and STEM) to boost the development of new radiation amplifiers in assessing the following parameters:

- The whole body distribution of the innovative NEs (radiolabelling and TEP),
- The quantification of the NEs (MRI) and specific tissue targeting (including vectorization of the NEs by MRI guided ultrasound induced blood-tumor permeation)
- The cellular and tissue distribution of NEs depending on tumor heterogeneity and ultrastructural chemical imaging (nano-SIMS and analytical transmission electron microscopy)

Sub-task 2.1.4a. Biodistribution of NEs in entire body

PET is a whole body, non-invasive and quantitative nuclear imaging technique with unique detection sensitivity, which is a key-tool in drug development. NEs tagged with Fluorine-18 ($T_{1/2}$ = 109.8 min) or zirconium-89 ($T_{1/2}$=3.2 d) will be used to image and quantify the distribution of radiolabelled NEs with PET from a few hours with fluorine-18 to several days with zirconium-89 after injection. In addition, standard radiopharmaceuticals ([^{18}F] FDG for glucose metabolism or [^{18}F] F-MISO to measure tissue hypoxia) or biomarker-specific radiotracers can be used to evaluate tumor progression and radiotherapy efficacy.
MRI will be used to quantify gadolinium and iron NEs *in vivo.* Although less sensitive than PET, these sequences can provide more information about the heterogeneity of the tumor and of the NE distribution, without need of radiolabelling. This information will be correlated with observed clinical outcome. On a more fundamental side, MRI molecular imaging can be used to study the diffusion properties of NEs through tumors after delivery in order to optimize the time between injection of NEs and radiotherapy. We will also probe the use of ultrasound induced vascular wall permeation to increase the uptake of NEs to tumors, especially in the brain where the blood-brain barrier still restricts the local concentration of NEs when they are delivered intravenously. This technique is well controlled using an MRI guided motorized focused ultrasound system for rodents developed at UPSay (CEA-Neurospin).
The Vevo Imaging apparatus (to be purchased in this project and installed in the RadExp platform for access to all partners) is an animal dedicated imaging device, which allows image guided injections (of cells, drugs, genetic material or metabolic agents) into tumors or organs of small animal, and follow-up of nanoparticles pharmacokinetics via multispectral imaging. It allows monitoring the tumor response thanks to the visualization of microscopic margins of anatomical structures and live hemodynamic changes. The results will be compared to the data at cellular level obtained by intravital microscopy (facility available on the PIMPA platform-CNRS). This technique will allow to monitor the diffusion and the retention of NEs in tumors and surrounding tissues in animal models.

Sub-task 2.1.4b. Localization of NEs in tissues and in cells

Ultrastructural chemical imaging by nanoscale secondary ion mass spectrometry (nano-SIMS) and analytical transmission electron microscopy (analytical TEM: EELS, EFTEM and STEM) will be used to characterize the cellular and tissue distribution of NEs. To this end, image-guided biopsies will be withdrawn and tissues will be specifically prepared to locate elements such as F, Gd, Mn, Fe, Pt, Au and other metals. The location of biopsies will be guided by (or co-located with) *in vivo* imaging. They will be correlated with tissues

characteristics (*e.g.* microvascular permeability, cell density, hypoxia, proliferation…), the treatment response and to early therapy effects.
The localization of NEs with cell organelles will be performed using advanced optical microscopy techniques such as FLIM microscopy.

Sub-task 2.1.4c. Pharmacokinetics of NEs

A quantitative evaluation of the NEs by adapted analytical techniques will be carried out to determine the pharmacokinetics parameters and biodistribution of the NE's. For this purpose animals will be placed in metabolic cages. At first the pharmacokinetics PK will be followed by sampling blood and assaying the NE's in the plasma at time 5, 10, 30, 60, 120, 180, 360, 480, 1440 and 2880 minutes after intravenous administration. Modelization of PK profiles will be carried out in order to calculate the area under the curve, elimination half-life, clearance and volume of distribution. Urine and feces will be collected in order to determine the elimination pathway. Finally, biodistribution in healthy tissue and tumors will carried out after extracting tissues at time 1, 24 and 48 hours. After extracting the NE's, they will be analyzed in each tissue as well as in the carcass. These data together with the biodistribution information obtained in sub-task 2.1.4a will allow to modelize the pharmacokinetics of the NEs and drugs and to optimize the protocols of association.

