FLASH Radiotherapy with hIgh
Dose-rate particle beAms

# FRIDA WP1 – Radiobiology

## *Overview of LNS-TIFPA in vitro and in vivo experiments*

## IBSBC-CNR (LNS-CT) & CIBIO (TIFPA)

FRIDA conclusive meeting
4 March 2026

**Giusi Forte, PhD**
**IBSBC-CNR (Cefalù, PA) & INFN-LNS (Catania)**

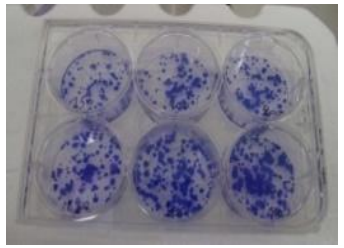
# WP1 – Radiobiology
# IBSBC-CNR (LNS-CT) & CIBIO (TIFPA)

*In vitro characterization of FLASH-dependent biological effects*

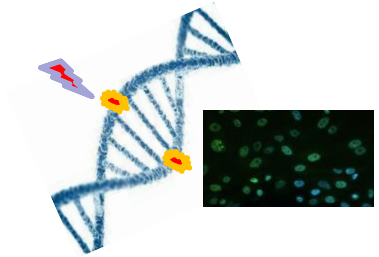
- **Cell models and Experimental Conditions**

| **Cell Lines:** | **Electron Dose Rate:** | **Dose range:** | **Oxygen Concentration:** |
|---|---|---|---|
| Non tumorigenic MCF10A<br>Tumorigenic MDA-MB-231 | Conventional: 6 Gy/min<br>FLASH: 230 Gy/s | 2-4-6-9-15 Gy | 20% [$O_2$] Normoxia |

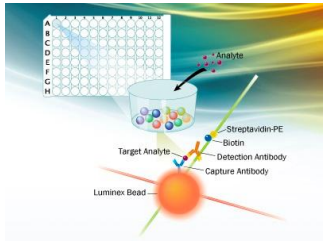
- **Biological endpoints**

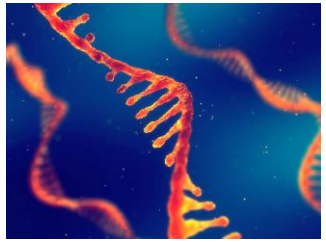


**1)** Cell survival by clonogenic survival assay.

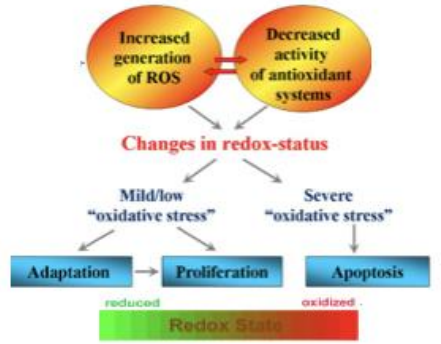


**2)** DNA damage analysis 30', 1h, 24 h post irradiation with 2 Gy and 5 Gy.



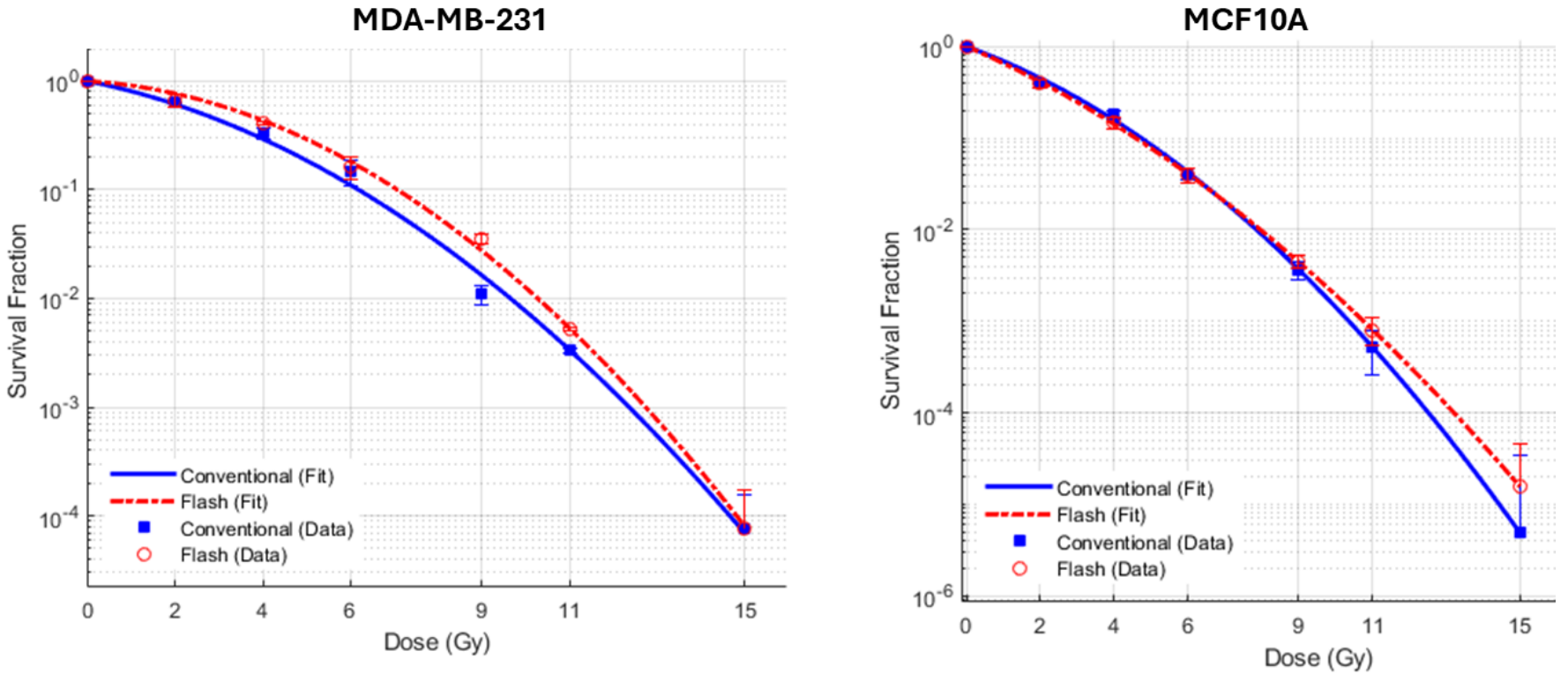



**3)** Molecular analysis, cells were irradiated with 2, 9, 15 Gy (*conv. Vs FLASH*). Conditioned medium and pellets were collected at 24h, 48h and 72h post RT for immunological and gene expression profiling




