**Effects of Conventional and FLASH radiotherapy on glioblastoma and melanoma cells**

Giulia Rosini1, Riccardo Nieri1, Elisa De Santis1, Matteo Paolini1, Andrea Cavalieri2,3,4, Fabio Di Martino2,3,4, F. Paiar2,4, Mario Costa1,2,5 and Beatrice D’Orsi1,2

1Institute of Neuroscience, Italian National Research Council, Pisa, Italy; 2University of Pisa, Center for Instrument Sharing of the University of Pisa (CISUP), Pisa, Italy; 3National Institute of Nuclear Physics, Section of Pisa, Pisa, Italy; 4Azienda ospedaliero universitaria pisana, U.O. Fisica Sanitaria, Pisa, Italy; 5Laboratory of Biology “Bio@SNS”, Scuola Normale Superiore, Piazza dei Cavalieri, Pisa, 56124, Italy.

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Radiotherapy is one of the most effective anti-tumor therapies, used in more than 60% of cancer patients at some point in their oncological treatment to eliminate/reduce the size of the tumor. At present, conventional radiotherapy (CONV-RT), the main approach used in clinic, presents some limitations, including the dose fractionation into several daily sessions and the risks for the surrounding normal tissue. Recently, an ultra-high-dose rate electron irradiation method termed FLASH radiotherapy (FLASH-RT) selectively spares healthy tissue while leaving unchanged the therapeutic effect on tumor cells. The radiobiological mechanism of FLASH-RT responsible for the FLASH effect have yet to be fully explored. Recent studies have shown that FLASH-RT can induce the protection of mammalian cells through transient hypoxia, potentially reducing reactive oxygen species (ROS) formation and DNA damage, thus decreasing toxicity to healthy tissues. Moreover, a deeper understanding of how calcium signaling is remodeled in some cancers and the consequences of calcium signaling on key events, such as proliferation, invasion and sensitivity to cell death, is needed. In fact, mitochondrial Ca2+ controls ATP synthesis, apoptosis, ROS generation and biosynthesis, and can determine the fate of the cell.

We aimed to examine the FLASH-RT-induced radiobiological damage occurring during and post irradiation, to understand the key processes involved and FLASH potential in therapy. Using combined population-based and single-cell imaging approaches, we investigated Ca2+ homeostasis, ROS formation, cell death, mitochondrial and cellular markers, gene expression and cancer metabolic reprogramming in murine glioma GL261 and melanoma B16-F10 cells compared to "non-tumoral control" cells using three increasing total irradiation doses (4, 8, 16 Gy).

Interestingly, our data demonstrated that both FLASH and CONV mode produced a significant raise in cellular ROS compared to the control cells in a dose dependent manner. At any dose and in all cells, CONV induced higher ROS production than FLASH treatment. Moreover, both FLASH and CONV resulted in a significant increase in intracellular Ca2+ compared to the control cells in different ways, at lower doses (4 and 8 Gy) for GL-261 cells, and only at 8 Gy for B16-F10 cells. Moreover, cell injury, significantly increased 72 h following both CONV- and FLASH-RT in a dose-dependent manner, indicating that both treatments induce damage to GL-261 and B16-F10 cells. However, FLASH-RT triggered more cell death at 4 Gy compared to CONV-RT. We next investigated the temporal (0 to 72 h post-irradiation) transcriptional activation of several genes involved in cell death, cell cycle arrest, senescence and autophagy observing that FLASH-RT induces greater transcriptional activation than CONV-RT 24h post-irradiation. Finally, different mitochondrial and cellular markers, and cancer metabolism were assessed using Flow Cytometry and Seahorse analysis highlighting the differences between FLASH and CONV mode and between non-tumoral control and cancer cells.