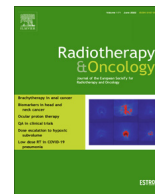




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## Original Article

## Pencil beam scanning proton FLASH maintains tumor control while normal tissue damage is reduced in a mouse model

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## ABSTRACT

**Purpose:** Preclinical studies indicate a normal tissue sparing effect when ultra-high dose rate (FLASH) radiation is used, while tumor response is maintained. This differential response has promising perspectives for improved clinical outcome. This study investigates tumor control and normal tissue toxicity of pencil beam scanning (PBS) proton FLASH in a mouse model.

**Methods and materials:** Tumor bearing hind limbs of non-anaesthetized CDF1 mice were irradiated in a single fraction with a PBS proton beam using either conventional (CONV) dose rate (0.33–0.63 Gy/s field dose rate, 244 MeV) or FLASH (71–89 Gy/s field dose rate, 250 MeV). 162 mice with a C3H mouse mammary carcinoma subcutaneously implanted in the foot were irradiated with physical doses of 40–60 Gy (8–14 mice per dose point). The endpoints were tumor control (TC) assessed as no recurrent tumor at 90 days after treatment, the level of acute moist desquamation (MD) to the skin of the foot within 25 days post irradiation, and radiation induced fibrosis (RIF) within 24 weeks post irradiation.

**Results:** TCD<sub>50</sub> (dose for 50% tumor control) was similar for CONV and FLASH with values (and 95% confidence intervals) of 49.1 (47.0–51.4) Gy for CONV and 51.3 (48.6–54.2) Gy for FLASH. RIF analysis was restricted to mice with tumor control. Both endpoints showed distinct normal tissue sparing effect of proton FLASH with MDD<sub>50</sub> (dose for 50% of mice displaying moist desquamation) of <40.1 Gy for CONV and 52.3 (50.0–54.6) Gy for FLASH, (dose modifying factor at least 1.3) and FD<sub>50</sub> (dose for 50% of mice displaying fibrosis) of 48.6 (43.2–50.8) Gy for CONV and 55.6 (52.5–60.1) Gy for FLASH (dose modifying factor of 1.14).

**Conclusions:** FLASH had the same tumor control as CONV, but reduced normal tissue damage assessed as acute skin damage and radiation induced fibrosis.

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Radiotherapy (RT) is an important part of treatment in clinical cancer therapy. A key challenge in RT is to maximize the therapeutic ratio; the balance between cure and toxicity of treatment, by increasing the radiation effect in the tumor, while minimizing damage to surrounding healthy tissue. FLASH radiotherapy, in which the radiation dose is delivered with ultrahigh dose rate, above 40 Gy/s, has received a lot of attention, since it was demonstrated to induce less damage to normal tissue relative to conventional dose rates, while the effect in the tumor was unaltered [1–3]. Most data so far have been focused on electron FLASH, but data with proton FLASH are emerging [4,5].

The normal tissue sparing effect has been demonstrated in animal models for a large panel of radiation toxicities using both early

and late endpoints [2]. These include early apoptosis in the lung [6] and intestinal crypt regeneration and gut function in mice [7], skin toxicities in mice [5,8–12] and mini-pigs [13], lung fibrosis in mice [6], and loss of neural stem cells and memory in mice [14].

Most of the current in vivo studies of the FLASH effect in normal tissues are single dose studies, from which it is not possible to quantify the FLASH factor, the dose modifying factor between conventional and FLASH dose rate. However, some studies with electron FLASH allow for dose modifying factor determination, such as Hendry et al. [10], Vozenin et al [13] and Ruan [15], demonstrating dose modifying factors in the range of 1.1–1.4. We recently conducted an in vivo study validating the effect of pencil beam scanning (PBS) proton FLASH on acute skin toxicity, and demonstrated that a 44–58% higher dose was needed to obtain the same biological response when using proton FLASH [5].