Distribution in the body of nanoagents (NEs and all the large molecules such as Dbait described in task 2.2.2) may dependent on the vasculature. Indeed these are too large to easily diffuse out of tight junctions and show specific distribution in tumors. The intravital microscopy facility of the PIMPA platform (CNRS UPSAy) will allow monitoring the diffusion and the retention of these nanoagents in tumors and surrounding tissues in animal models. These data together with the biodistribution information obtained in sub-task 2.1.4a will allow to modelize the pharmacokinetics of the NEs and other drugs.

Task 2.1.5. Preclinical evaluation of NEs and radiation protocols

Purpose*: This task is devoted to the preclinical evaluation in animal models of treatment protocols based on irradiation by medical sources combined with NEs developed in this project.*

For the NEs selected in 2D and 3D cell experiments (task 2.1.2), and by pharmacokinetics (task 2.1.4), the improvement of radiation effect on tumors will be evaluated on xenograft immuno-deficient mouse models. NEs will be injected in an orthotopic (Glioblastoma in brain) and/or systemic way in HNSCC subcutaneously xenografted mice. The mice will be treated by different radiation modalities. The antitumor effect will be evaluated through tumor time-dependent size and mass assessing and animal survival.

WP 2.2. Mechanisms of resistance to radiotherapy and radiotherapy induced pathologies

To increase the effect on the tumor or to decrease the damage on normal tissues are two different approaches to improve cancer treatment. We aim to study these two options using different drugs. In a first approach normal tissues will not be sensitized and in a second approach the tumor will not be protected. Two factors have made such approaches feasible: an improved understanding of the molecular response of model organisms to ionizing radiation and a new appreciation of the exploitable genetic alterations in tumors. These have led to the development of treatments combining pharmacological interventions with ionizing radiation that more specifically target either tumor or normal tissue, leading to improvements in efficacy.
Four main biological factors of tumors have been shown to affect outcome after radiotherapy: the extent of the hypoxia; the ability of the surviving cells to repopulate within a treatment time of 6–7 weeks; the intrinsic radioresistance of the tumor cells; and the immune response[34]. Knowledge of the importance of such processes has led to the development of strategies to overcome or modulate them in order to increase ionizing radiation-induced killing of tumor cells. The NanoTheRad project aim to cover these different strategies as that will be illustrated by some work already in progress at UPSay.

[34] *Park B., et al. Int J Mol Sci.* ***2014*** *Jan; 15(1): 927–943.*

Task 2.2.1. Radiotherapy induced pathologies

Purpose: *Radiotherapy is facing a double threat with risk of local recurrence at the edge of the treated site and patient developing a second cancer induced by treatment. Actually advances in medical technologies have led to more cancer patients being cured and increased survival times. But with cancer survivors living longer, there's a growing concern regarding the risk of radiation-induced second cancers in patients treated with ionizing radiation - particularly children. This is thus of major interest to investigate the impact of innovative treatments on the induction of secondary cancers.*

Sub-task 2.2.1a. Radioinduced mutagenesis/tumorogenesis

Irradiation is mutagenic as DNA damage can be repaired by error prone systems which will modify irreversibly the genetic content of the cells. To apprehend the risk associated to innovative protocols we will monitor mutagenic effects of the various associations using the hypoxanthine phosphorybosyl transferase (HPRT) test recommended in the guidelines for mammalian gene mutation tests (Organization for Economic Co-operation and Development-OECD (1997)).

As the long term risk of mutagenesis is apparition of tumors, we will monitor the frequency of radio-induced tumor in mice treated with local irradiation (using advanced sources developed in this project, such as plasmas) and subcutaneous injections of nanoagents (Dbait or NEs). To increase the probability of tumor development, P53 deficient transgenic mice will be used as animal model. This study will allow to compare combined protocols to determine best combined protocols with lowest risks of radio-induced tumors.

In parallel, mice models highly susceptible to develop Melanoma will be used to monitor radio-induced cutaneous tumors.