**4)** GSH/GSSG ratio at 9 and 15 Gy (*conv. Vs FLASH*)

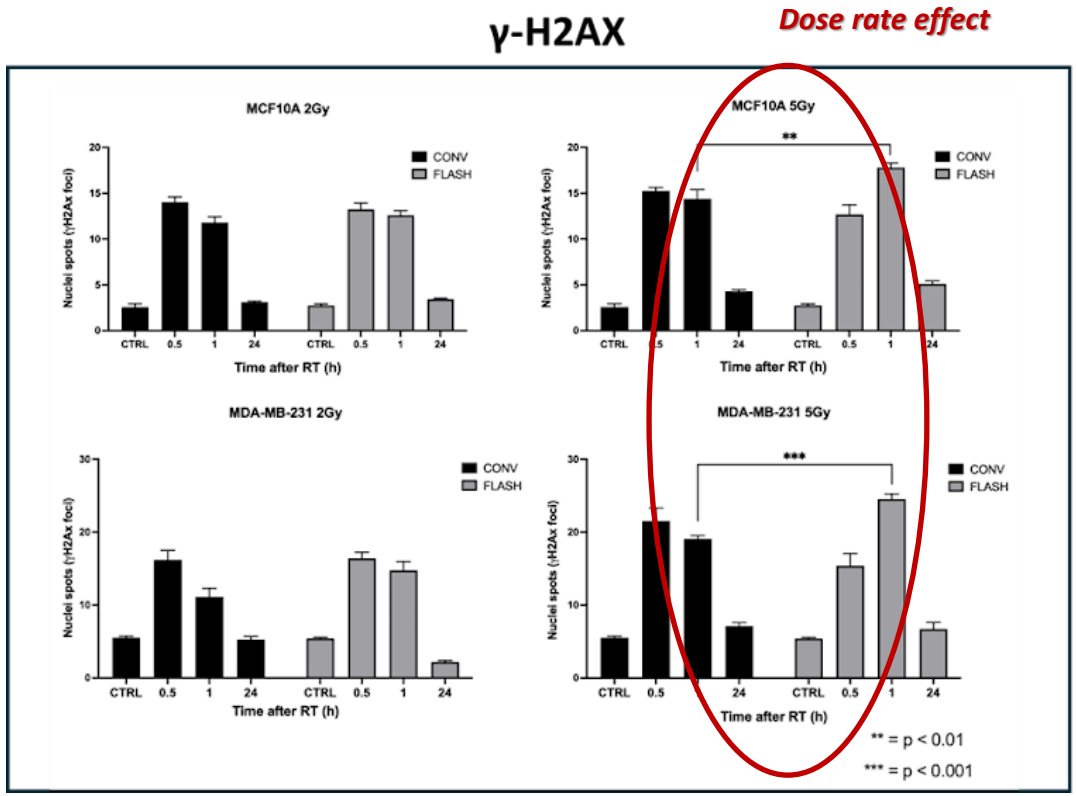
## *Survival curves*

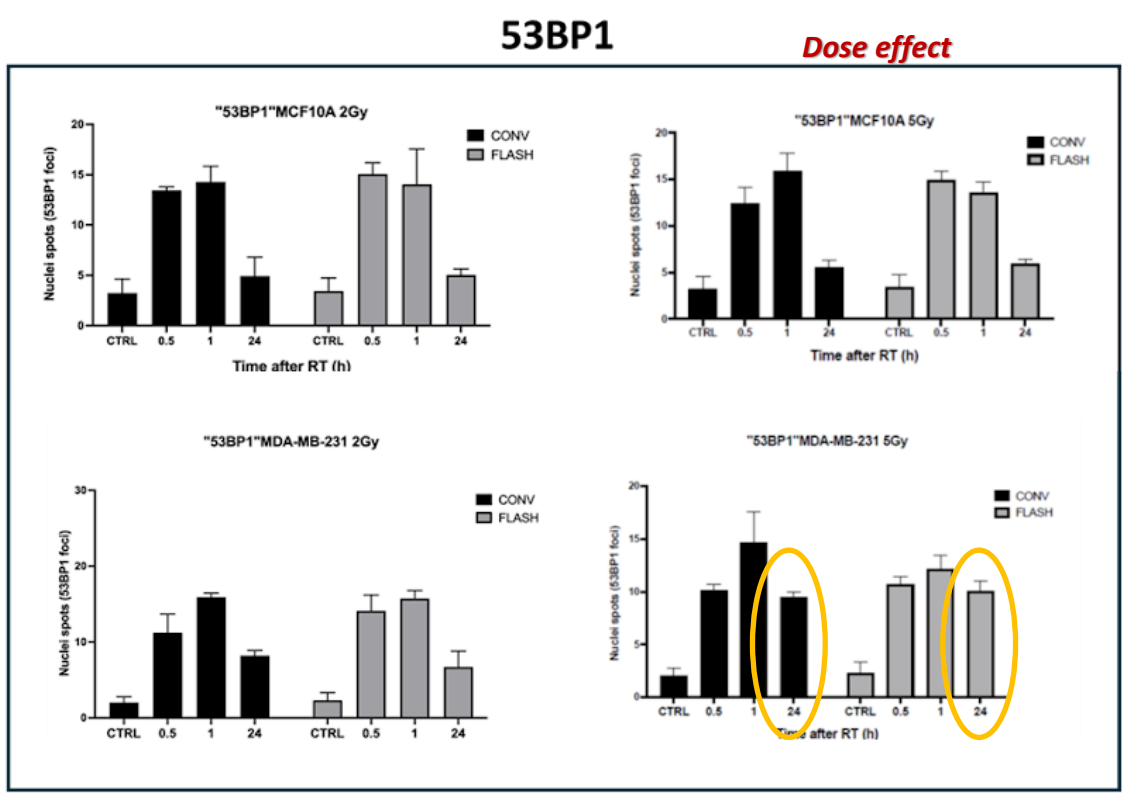



Average curves obtained using mean of fitting values from surivial curves resulting from two indipendent experiments for each cell lines (four replicates for each dose).

- No significant differences in survival induced by FLASH-RT (230 Gy/s) vs Conv-RT (6 Gy/min), on MCF10 (non – tumorigenic) and MDA-MB-231 (tumorigenic triple negative BC) cell lines in normoxic conditions.
- Previous experiments on MDA-MB-231 conducted in normoxic condition by the group of Adrian et al. (2021) showed increasing survival in responce to FLASH-RT **using 800 Gy/s**

# DNA Damage

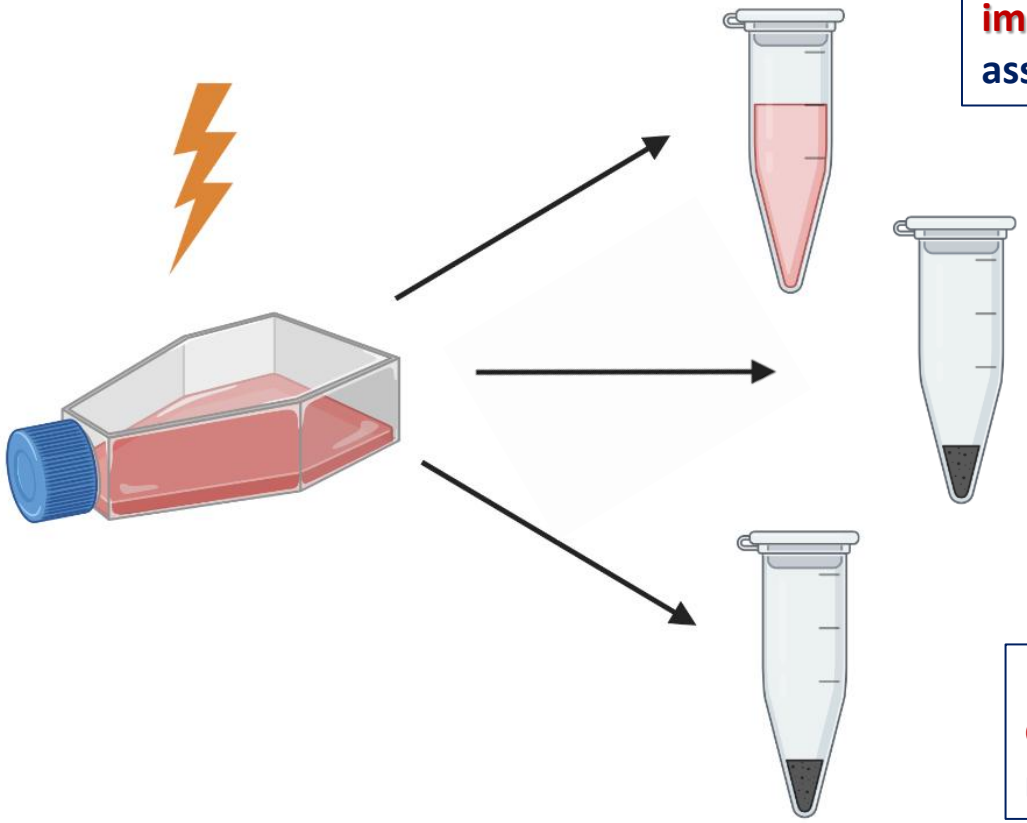



A significant major presence of γ-H2AX foci in 5 Gy treated samples with FLASH RT 1h post treatment respect to conv RT, not observable for 53BP1. Overall, no significant differences in DNA damages induced by the two dose rates at 24h.

# Molecular investigations

Electrons FLASH and CONV



Conditioned media were collected at 24h, 48h and 72h post RT for comparison of **immunological profiling** by using Luminex assay (stored -80°C)

Pellets were collected at 24h, 48h and 72h post RT for **gene expression profiling** by using NGS (stored -80°C)

Pellets were collected at 24h post RT for **GSH/GSSG ratio quantification** by using metabolomics methods (stored -80°C)