Current data on the effect of FLASH on tumor response have mainly focused on tumor regrowth and mouse survival data from

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tumor models from various cancer sites. For electron FLASH, this includes xenograft tumor models from mammary carcinoma, laryngeal carcinoma, glioblastoma and T-cell acute lymphoblastic leukemia, and syngeneic models of mouse lung carcinoma and murine glioblastoma [2,6,16,17]. For proton FLASH, the effect on tumor response has been demonstrated for tumor regrowth in syngeneic pancreatic flank tumors, in head and neck cancer models and in a sarcoma model [11,12,18]. Tumor control has not yet been studied as endpoint in a comparison study of FLASH effects. However, compared to tumor regrowth assays, tumor control is a more clinically relevant endpoint, and full dose–response curves are essential for a proper comparison between FLASH and conventional dose rate, CONV, effects.

The primary aim of the present study was to establish the effect of PBS proton FLASH on local tumor control, using a mouse mammary carcinoma. Furthermore, the level of normal tissue sparing effect with FLASH was compared between early skin damage and radiation induced fibrosis in the same animals.

## Method

### Animal and tumor models

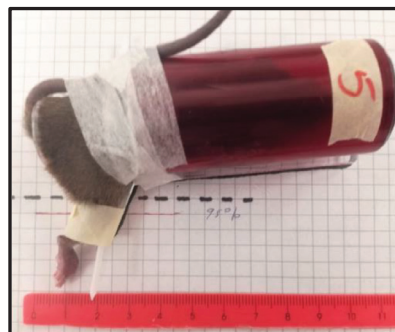
Experiments were performed on 16–20-week-old female CDF1 mice with the different ages distributed between the treatment groups. The tumor model used was the C3H mouse mammary carcinoma. Its derivation and maintenance has been described previously [19]. Experimental tumors were produced following sterile dissection of large flank tumors (1000–1200 mm<sup>3</sup>). Macroscopically viable tumor tissue was minced with a pair of scissors, and 5–10 µl of this material were injected subcutaneously using a Hamilton syringe in the foot of the right hind limb of the animals. The tumor take rate was 100%. Treatments were carried out when tumors had reached a tumor volume of about 200 mm<sup>3</sup>, which occurred 13–22 days after inoculation. To ensure no systematic difference of initial tumor growth in the treatment groups, the dose groups were randomly distributed. All experiments complied with the ARRIVE guidelines and were performed under the Institutional and National Guidelines for Animal Welfare. The study was approved by the local Experimental Institutional Review Board.

### Mouse setup and response

The procedure for radiation treatment has previously been described [5,20]. Irradiations were performed locally to the tumor bearing leg of non-anesthetized mice restrained in a Lucite jig. The tumor-bearing legs were exposed and loosely attached to the jig with tape, without impairing the blood supply to the foot (Fig. 1A). The mouse jigs were placed on a lucite plate fixed on the top of a water bath, with the target leg placed through a hole into the water bath (Fig. 1B). The temperature of the water bath was kept at 25 °C and monitored throughout the experiments. All mice were treated in the same time interval of the day (16:00–22:00). After treatment the leg was detached from the jig, and the mice were returned to their cage for follow up. Tumor growth was followed with tumor volume determined on a daily basis using the formula  $D_1 * D_2 * D_3 * \pi / 6$ , where the  $D$  values represent the three orthogonal diameters.

The response of tumors to treatment was assessed using a local tumor control assay, where the mice were followed at weekly intervals up to 90 days post-treatment. Response was calculated as the percentage of animals in each dose group showing no recurrent tumor at 90 days after treatment. Mice with tumor regrowth were sacrificed.

(A)



(B)



**Fig. 1.** Setup of mice for treatment. (A) Tumor bearing mouse restrained in jig. (B) Three mice are irradiated at the time with individual fields. The mouse body is shielded with brass.

In each experiment, extra mice were set up as back up mice. The tumor growth of these were followed and is presented as control mice in [Supplementary Fig. 1](#).