Subtask 2.2.1b. Screening of inhibitors of radio-induced migration

One cause of the radiotherapy failure is the spreading of the primary tumor to other organs by diffusion of metastasis. Using a platform automated cell imagery system, we will search for potential inhibitors of cell migration, using genome-wide and gene expression profiling (GEP) analysis of primary tumors and metastasis. Such approach has been already tested with Uveal Melanoma (a common primary intraocular malignancy mainly treated by protontherapy), and lead to identification of the *PTP4A3/PRL-3* (Protein Tyrosine Phosphatase 4A3/Phosphatase of Regenerating Liver-3) phosphatase that can be inhibited by numerous small molecule inhibitors including the antiprotozoal pentamidine. These molecules will be tested for their ability to prevent radio-induced tumor cell migration and sensitize uveal melanoma tumors to irradiation by different modalities. This study will allow to confirm the role of tumor cell migration in the local recurrence and to develop new biostatistics methods and assays for screening drugs inhibiting cell migration and increasing radiotherapy efficacy.

Task 2.2.2. Oxydative damage and radiotherapy

Purpose: *Ionizing radiation, cold plasmas and most of the NEs are thought to act by increasing oxidative damage in the cells. However, tumor cells can survive in hypoxic conditions (a hallmark of cancer). Hypoxia is a major factor in radio-resistance. Several studies have shown that reducing hypoxia as well as increasing oxidative stress could improve the outcome of radiotherapy[35]. There is thus an urgent need to identify new molecules reducing hypoxia.*

Sub-tasks 2.2.2a. Efficacy of aminoflavine combination with radiotherapy

Aminoflavone (AF; NSC 686288), which is the active component of a prodrug (AFP464) is in clinical trials in the US (trial NCT01233947). This drug induces oxidative DNA damage and apoptosis [36] and inhibits HIF-alfa expression[37]. It is thus a good candidate for reducing hypoxia and improving tumor sensitivity to radiotherapy. Within this project breast cancer xenografted tumors will be tested for their sensitivity to the combined treatment (survival analysis and proliferation, apoptosis monitoring). In addition, using the new echography facility on the RadExp platform the hypoxia and free radicals content in the tumors will be

[35] *Horsman et al. J Radat Res.* ***2016****. Mar 16. pii: rrw007*

[36] *McLean et al. Int J Cancer* ***2008****. 122 (7) : 1665-1674*

[37] *Terzuoli et al Cancer Res.* ***2010*** *: 70 (17) : 6837-6848*

followed during and after treatment. The results will be confirmed by immunostaining of tumor samples using specific markers specific to stress response to hypoxia, DNA damage and oxidative stress.

Sub-tasks 2.2.2b. Nucleotides pool and radiosensitivity

Cytidine deaminase (CDA) expression is frequently down regulated in cancers. About 60 % of cancer cells and tissues are expressing low level of CDA in six major types of cancer[38]. CDA is involved in pyrimidine salvaging and could indirectly influence DNA repair via controlling the pool of pyrimidine. CDA-deficient tumor cells have been shown to be highly sensitive to aminoflavone [39]. To test if this sensitivity is related to the property of the drug to induce oxidative stress, we will compare the response of breast cancer cell lines (with different level of CDA) to different treatments by NEs and different irradiation modalities. They will be studied in nucleotides content, DNA repair activities and hypoxia status will be characterized as described above.