# Gene expression profiling

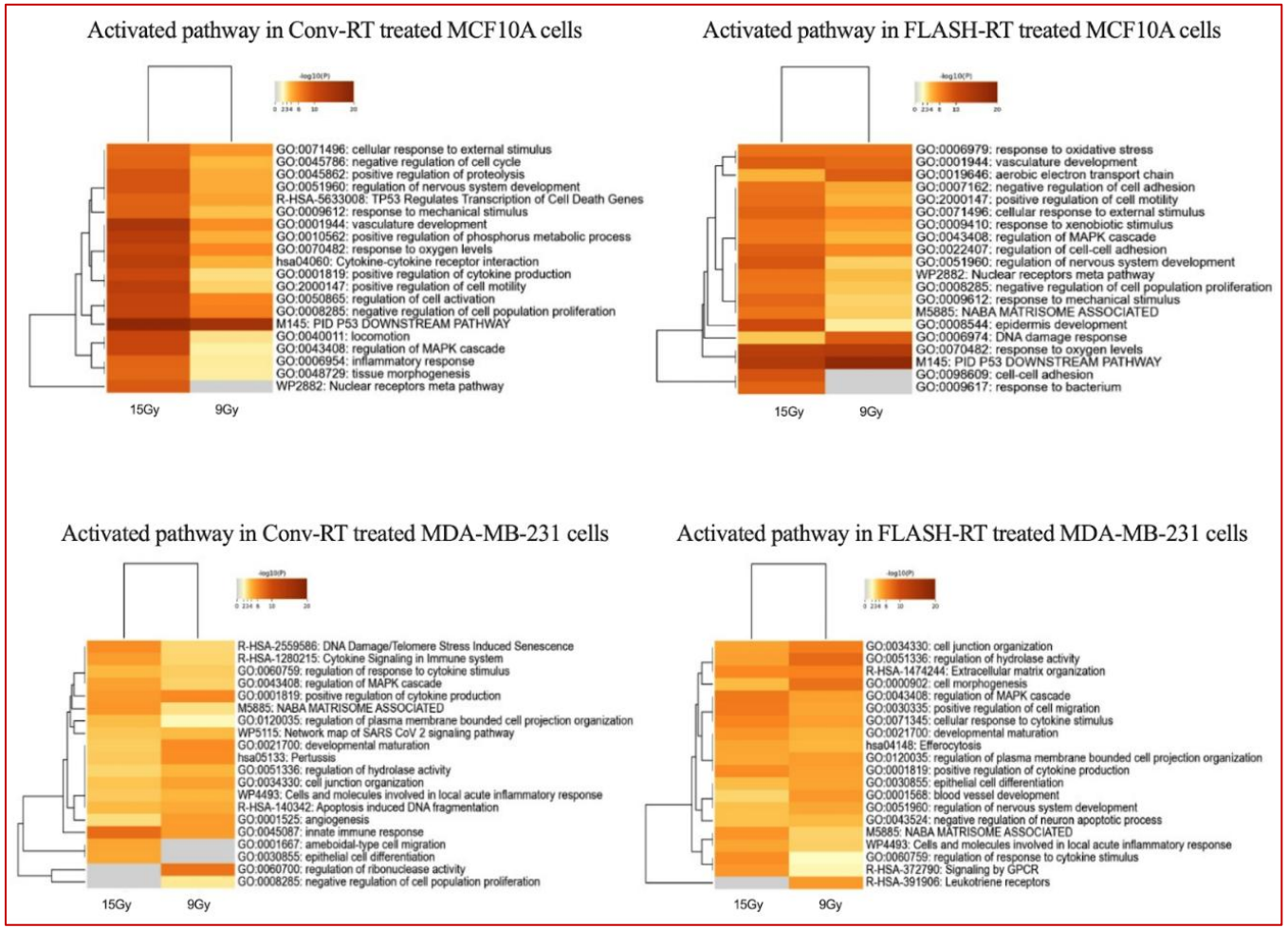
**TABLE 1**

**Treated vs Untreated comparisons**

| MCF10 | Down | Up | Total |
|---|---|---|---|
| MCF10_ 9 Gy Conv-RT vs untreated | 38 | 179 | 217 |
| MCF10_ 15 Gy Conv-RT vs untreated | 58 | 335 | 393 |
| MCF10_ 9 Gy FLASH-RT vs untreated | 51 | 233 | 355 |
| MCF10_ 15 Gy FLASH-RT vs untreated | 67 | 448 | 515 |
| **MDA-MB-231** | **Down** | **Up** | **Total** |
| MDA-MB-231_ 9 Gy Conv-RT vs untreated | 28 | 121 | 149 |
| MDA-MB-231_ 15 Gy Conv-RT vs untreated | 29 | 198 | 227 |
| MDA-MB-231_ 9 Gy FLASH-RT vs untreated | 29 | 240 | 269 |
| MDA-MB-231_ 15 Gy FLASH-RT vs untreated | 25 | 263 | 288 |

**FLASH-RT vs Conv-RT comparisons**

| MCF10 | Down | Up | Total |
|---|---|---|---|
| MCF10_ 9 Gy FLASH-RT vs 9 Gy Conv-RT | 0 | 18 | 18 |
| MCF10_15 Gy FLASH-RT vs 15 Gy Conv-RT | 0 | 6 | 6 |
| **MDA_MB-231** | **Down** | **Up** | **Total** |
| MDA-MB-231_ 9 Gy FLASH-RT vs 9 Gy Conv-RT | 0 | 13 | 13 |
| MDA-MB-231_15 Gy FLASH-RT vs 15 Gy Conv-RT | 0 | 0 | 0 |

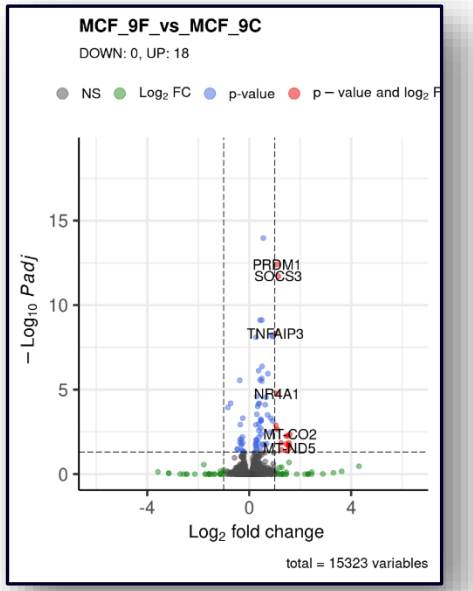


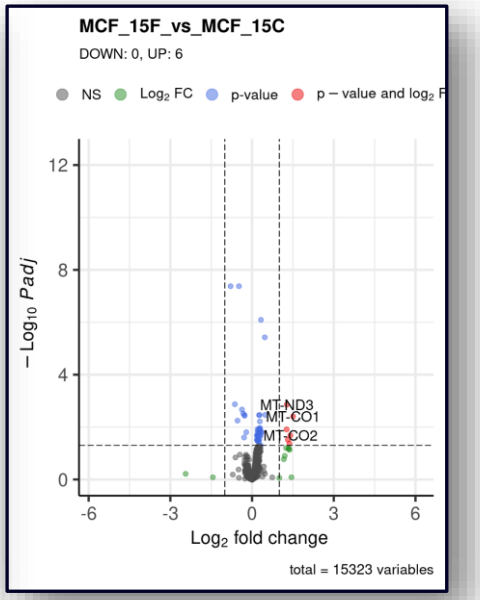

The gene expression profiling revealed a major number of activated genes in response to FLASH RT, especially with the higher dose.

# *MCF10 Gene expression profiling*

## MCF10 **FLASH-RT** vs **Conv-RT**

| Name | Total | Unique | Color Code |
|---|---|---|---|
| MCF_15F_vs_MCF_15C | 6 | 6 | |
| MCF_9F_vs_MCF_9C | 18 | 18 | |




## MCF10 UP-REGUALTED GENES BY FLASH- *vs* CONV-RT -

| MCF10 9Gy FLASH vs CONV | | | | MCF10 15 Gy FLASH vs CONV | | | |
|---|---|---|---|---|---|---|---|
| symbol | description | pvalue | diffexpressed | symbol | description | pvalue | diffexpressed |
| PRDM1 | PR/SET domain 1 | 5,60188E-17 | UP | MT-ND3 | mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 3 | 1,98E-06 | UP |
| SOCS3 | suppressor of cytokine signaling 3 | 4,21704E-16 | UP | MT-CO1 | mitochondrially encoded cytochrome c oxidase I | 1,06E-05 | UP |
| TNFAIP3 | TNF alpha induced protein 3 | 2,07804E-12 | UP | MT-CO3 | mitochondrially encoded cytochrome c oxidase III | 5,07E-05 | UP |
| NR4A1 | nuclear receptor subfamily 4 group A member 1 | 2,12273E-08 | UP | MT-CO2 | mitochondrially encoded cytochrome c oxidase II | 0,00014 | UP |
| IL6 | interleukin 6 [Source:HGNC Symbol;Acc:HGNC:6018] | 3,72232E-06 | UP | MT-CYB | mitochondrially encoded cytochrome b | 0,000248 | UP |
| NR4A2 | nuclear receptor subfamily 4 group A member 2 | 7,06213E-06 | UP | MT-ND4L | mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4L | 0,000408 | UP |
| MT-CO2 | mitochondrially encoded cytochrome c oxidase II | 1,50046E-05 | UP | | | | |
| MT-CYB | mitochondrially encoded cytochrome b | 1,94524E-05 | UP | | | | |
| MT-CO1 | mitochondrially encoded cytochrome c oxidase I | 1,98701E-05 | UP | | | | |
| MT-ND4L | mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4L | 6,09557E-05 | UP | | | | |
| MT-CO3 | mitochondrially encoded cytochrome c oxidase III | 6,4526E-05 | UP | | | | |
| MT-ND2 | mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 2 | 8,36561E-05 | UP | | | | |
| MT-ATP6 | mitochondrially encoded ATP synthase membrane subunit 6 | 9,69581E-05 | UP | | | | |
| MT-ND1 | mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1 | 0,000103213 | UP | | | | |
| MT-ND4 | mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4 | 0,000111211 | UP | | | | |
| MT-ND5 | mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 5 | 0,000166754 | UP | | | | |
| MYLIP | myosin regulatory light chain interacting protein | 0,000223644 | UP | | | | |
| MT-ATP8 | mitochondrially encoded ATP synthase membrane subunit 8 | 0,000274814 | UP | | | | |

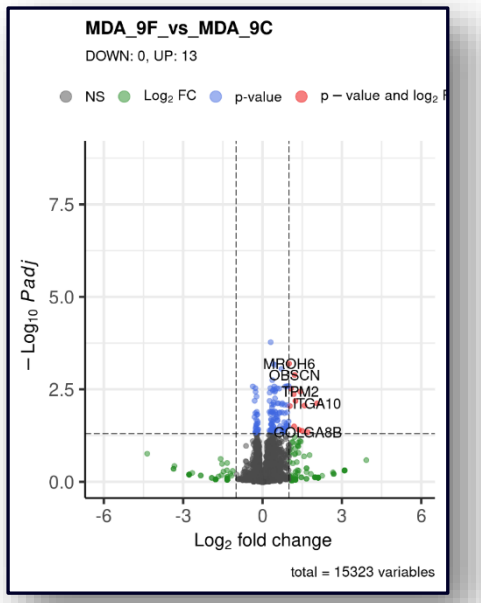
Up regulation of mitocondrial genes concerning aerobic cellular metabolism