The skin toxicity was assessed in the irradiated mice using a previously published skin score table [21] as described in [5] ([Supplementary Table 1](#)). The skin damage in an area covering the whole foot including the tumor was evaluated, and scored in steps of 0.5, with 3.5 being the maximum value. The mice were observed on a week-daily basis between 11 and 25 days following treatment, and the percentage of animals in each dose group with a score of 1.5, 2.0, 2.5, 3.0 or 3.5 was determined.

Radiation-induced fibrosis (RIF) was assessed in the irradiated mice using a modification of the leg contracture model described by Stone [22]. Based on the degree of extensibility of the irradiated leg, the endpoint for RIF was defined as a permanent reduction in extensibility of at least 75% relative to the untreated leg. This endpoint was considered to correspond to severe irreversible subcutaneous fibrosis. The extensibility of the irradiated legs was measured every second week from Day 30 to 24 weeks after irradiation. Mice that died before developing fibrosis were not included in the analysis.

A total of 162 mice allocated to different dose groups were irradiated in four separate experiments with doses in the range of 40.4–60.2 Gy for FLASH and 40.1–59.7 Gy for CONV. FLASH and CONV were treated on the same days. The selected dose levels were based on previous studies of response for this tumor model [20]. For all three assays, all scorings and evaluations were performed blinded to the given dose and modality.

### Dose delivery

All treatments were performed with proton PBS at the fixed horizontal proton beam line at the Danish Centre for Particle Therapy at Aarhus University Hospital (ProBeam, Varian Medical Systems, Palo Alto, CA, USA). The mouse setup and treatment fields have been described previously [5] and will only be summarized briefly here. The beam energy was 244 MeV for CONV and 250 MeV for FLASH. Up to three mice with 3.5 cm center-to-center separation were irradiated per round (Fig. 1.B). The irradiated mouse leg was placed in the entrance plateau of the beam in a treatment depth of 13.5 cm with the mouse bodies shielded from direct radiation by a brass block. The entrance surface of the water bath was aligned with the isocenter plane using positioning lasers. For both CONV and FLASH, each mouse was irradiated with a  $2 \times 3$  cm field that consisted of  $5 \times 7$  spots with 5 mm vertical separation and 5.1 mm horizontal separation. The CONV field was delivered in clinical mode with a low beam current of 1.1 nA and beam pauses of a few milliseconds between each spot [5]. The spot pattern was repainted every 0.12 Gy to reduce the field dose rate and improve the robustness to mouse leg motion. The number of repaintings was 327–488 depending on the dose level.

The FLASH field was delivered without repainting using a requested beam current of 215 nA in a research mode based on a prototype integrated ultra-high dose rate beam monitoring and control system [23].

For absolute dose determination, the dose of both CONV and FLASH fields at the mouse depth were measured with an Advanced Markus chamber that was cross-calibrated against a reference Roos ionization chamber in a 244 MeV beam under reference setup conditions. The Advanced Markus chamber showed minimal ion recombination effects for FLASH, and the applied ion recombination correction factor  $k_s$  was in the range 1.001–1.002. The conversion factors from monitor units (MU) to dose measured with the Advanced Markus chamber were used to determine the mouse dose assuming reproducible beam delivery for both CONV and FLASH. Advanced Markus chamber measurements showed that the scattered dose to a mouse caused by irradiation of its neighbor mouse in the water bath was approximately 1.5% for CONV and 1.7% for FLASH. This dose contribution was neglected in this study.

For all irradiations, the duration of each spot delivery was extracted from machine log files [24]. The field dose rate was determined as the total dose divided by the total field duration. The duration of the beam pauses between spots for CONV was not directly available in the machine log files. Based on previous measurements it was assumed to be 4.3 ms. The local PBS dose rate for 95% dose,  $DR_{PBS95\%}$ , was defined as 95% of the dose in a point divided by the time interval between reaching 2.5% and 97.5% of the dose in the point [25]. For FLASH irradiations,  $DR_{PBS95\%}$  was determined in the plane of the mouse leg target by simulating the treatment deliveries using logged spot durations as explained in [5]. For CONV irradiations,  $DR_{PBS95\%}$  was essentially equal to the field dose rate because repainting distributed the dose delivery in any point over the total field delivery time.