Task 2.2.3. DNA repair and treatment resistance

Purpose: *To understand radioresistance mechanisms and counter them.*

Sub-task 2.2.3a. Radiation resistance in microorganisms and NEs

Deinococcus radiodurans is the most radioresistant organism isolated so far. It belongs to a family of bacteria characterized by an exceptional ability to withstand the lethal effects of DNA-damaging agents, including ionizing radiation, ultraviolet light and desiccation. *D. radiodurans* can survive extremely high doses of radiation such as 5,000 Gy γ-irradiation generating approximately 200 DNA double-strand breaks, and other damages without loss of viability. It is now admitted that radioresistance in bacteria is a combination of physiological tools and many different mechanisms including an efficient protection of proteins against oxidation, efficient DNA repair mechanisms, and a condensed nucleoid contribute to radiation resistance enabling survival from stresses. In a collaborative work, partners of NanotheRad (P3, P27), have shown that NEs such as platinum and gold nanoparticles enter the *Deinococus* cells and amplify radiation effects of gamma rays. Therefore this organism can be used to rapidly test the efficiency of NEs alone or when combined with different sources of irradiation. Moreover, to better characterize their targets into the cells and the radioresistance mechanisms, there is to our disposal a mutant library of *D. radiodurans* constructed by transposition with several hundred of mutants of specific genes involved in DNA repair mechanisms, protection of protein from oxidization, chromosome structure, etc that can be tested by series on plates and/or in liquid.

Sub-task 2.2.3b. Radiation resistance of stem cells

Proliferative cells may become quiescent, and vice-versa. This phenomenon is known as phenotypic switch. This switch is crucial to appreciate tumor resistance as quiescent cells (i.e. cancer stem cells which are able to induce new tumor development) are not well killed by conventional treatment. Within this project we propose to overcome this limitation. We have shown that melanocyte stem cells are sensitive to electrons irradiation (not published). It is now required to better understand how melanocyte and melanoma stem cells acquire their stem-like properties and to identify factors controlling the underlying cellular mechanisms. This project aims at using new protocols of radiotherapy and/or immunotherapy for targeting proliferative cells and quiescent cells to improve anti-melanoma therapeutic strategies.

Sub-task 2.2.3c. DNA repair inhibitors and innovative treatments

A novel DNA repair inhibitor, the Dbait, has been discovered and recently developed at UPSay. It has undergone a first-in-man Phase I clinical trial on cutaneous melanoma with radiotherapy[40], with no evident toxicity in the patients but a promising radiosensitizing effect. These molecules trigger false DNA damage signaling and hijack key damage signal transducers such as DNA dependent protein kinase (DNA-PK)[41] and PARP[42]. Consequently, the DNA repair enzymes are diverted from their primary objective, the double-strand

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[38] *Mameri et al.* ***2016****. (in revision)*

[39] *https://clinicaltrials.gov/ct2/results?term=aminoflavone&Search=Search*

[40] *Le Tourneau C. et al. Br J Cancer,* ***2016****. 114(11) : 1199-1205*

[41] *Quanz M. et al. Clin Cancer Res* ***2009*** *15 (4): 1308-1316 ; Quanz M. et al. Plos One* ***2009.*** *4 (7):e6298*

[42] *Croset A. al al. Nucleic Acids Res. 2013. 41 (15) :7344-7345*

breaks on chromosomes, inhibiting DNA repair and leading to cell death. Preclinical studies have demonstrated that this strategy sensitizes tumors to DNA damaging treatments such as conventional radiotherapy and chemotherapy. However Dbait mainly inhibits double-strand break repair whilst conventional PARP inhibitors (as olaparib) inhibits base excision repair. Thus we expect these inhibitors to sensitize the cells differentially when treated by NEs and different radiation modalities. The effects will be tested on cell and animal models with the aim to decipher the damage that are induced by these treatments. This work will help us choose the best combined protocol.

Task 2.2.4. Immune response and radiotherapy

Purpose: *To analyze the abscopal effects with different protocols and identify the best combination with immunotherapy*.

Since a long time, delivery of localized radiation to tumors has been reported as responsible for systemic responses at distant sites, a phenomenon known as the abscopal effect. This has been attributed to the induction and the enhancement of the endogenous anti-tumor innate and adaptive immune response. With the emergence of immunotherapy (mainly via the development of checkpoint inhibitors anti-PDL1, anti-CTLA4) the role of radiotherapy in support to these treatments is under investigation in numerous laboratories.

The IC and the IGR have leader teams working in the field of immunology (for example S. Amigorena, G. Kremer and L. Zietvogel teams). Several collaborations already exist between these teams and the radiobiologists involved in NanoTheRad. The content of the task will be defined at the end of the first year after the community will provide the first results on the effect on cells of new radiation modalities and/or nanoagents. The immnunologists will participate in the steering committee of the NanoTheRad.