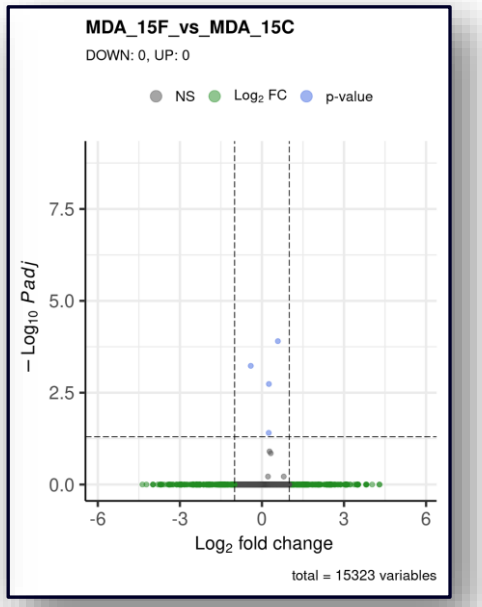
Not QUANTITATIVE but QUALITATIVE differences in FLASH vs conv- RT activated genes

# *MDA-MB-231 Gene expression profiling*

MDA-MB-231 **FLASH-RT** vs **Conv-RT**

| Name | Total | Unique | Color Code |
|---|---|---|---|
| MDA_15F_vs_MDA_15C | 0 | 0 | |
| MDA_9F_vs_MDA_9C | 13 | 13 | |



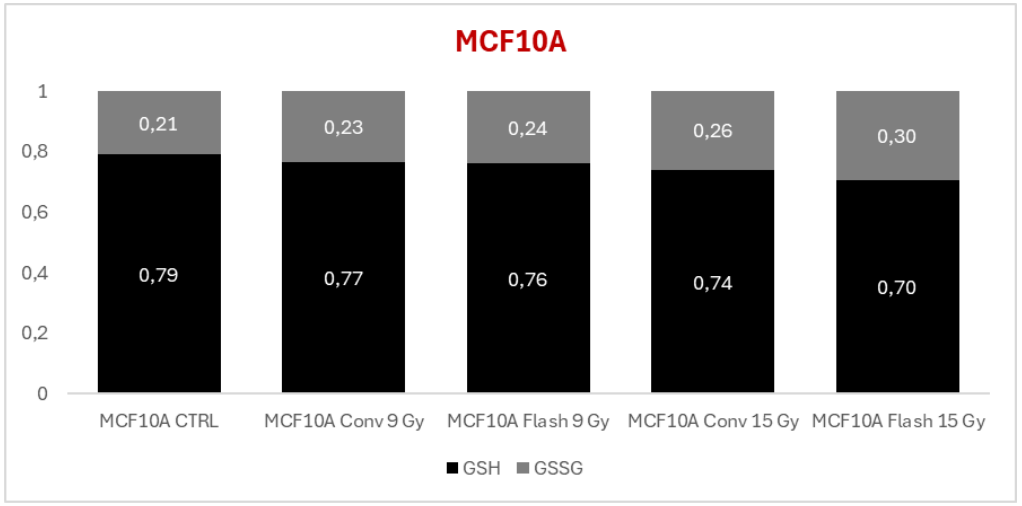

MDA-MB-231 UP-REGUALTED GENES BY FLASH- *vs* CONV-RT -

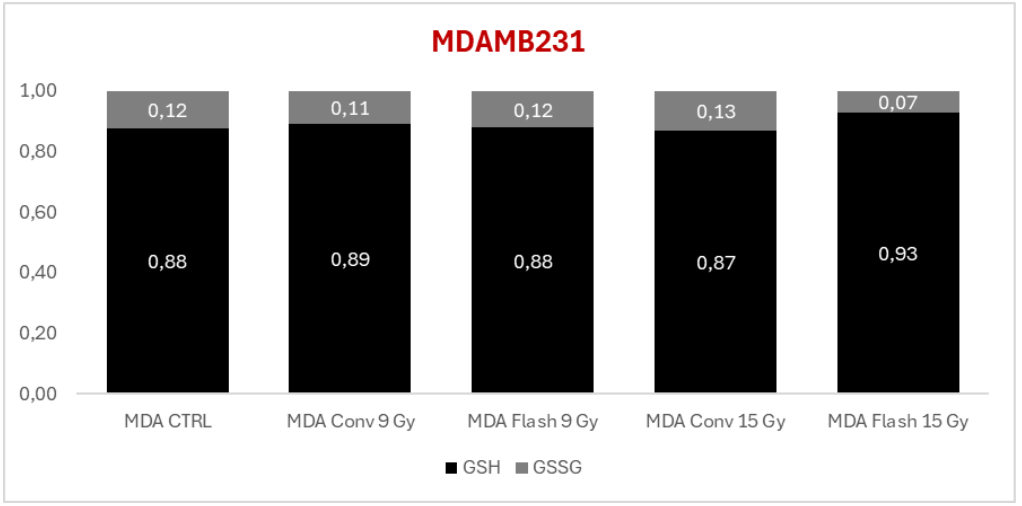
| MDA-MB-231 9Gy FLASH vs CONV | | | |
|---|---|---|---|
| **symbol** | **description** | **pvalue** | **diffexpressed** |
| MROH6 | maestro heat like repeat family member 6 | 1,36843E-07 | UP |
| OBSCN | obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF | 6,51718E-07 | UP |
| FBXW9 | F-box and WD repeat domain containing 9 | 3,87945E-06 | UP |
| TPM2 | tropomyosin 2 | 5,72543E-06 | UP |
| PLXNB3 | plexin B3 | 7,89776E-06 | UP |
| MAPK8IP3 | mitogen-activated protein kinase 8 interacting protein 3 | 1,40913E-05 | UP |
| ITGA10 | integrin subunit alpha 10 | 1,96274E-05 | UP |
| TTLL3 | tubulin tyrosine ligase like 3 | 2,67791E-05 | UP |
| WDR27 | WD repeat domain 27 | 2,81398E-05 | UP |
| IL18BP | interleukin 18 binding protein | 0,00021973 | UP |
| CDK5RAP3 | CDK5 regulatory subunit associated protein 3 | 0,000320429 | UP |
| RNF207 | ring finger protein 207 | 0,00038352 | UP |
| GOLGA8B | golgin A8 family member B | 0,000457947 | UP |

Up regulation of structural and signal transduction genes

Not QUANTITATIVE but QUALITATIVE differences in FLASH vs conv- RT activated genes