Alanine *in vivo* dosimetry was performed for each individual mouse using one alanine pellet placed in the middle of the field at the beam entrance on the water bath and another alanine pellet, wrapped in parafilm, placed with double-sided tape on the mouse leg holder. The measured alanine entrance dose was compared with the planned mouse foot dose scaled to the beam entrance position using scaling factors established in experiments with simultaneous measurements of the entrance dose by alanine and the mouse depth dose with an Advanced Markus Chamber. The measured alanine dose on the mouse leg holder was compared directly with the planned mouse foot dose.

The alanine pellets (Batch BX608 from Harwell Dosimeters) consisted of 91% L- $\alpha$ -alanine powder and 9% paraffin wax. The pellets had 4.8 mm diameter, 2.8 mm height and a mass density of 1.15 g/cm<sup>3</sup>. Electron paramagnetic resonance (EPR) measurements were carried out on a Bruker EMXmicro spectrometer operating at 9.53 GHz [26]. The EPR signal from each alanine pellet was corrected for pellet mass, temperature during irradiation and fading between irradiation and read-out. A beam quality correction factor of  $k_Q = 1.022$  [27] was applied to correct for the difference in alanine response between the proton beam plateau and the <sup>60</sup>Co calibration beam quality.

### Data analysis

Dose response curves for both tumor control and normal tissue toxicity were fitted to the data using logit analysis. Differential response was assessed using a Fisher's exact test with  $p < 0.05$  as significance level. The dose to give a 50% response (TCD<sub>50</sub>, MDD<sub>50</sub> or FD<sub>50</sub>) was used to assess the FLASH dose modifying factor.

### Results

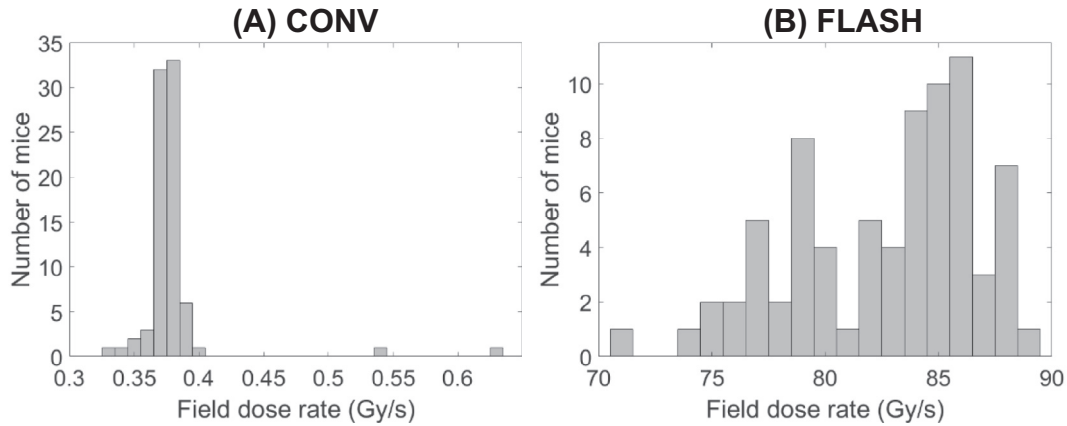
One CONV mouse was excluded immediately after treatment since only 55% of the treatment was delivered due to technical problems. Furthermore, the CONV irradiation was interrupted and then resumed after 24 minutes and 26 minutes for two other mice. One of these mice did not receive the last 1.4% of its treatment (0.76 Gy). These two mice were both included in the study. Three FLASH mice, which were treated in the same round, were excluded because a technical error resulted in a much higher dose than intended.