3. CONCLUSION

This program present works which aim to become the founders of long-term technical and scientific developments. The projects will last longer than the 3 years period and, thanks to the setting of strong interdisciplinary collaborations, should lead to important progress in cancer treatment.

At the end of 2017, the **scientific milestones** will be:

Milestone 1 (WP1.1): Achievement of the Detector Calibration for dosimetry in FLASH protocol allowing the accurate determination of the dose for UHDR radiation exposition and quantification of the effect on tumor and healthy tissues,

Milestone 2 (WP1.1): Achievement of the fluorescence detection in-line for the fs-electron source using the ultra-sensitive camera (IemCCD) allowing accurate dosimetry of ultimate dose rate irradiation,

Milestone 3 (WP1.2): Achievement of the UHDR proton dosimetry,

Milestone 4 (WP1.3): Proof of efficacy of cold plasma treatment on cell culture,

Milestone 5 (WP 2.1): Selection of a 1st set of new nano-enhancers (NEs) soluble and stable in biocompatible solvents and able to amplify the physical effects of x-rays and fast ions.

Milestone 6 (WP 2.1): Validation of the efficiency of nano-enhancers to amplify radiation effects in human cell lines treated by x-rays or fast ions

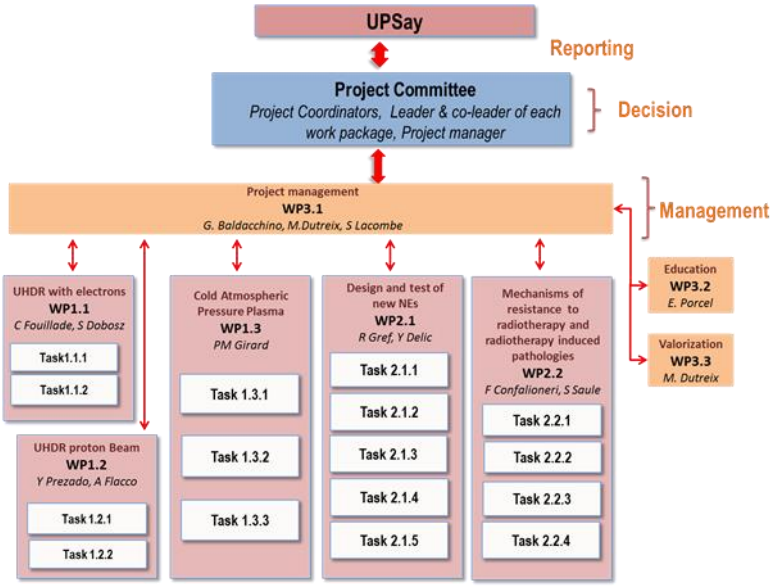
Milestone 7 (WP 2.1 & WP2.2): Safety of innovative irradiation treatments, NEs, and other active drugs including mutagenesis and toxicity in cell cultures as well as pharmaco-kinetics.

Milestone 8 (WP 2.2): identification of new biological targets for drug development and biomarkers for selecting patients to be treated with Dbait and radiotherapy. Workshop on Immunology/radiotherapy.

III- COORDINATION, TEACHING AND VALORISATION

WP 3.1: Project management

The coordination will be ensured by the **Project Committee** composed of the three main coordinators (Dr Baldacchino, Dr Dutreix and Pr Lacombe,) together with leaders and co-leaders of each WP. The comittee will be supported by a project manager. This committee will be in charge of the project supervision including the coordination between all the partners, the budget management, the education and dissemination. These tasks will be ensured via a consortium agreement that defines the respective participants' contributions, WP objectives, deliverables, and milestones and possible hurdles/revised strategy for the forthcoming period. Ethical, intellectual property and administrative aspects of the project (decision-making structure of the consortium, responsibilities of each partner, work packages and activities, financial and industrial property rules, publication policy) will be defined. The project will start with a kick-off meeting at month 1 (as described in the project Gantt chart in annex 3). The scientific outcomes will be followed by the leaders and co-leaders of WP and supervised by the three main coordinators. The project committee will meet on a basis of 1 meeting every 6 months and progress reports will be transmitted to UPSay.



The education WP will be followed by a teacher-researcher of UPSud (Dr E. Porcel). The valorization WP will be followed by M. Dutreix who has experience in start-up development.