# *GSH/GSSG ratio*

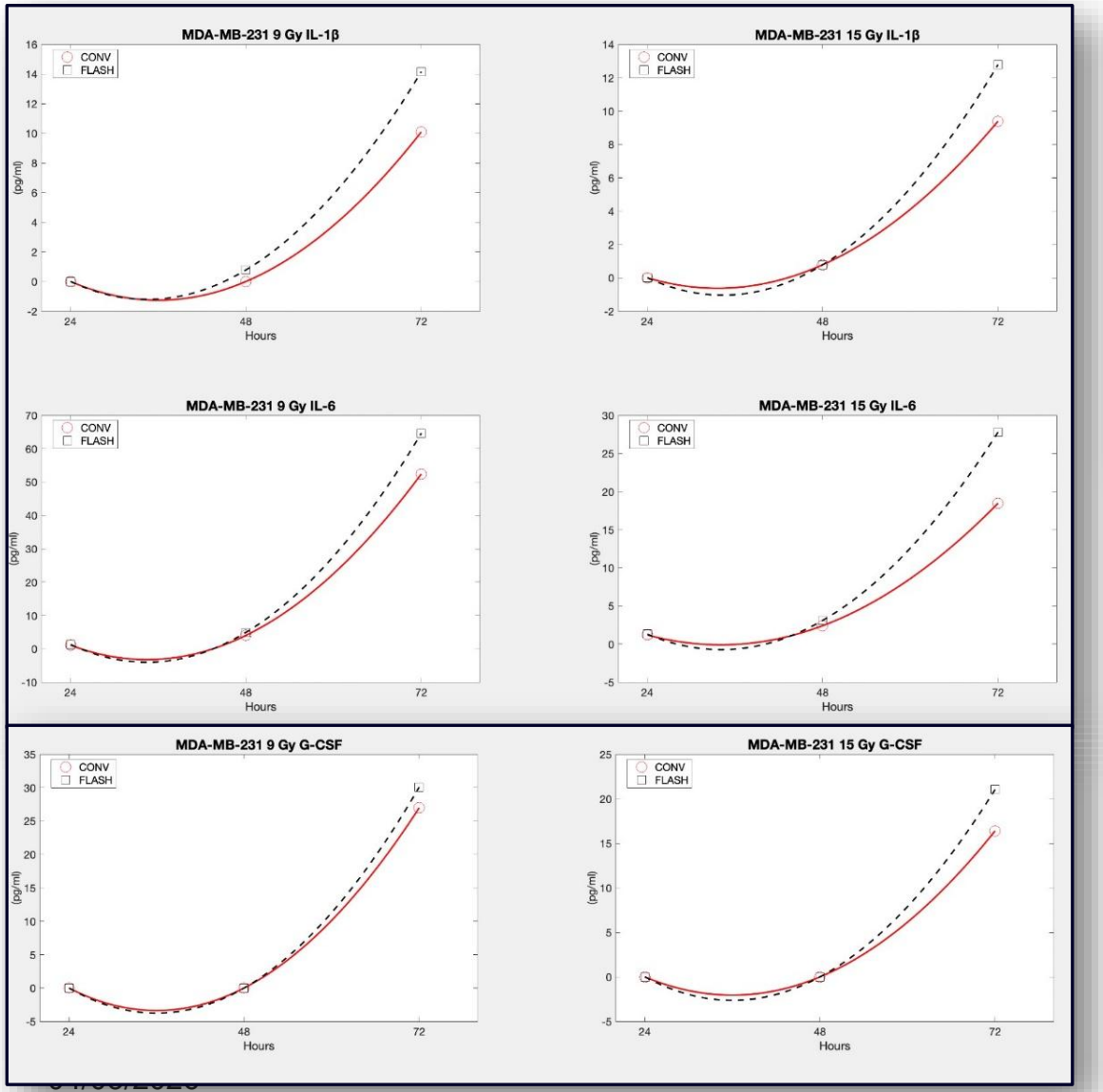


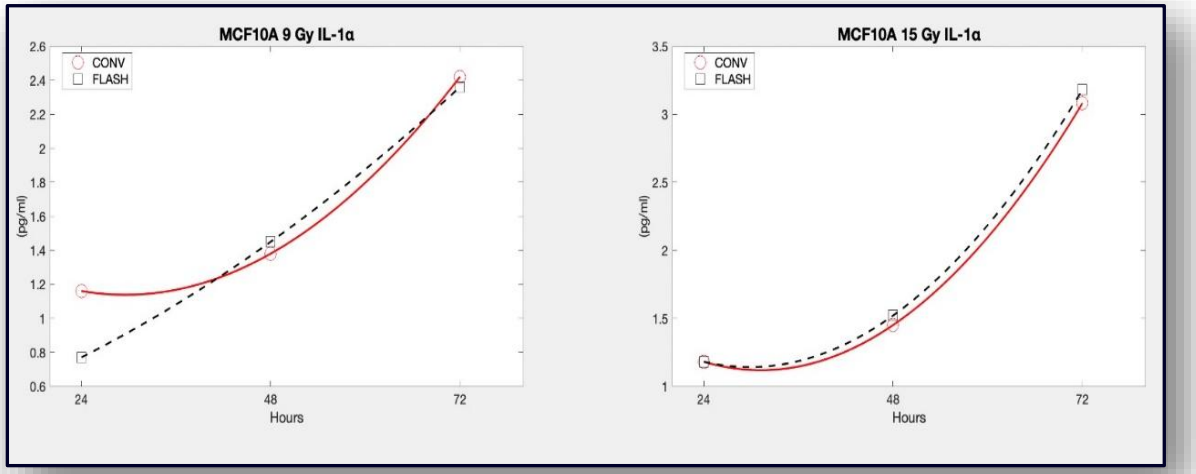





Percentage distribution of GSH/GSSG intracellular content expressed in nmol/mg of total protein from 6 biological samples of MCF10A and MDA-MB-231 cell lines, collected **24 hours post RT treatment**.

# *Immunological profiling*




The immunological profiling revealed a different kinetics of some inflammatory cytokines (IL-6, IL-1b, G-CSF, MCP-1, TGF-β1, TGF-β2) in MDA-MB-231, with increasing levels in response to higher doses of 9 and 15 Gy of FLASH-RT especially in late time points.

No differences were observed for MCF10 cells in conv vs FLASH-RT irradiation

## *Evidences from in vitro results in normoxic conditions*

CONV-RT

Dose rate:
6 Gy/min

FLASH-RT

Dose rate:
230 Gy/sec

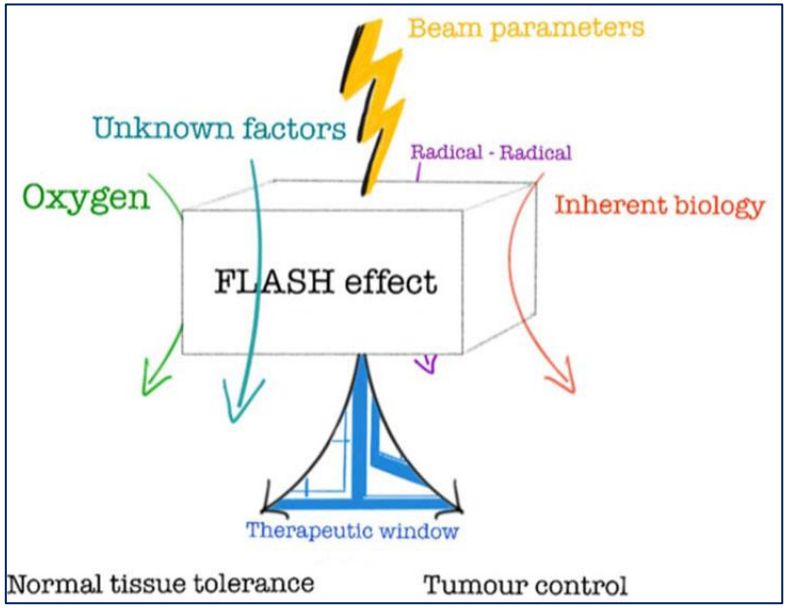
oteins
expression)

The final balance of biological processes could be affected by increased gene and protein expression, however there is cell and tissue dependence of the response

# Conclusive remarks

Our study provided **novel insights into the early biological effects induced by very high dose-rates in normoxic conditions, within 24 hours post-treatment**. Specifically,

- ✓ The DNA repair kinetics showed significantly greater γ-H2AX foci within 1 hour post treatment with 5 Gy of FLASH-RT in the MDA-MB-231 and MCF10A cell lines, which was reversed later at 24 hours.
- ✓ The healthy MCF10A transcriptomic response 24 hours post FLASH-RT was featured by the upregulation of mitochondrial respiratory chain genes, revealing an early response to oxidative stress caused by FLASH-RT.
- ✓ The GSH/GSSG balance was not affected at 24 hours in both cell lines.
- ✓ Increased rate of gene expression and cytokine production in response to FLASH-RT, as sign that the dose rate effect is perceived as a more intense stimulus by both the cell lines
- ✓ No difference in survival curves were obtained after 10 days post treatment by clonogenic assay, suggesting that the dose-rate effect may modulate the early cellular response.
- ✓ Experiments performed on Zebrafish embryos reveled a tissue dependence of FLASH effect appearance.




*Adrian G et al. Exp Rev in Mol Med (2022) 24, e10, 1-12*

# Updates on Results from Zebrafish experiments

cancers

MDPI

Article

**Investigating the Influence of Conventional vs. Ultra-High Dose Rate Proton Irradiation Under Normoxic or Hypoxic Conditions on Multiple Developmental Endpoints in Zebrafish Embryos**

Alessia Faggian [1,2,†], Gaia Pucci [3,†], Enrico Verroi [4], Alberto Fasolini [1], Stefano Lorentini [4,5], Sara Citter [1,5], Maria Caterina Mione [2], Marco Calvaruso [3], Giorgio Russo [3], Emanuele Scifoni [1,4], Giusi Irma Forte [3], Francesco Tommasino [1,4,*,‡] and Alessandra Bisio [2,4,‡]

## Experimental conditions and setting

***In vivo* model:**
Zebrafish embryos (WT AB), up to 120 hpf

**Oxygen Concentration:**
- ~20 % [$O_2$], Normoxia
- ~2,5 % [$O_2$], Hypoxia

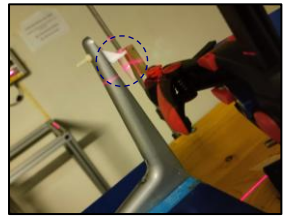
**Proton Dose Rate:**
- Conventional (0,25 Gy/sec)
- FLASH (270 Gy/sec)

**Dose range:**
30 Gy

**Radiation Modifier:**
Curcumin (5 µM)

*Embryo handling and positioning* ***in* 0,5 mL eppendorf tubes filled with:**
- **300 µL E3 *medium***
- **embryos (1,5 mm thickness)**
- **200 µL *Low Melting Agarose* (LMA), 1 %**


*Hypoxia induction* ***by the use of an hypoxic chamber (0.1% $O_2$)***



**Irradiation site:**
**Trento Proton Beam Line Facility**

**After irradiation, embryos were separated 1/well into 96-well plates for subsequent daily analysis**

## Analysis (until 120 hpf)

**From 48 hpf**
- **Mortality rate (%)**
- **Malformed embryos rate (%)**

**From 72 hpf**
- **Morphological alterations rate (%) [1]**
- **Morphometric measurements (mm) [2]**

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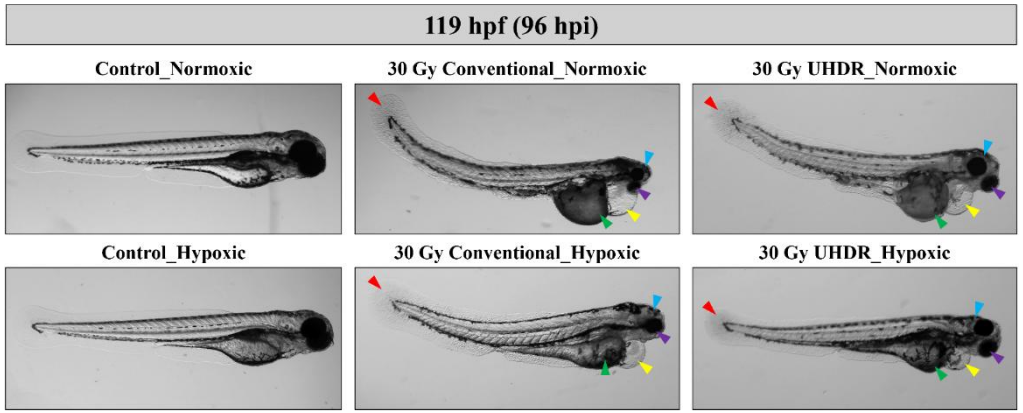
# Results:



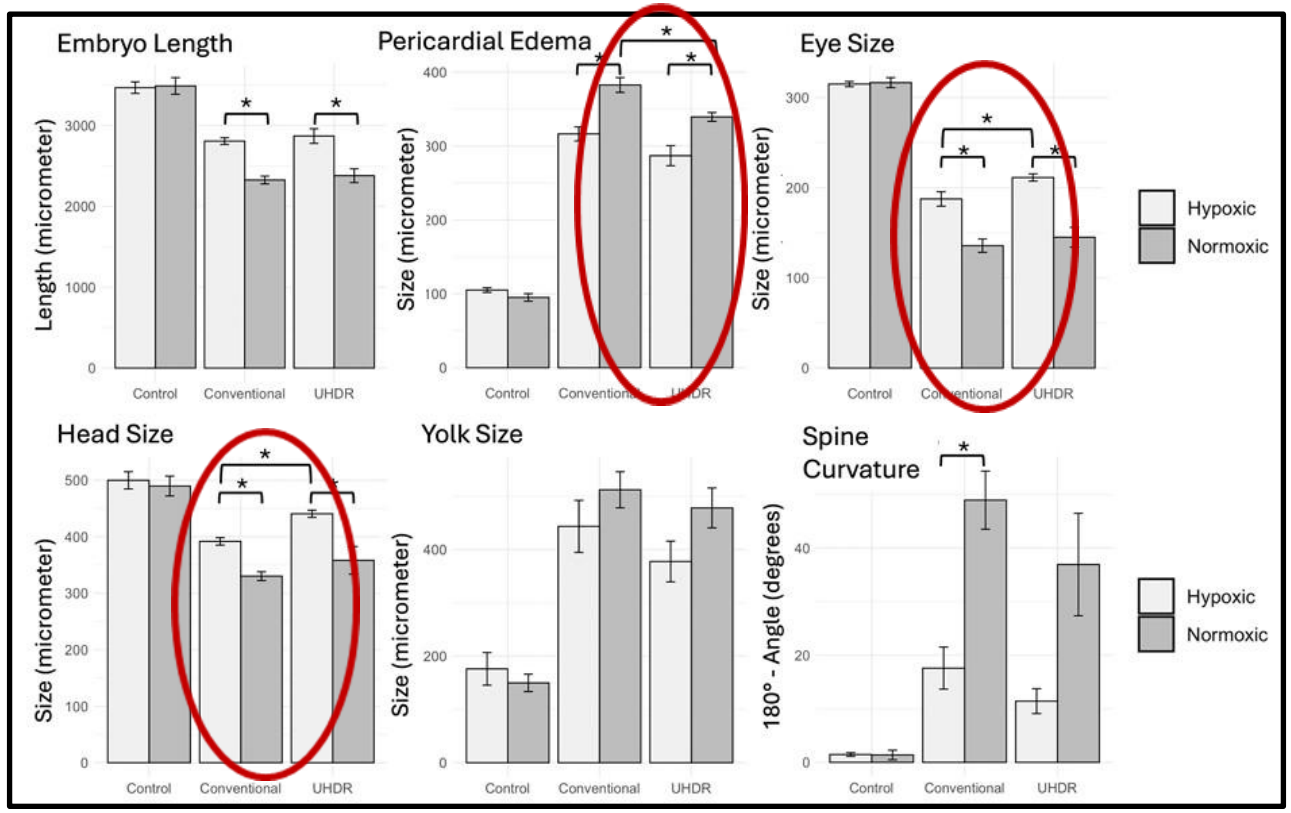

**General trend of significantly reduced toxicity after exposure to a UHDR vs CONV regimes:**

- ✓ **Differences are significant for the levels of PE induced by a UHDR *vs* CONV irradiation in normoxic conditions, and for eye and head size in hypoxic conditions;**
- ✓ **The toxicity scoring analysis shows a tendency toward a protective effect of the UHDR, with a lower percentage of embryos in the high score categories.**

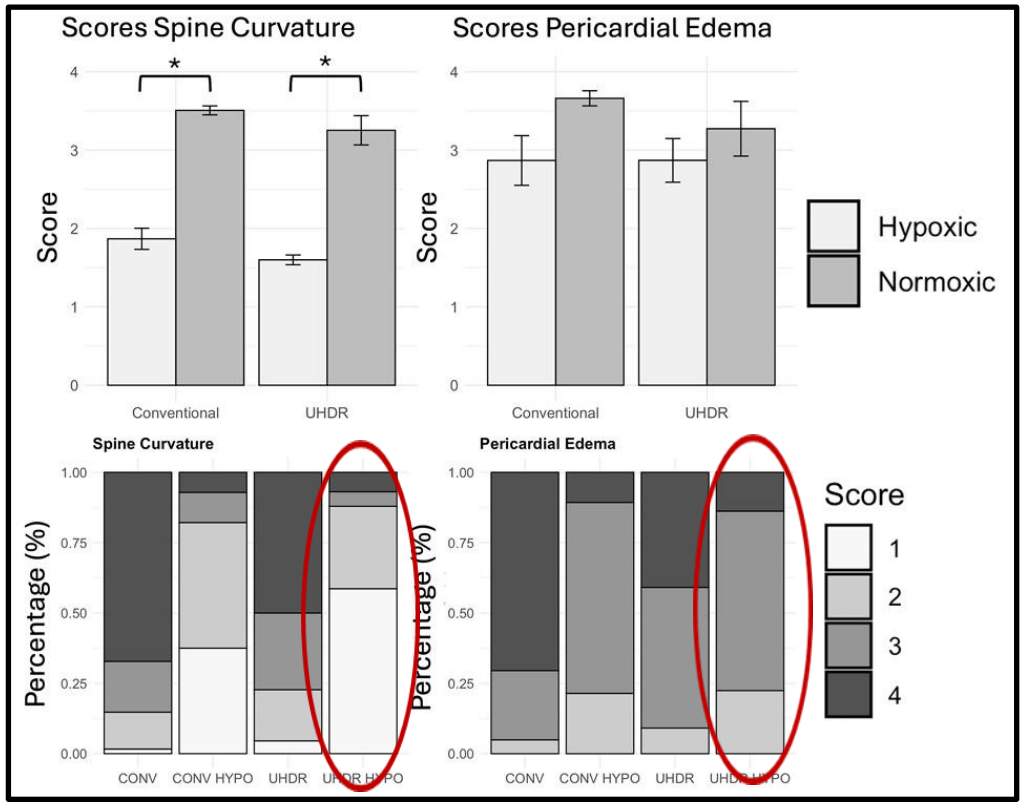
FUTURE

- **Gene expression analysis**
- **Role of as curcumin**

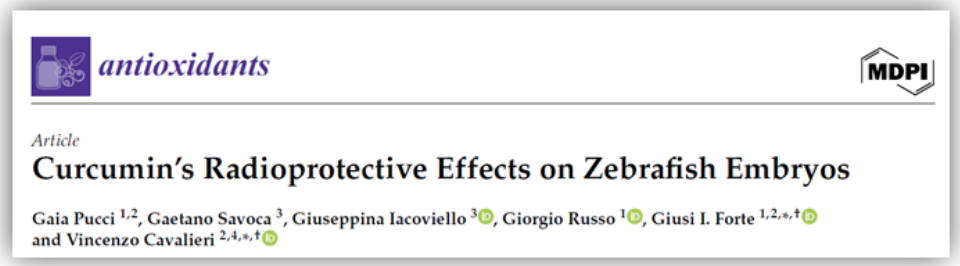
***Quantitative comparisons of malformations***




***Average toxicity scores and percentage distribution***



# Updates on Results from Zebrafish experiments


antioxidants

MDPI

Article

Curcumin's Radioprotective Effects on Zebrafish Embryos

Gaia Pucci [1,2], Gaetano Savoca [3], Giuseppina Iacoviello [3], Giorgio Russo [1], Giusi I. Forte [1,2,*,†] and Vincenzo Cavalieri [2,4,*,†]

Future

Paper in preparation on the Curcumin radioprotective effects on UHDR-RT treated zebrafish embryos

## Experimental conditions and setting

***In vivo* model:**
Zebrafish embryos (WT AB), up to 120 hpf

**Oxygen Concentration:**
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**Dose range:**
10 and 30 Gy

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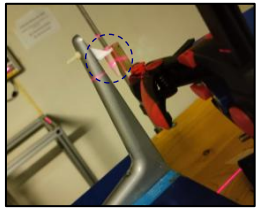