For CONV, the field duration was 95–173 s and the average field dose rate was 0.38 Gy/s (range: 0.33–0.63 Gy/s). The field dose rate was stable in the range 0.33–0.40 Gy/s for all CONV treatments except two cases with higher field dose rates of 0.54 Gy/s and 0.63 Gy/s (Fig. 2A). The field duration for FLASH was 0.46–0.84 s and the average field dose rate was 83 Gy/s (range: 71–89 Gy/s) (Fig. 2B). In the region receiving 95% or higher dose, the mean PBS dose rate  $DR_{PBS95\%}$  varied from 171 Gy/s to 214 Gy/s across the FLASH mice. Within the region receiving 95% or higher dose,  $DR_{PBS95\%}$  ranged from 71% to 115% of its mean value [5].

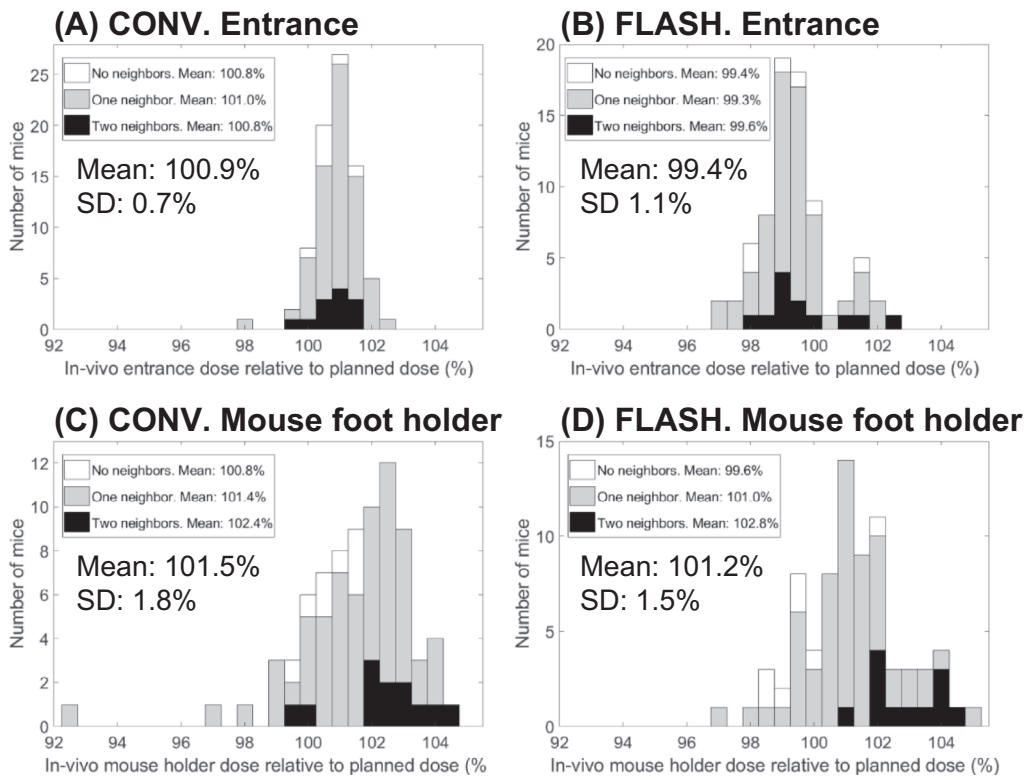
*In vivo* doses were measured with alanine pellets in the entrance and on the mouse foot holder for all treatments except for two missing entrance dose measurements and three missing mouse foot holder dose measurements. The mean entrance alanine doses agreed with the planned mouse dose scaled to the beam entrance position within 1% (Fig. 3A–B). The mean alanine doses on the mouse foot holder agreed with the planned mouse dose within 1% for mice irradiated without contaminating dose contributions from neighboring mice, while the alanine dose was approximately 1% and 2% higher for mice with one and two co-irradiated neighbor mice, respectively (Fig. 3C–D).

Tumor response was assessed as tumor control: no recurrent tumor at 90 days after treatment. Of the included mice, 9 FLASH treated and 7 CONV treated mice died during follow up before potential tumor regrowth. These mice were excluded from the analysis. The mice were either found dead or euthanized due to general bad state, and in 5 of these mice metastases were found. The final analysis included 72 CONV mice and 70 FLASH mice with 8–12 mice per dose group (Table 1).