The **budget** and the distribution procedure are fully described in Annex 2. Briefly the current funding is distributed as function of the involvement of the research teams (Annex 1) and schedule of the project (Gantt chart in Annex 3).

WP 3.2: Education

In order for UPSay to fully realize the associated healthcare and economic benefits there is an urgent need to train a new generation of researchers and experts to be familiar with all aspects of innovative advances in nanoagents and innovative sources and protocols for radiotherapy and cancer treatment. This includes early stage students (Bachelors and masters) up to early stage researchers (PhDs, postdocs).
This includes academic and practical courses as follows:

- Lectures, lab training and seminars in the European Master Course SERP+ EMJMD – module "Medical application of radiations" (coordinated by S. Lacombe),
- Lectures in the "Master Cancérologie",
- Lecture on "introduction à la physique médicale" in the Magister de physique fondamentale et physique appliquée (3rd year of Bachelor),
- Lectures in the "Diplôme Universitaire (DU) de radiothérapie",
- Seminars in the doctorate school "cancérologie",
- Lectures in the "Master de Nanoscience".

Seven master students will be recruited between January and March 2017 to be trained in the interdisciplinary topics. Two PhD students will be recruited in September or October 2017 to start co-supervised thesis (One in Chemical Physics and one in Nanoscience). In addition three postdocs (from Biology or Medical Physics background) will be recruited in 2017 to act as multidisciplinary ambassadors between the different laboratories of UPSay to promote the exchanges and expertises between the different laboratories of UPSay.

WP 3.3: Valorization

To accelerate the transfer of new technologies and the development of new drugs, various contacts with the industrial companies involved in clinical irradiation instruments manufacturing or in drug development have been initiated. When possible, collaborative programs will be developed with these companies. Moreover, several start-ups (spin-off of UPSay laboratories) have already emerged in the field of drug associated radiotherapy. The most advanced are Nanobiotix and DNA therapeutics that both have carried basic research discoveries (NEs and DNA repair inhibitors respectively) to clinic. Numerous patents in this field have been applied by the NanoTheRad teams giving the site a potential strength to become a reference for the companies in the domain. Contacts will be established to build new instruments according to specificities identified in the NanoTheRad program.

Besides the economic benefits from patent licensing, will be added the socioeconomic benefits for the patients and for the health care system thanks to higher efficiency of radiotherapy treatments and thus better control of associated costs. Partners will be sensitized to the patenting, licensing, and start-up creation by specific lectures during internal meetings.

IV- CONTRIBUTION TO UPSAY STRUCTURATION AND VISIBILITY

This highly interdisciplinary project will by nature play **a structuring role for UPSay** with the participation of approx. 80 researchers from 3 departments: Chemistry, Physics (Physique Ondes et Matière - PHOM), and Biology (Département Sciences de la Vie - SdV). The community will be gathered through a kick-off meeting at the project start, and annual steering meetings (see annex 3).

This project brings together themes and participants of Excellence Laboratories (**Labex)** including LERMIT, PALM and NanoSaclay. It will benefit from their support through co-financing. It will also gain from the existence of local technical platforms (RadExp and **Equipex** Apollon-Cilex and ICGex, Investissement d'avenir France HADRON).

To strengthen the international visibility of the UPSay community in the field of innovative radiotherapies, a dedicated webpage hosted by the UPSay website will be implemented and workshops will be organized (see Gantt chart in annex 3).

V- PERSPECTIVES BEYOND THE PERIOD OF IDEX FUNDING

A major perspective of this project is to bring the community to apply for European funds such as ERC, H2020 programmes such as Marie Curie ITN (in particular EID), Euronanomed, and FET Open. It is noteworthy to mention that some members of the consortium have already strong experience in elaborating and coordinating such ambitious projects. The participants will benefit from the experience and will be encouraged to follow the trainings organized by UPSay on project writing. The structuration of the community will give new opportunities to apply for European as well as national grants in particular for interdisciplinary calls (ANR, INCA, Physi-Cancer).

The program will help to develop specific platforms and a dedicated animal facility for radiation studies. The large set of instruments available will be attractive for private companies that do not have the capacity to own such equipment and will stimulate future collaborations between academics and industrials.