*Embryo handling and positioning* ***in*** **0,5 mL eppendorf tubes filled with:**
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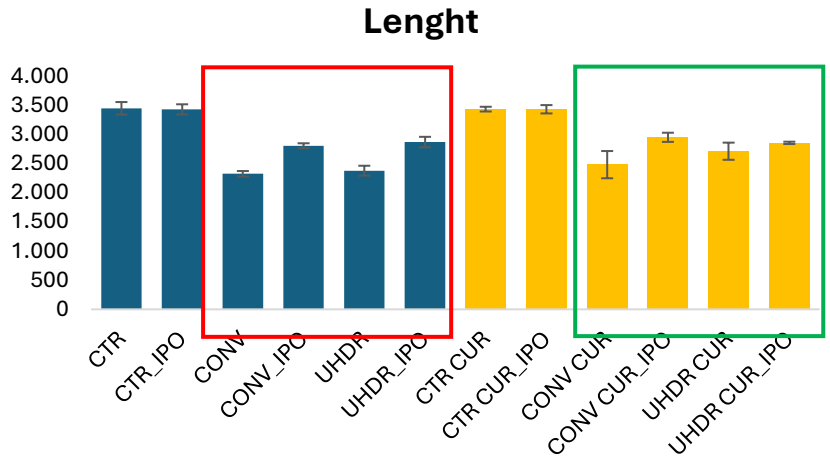
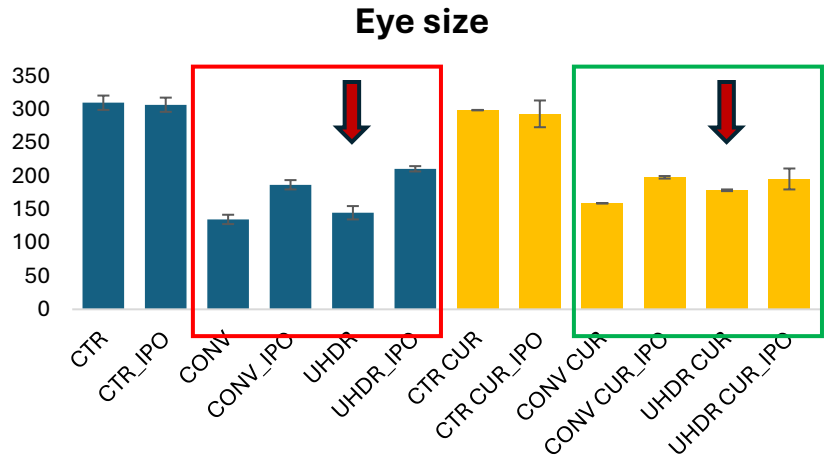
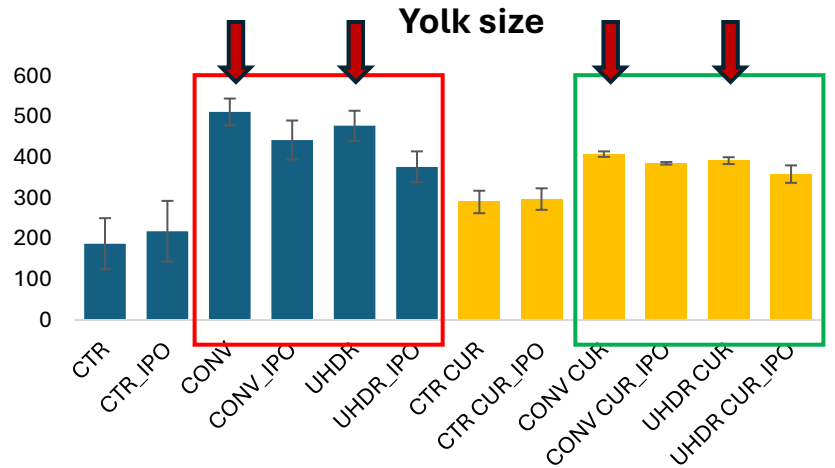
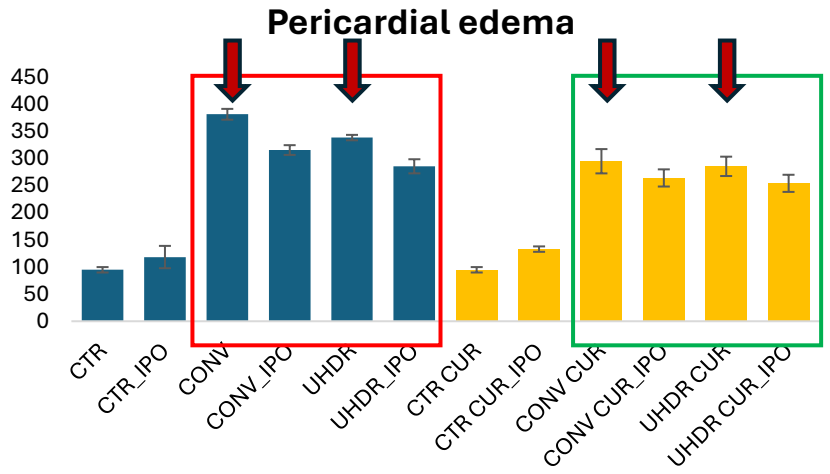
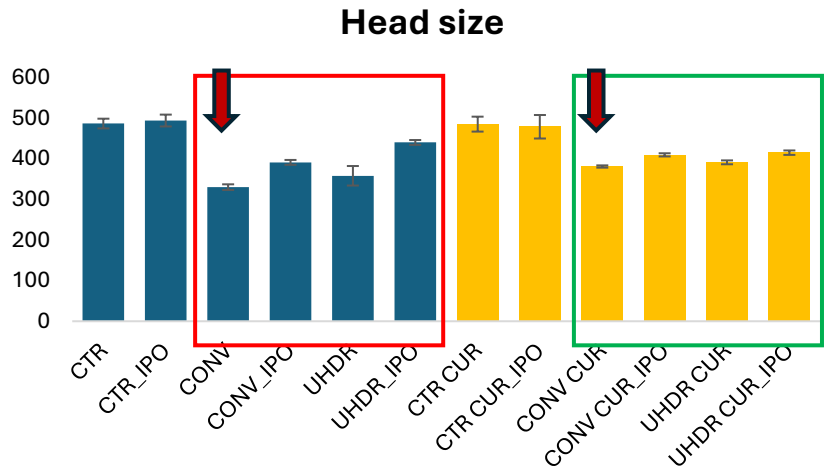



*The curcumin contribution can be observed in association with both CONV and FLASH irradiation; It appears greater in normoxic settings. It has more performances for yolk size and pericardial edema. Molecular analysis in progress*

# Comprensione dei meccanismi fisici e radiobiologici: l'effetto FLASH

Anthem

Ministero dell'Università e della Ricerca | Italiadomani | PNC

UNIVERSITÀ degli STUDI di CATANIA

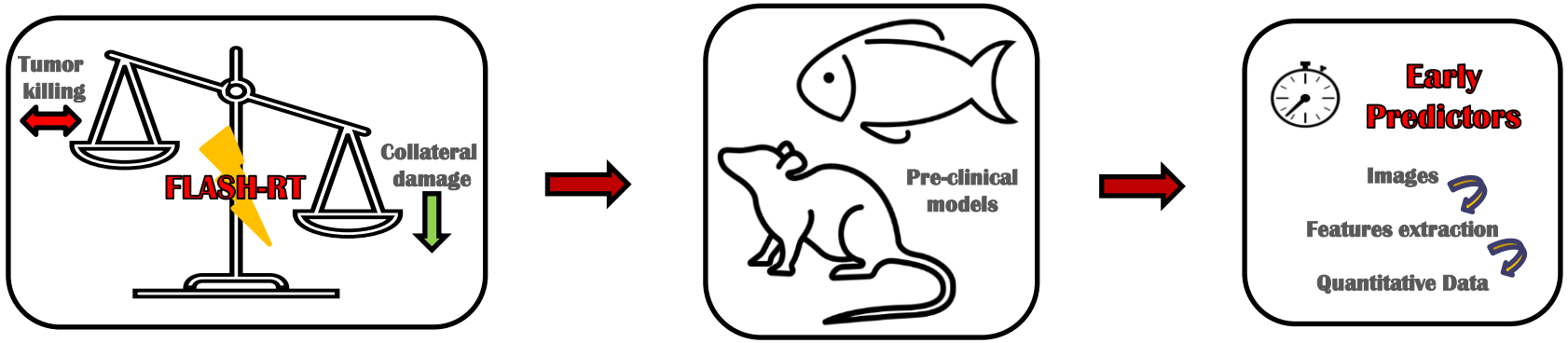
## *UnderstandinG the radIobiOlogical effeCts Of bNct anD flAsh therapy (GIOCONDA) (R&I)*

***A project responding to a call from Spoke 4 of the ANTHEM project inherently the B1 topic***

*In Sept. 2024*

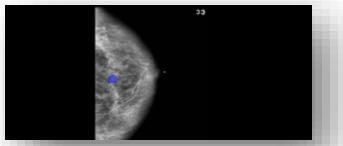
### *WP3 – Radiobiological insight into biological mechanisms and doseeffect relation in FLASH radiotherapy (CNR- IBSBC)*

**Objective 1 (RI)** Toxicity evaluation on preclinical models using radiomic tools to extract quantitative early predictors of FLASH effects




**Objective 2 (SS)** Modifying MATradiomic tool for its use in a Treatment Planning Software (TPS) for FLASH irradiation

matRadiomics



Università degli Studi di Napoli Federico II

Università di Pavia

Consiglio Nazionale delle Ricerche
Istituto di Bioimmagini e Sistemi Biologici Complessi

# Comprensione dei meccanismi fisici e radiobiologici: l'effetto FLASH

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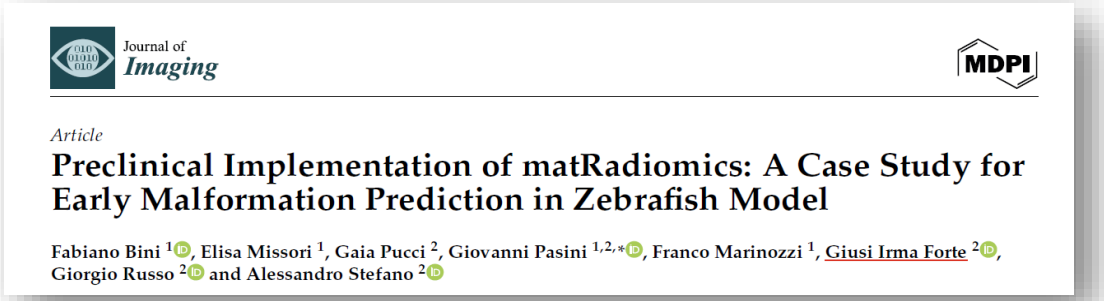