VI- SUPPORT BY THE DEPARTMENTS

NanoTheRad has been supported by three major research departments of UPSay, namely the department of PHOM, the department of Chemistry and SdV. Medical physicists and radiation oncologists of UPSay are fully involved. The exhaustive list of the laboratories dealing with the developments aiming at the improvement of radiotherapy included in this project is presented in the table (annex 1).

The research teams involved in this project are hosted by most of the institutions of UPSay including UPSud, CNRS, CEA, INSERM, Institut Curie, Ecole polytechnique, Université d'Evry, UVSQ, SOLEIL.

VII- LEADERSHIP QUALITIES OF THE PROJECT MANAGER

This project will be coordinated by three experienced researchers, senior expert, Research Director and Professor, internationally renowned in their field, coordinators of European projects and international networks. They are committed to this coordination during the 3 years of the project duration.

Gérard Baldacchino, PhD, is a senior expert at CEA Saclay in radiation chemistry with extreme conditions (T, P, pH, LET) and in ultrafast spectroscopy. He has collaborated with many research groups and industrial groups such as AREVA, EdF and ANDRA. He has also several collaborations with university groups in France and in Europe, in Japan, in the USA and in Canada especially for using large particle accelerators in pulsing mode (GANIL, HIMAC, ESRF). He has also used Monte Carlo codes for simulation. He is coordinating a competence network of ~100 experts of radiolysis in CEA. He has coordinated several ANR projects; He is currently coordinating Plan Cancer projects (BITMAP in 2016 – 255 k€). He has published 69 articles in high impact factor journals and book sections. He is regularly invited and chair in high level international conferences (Last: ICRR2015 in Kyoto, TSRP-APSRC 2016 in Mumbai). He is a visiting professor in Radiation Chemistry at the University of Nantes (Master 2 level) and University Paris-Descartes (Master pro level).

Marie Dutreix, PhD, Director of research CNRS, President of the Société Française du Cancer, co-founder of the biopharmaceutical company "DNA Therapeutics". She is an expert in DNA repair & genetic instability, cancer biology and radiobiology. She is leading the group "Recombination, Repair and Cancer" in the research unit of "Normal and pathological Signaling: from the Embryo to the innovative Therapy of Cancers**"** located at the Orsay campus of the Institut Curie. She is coordinator of "radio-oncology" of the SIRIC-Curie, member of the AVIESAN board for targeted therapy, member of the SAB CRUK/MRC Oxford Institute for Radiation. She develops new strategies to treat tumours resistant to conventional treatments. She discovered a new strategy (siDNA) to inhibit DNA repair in vivo (*www.youtube.com/watch?v=iEycs8yZSBE)*. Prizes: National special prize of «création d'entreprise de technologies innovantes» (2005); "valorisation of research" of University Paris Sud (2005); "tremplin" for biotechnology innovation of the Senate (2006); award from the "Ligue contre le Cancer" (2010). She was awarded in 2013 "Chevalier de l'ordre National du Mérite". 83 publications, more than 5 conferences as invited speaker/year.

Sandrine Lacombe, PhD, is Professor in chemistry at the University Paris Sud. She's leading a research group focused on the improvement of hadrontherapy by addition of nanoagents. She's coordinator of the European Marie Curie ITN project entitled ARGENT focused on "Advanced Radiotherapies GEnerated by Nanoproceses and Technologies" "(http://itn-argent.eu) and director of an Erasmus Mundus Master Course SERP Chem (http://serp-chem.eu). She also participate in an interdisciplinary ANR project (PROTON). She published more than 70 papers. She was a fellow of Prof. G. Ertl, Nobel Prize 2007, at the Max Planck Gesellshaft- Fritz Haber Institut (Berlin, Germany). She was invited for 1 year at CHU Sherbrook as invited Professor. She received the price of IRCA Award 2015 (Sydney University). She's member of the editorial boards of Cancer Nanotechnology (UK) and Advanced Physics X (UK). She participates as invited speaker at approx. 2 international conferences per year (ex: Gordon, EMBO, ESF conferences, Annual Conference of the American Radiation Research Society 2016).