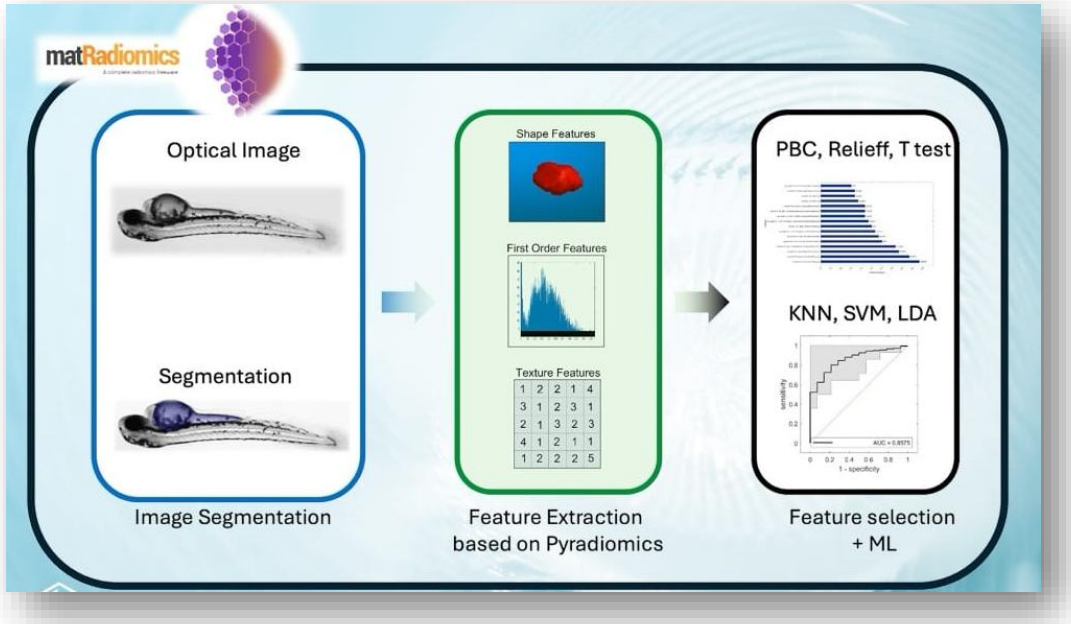
Journal of *Imaging*

MDPI

*Article*

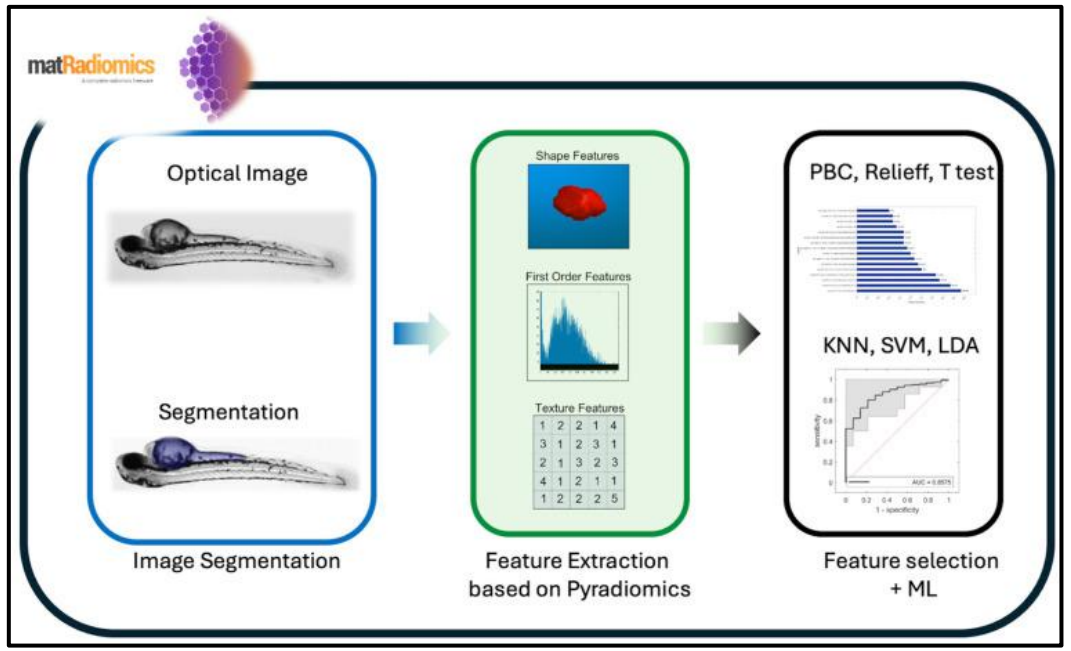
**Preclinical Implementation of matRadiomics: A Case Study for Early Malformation Prediction in Zebrafish Model**

Fabiano Bini [1], Elisa Missori [1], Gaia Pucci [2], Giovanni Pasini [1,2,*], Franco Marinozzi [1], Giusi Irma Forte [2], Giorgio Russo [2] and Alessandro Stefano [2]



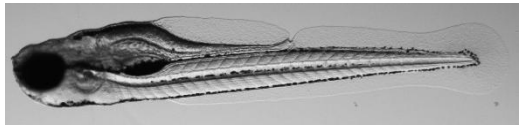
# First Results:

This study extends matRadiomics to preclinical settings and validates it through a case study focused on early malformation differentiation in a zebrafish model




CASE STUDY

To distinguish High (82 imm) vs Low (61 imm) malformation, in images of zebrafish larvae at an early time point of 72 hours post fertilization (hpf), following RT treatment at 30 Gy



Control


High Malformation

Low Malformation

RESULTS

| Mask Type | Images | Selected Features | AUC (ML) |
|---|---|---|---|
| All_fish |  | original_shape_SurfaceArea | 0.723 (SVM) |
| Heart |  | original_glrlm_GrayLevelNonUniformity | 0.704 (LDA) |
| Head |  | original_shape_MinorAxisLength | 0.708 (KNN) |
| Eye |  | original_gldm_DependenceNonUniformityNormalized | 0.706 (SVM) |
| Yolk |  | original_shape_MinorAxisLength | 0.693 (LDA) |
| Length |  | original_glszm_ZoneVariance, original_shape_MeshVolume | 0.718 (SVM) |

- The best result was obtained on the **whole-fish mask**, probably because the scoring method used evaluates the entire larva
- matRadiomics has a great potential as decision support tool in preclinical research

NEXT STEP

Extraction of features as quantitative early predictors of FLASH effects on healthy tissues:

- CONV (179 imm) vs FLASH (162 imm), at 72 hpf
- Are there any features that significantly differentiate embryos irradiated in CONV vs FLASH mode?
- In which anatomical regions will we find them?
- Will there be any consistency with what was seen at 119 hpf in our previous work (*Faggian A et al., 2025*)?

# Prodotti del Progetto FRIDA

**Tesi di dottorato:**

- "Analysis of the effects of innovative radiotherapy treatment in Zebrafish". Dottoranda Gaia Pucci del XXXV ciclo di dottorato dell'Università di Palermo.

**Comunicazioni a congresso:**

- Pucci G, et al. "Curcumin enhances the tissue sparing effect induced by ultra-high dose rate proton irradiation in a zebrafish embryo model" FRTPT Conference, 4-6 December 2024, Rome.
- Pucci G et al. "FLASH effect evaluation on Zebrafish model". 48th ERRS Annual Meeting, 2024, Aveiro, Portugal.
- Pucci G, et al. "INVESTIGATION OF THE FLASH EFFECT DOSIMETRIC DEPENDENCE BASED ON A ZEBRAFISH EMBRYO MODEL". (SIRR 2024 - XXI Convegno Nazionale SIRR Società italiana per le Ricerche sulle Radiazioni, 14-16 Ottobre, Pavia.
- Faggian A, et al. "Exploring the impact of dose rate and hypoxia on the development of zebrafish embryos after proton irradiation" PTCOG conference 2-7 June 2025
- Pucci G, et. al. "Zebrafish (Danio rerio): a small preclinical model with great potential for radiobiological studies. From basic analyses to future applications." SIRR International Day: 2025 – Simulation and Biological Damage Prediction". Catania – 6 Ottobre 2025
- Pucci G, et al. " Zebrafish (Danio rerio): a small preclinical model with great potential for radiobiological studies on FLASH effect understanding". Workshop on "High Power Lasers for Fundamental Science and Applications" (HPLA2025) – Catania 17-19 Novembre 2025.

**Articoli scientifici:**

- Pucci G, et al. "Curcumin's Radioprotective Effects on Zebrafish Embryos". Antioxidants 2024, 13, 1281.
- Faggian A. et al. "Investigating the Influence of Conventional vs. Ultra-High Dose Rate Proton Irradiation Under Normoxic or Hypoxic Conditions on Multiple Developmental Endpoints in Zebrafish Embryos" Cancers 2025, 17, 2564.
- Minafra L. et. al "Molecular changes induced by FLASH irradiation in breast cell lines" paper submitted to Frontiers in Oncology 2026

# *Thank for your attention!!*

Dott. Russo G.
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Dott. Minafra L.
Dott.ssa Bravatà V.
Dott. Cammarata FP.
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