Dose graded treatment groups were used, and the percentage of mice with tumor control in each dose group was calculated. Dose response curves are shown in Fig. 4A. The TCD<sub>50</sub> values with 95% confidence interval were 49.1 (47.0–51.4) Gy for CONV dose rate,



**Fig. 2.** Distribution of field dose rates for (A) conventional dose rate (CONV) and (B) FLASH dose rate treatments.



**Fig. 3.** Alanine-measured dose. Distributions of in vivo alanine entrance doses (A-B) and mouse foot holder doses (C-D) relative to the planned doses for conventional dose rate (CONV) and FLASH. Different colors indicate the distribution for mice irradiated with zero, one or two neighboring mice. The numbers specify the mean and standard deviation (SD) of the distributions.

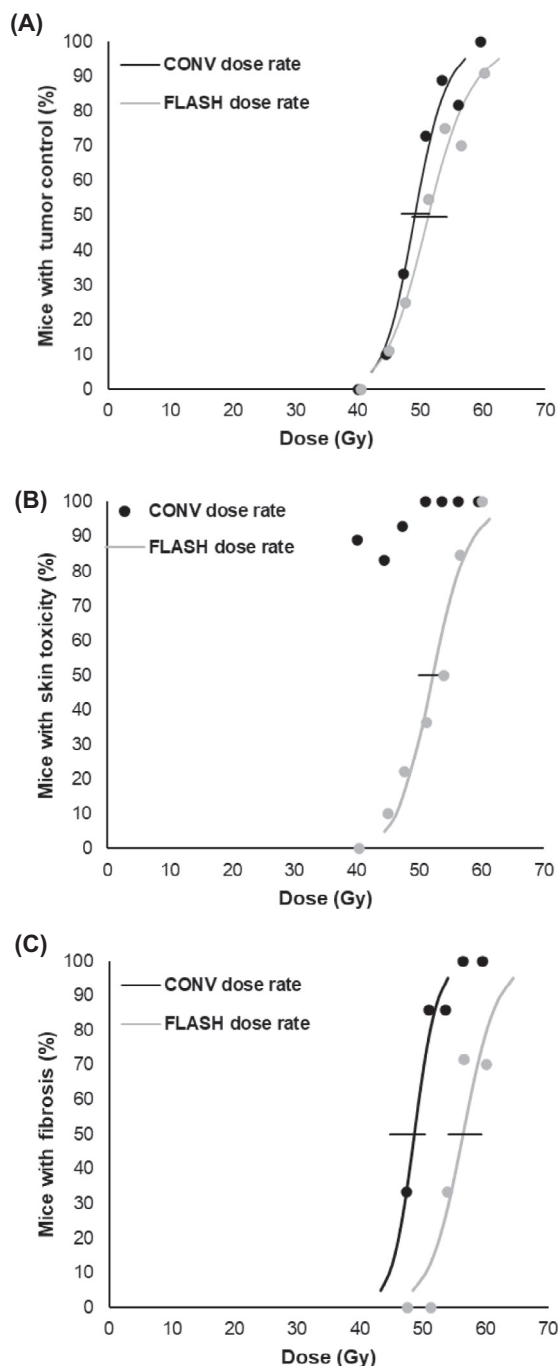
**Table 1**

No of mice included in the analysis of tumor control, skin toxicity and fibrosis.

Dose	Tumor assay		Skin toxicity assay		Fibrosis assay	
	FLASH	CONV	FLASH	CONV	FLASH	CONV
40	9	8	9	9	0	0
44	9	10	10	12	0	0
47	8	12	9	14	4	3
51	11	11	11	11	5	7
54	12	9	12	11	9	7
56	10	11	13	12	7	9
60	11	11	11	11	10	10
Total	70	72	75	80	35	36

and 51.3 (48.6–54.2) Gy for FLASH dose rate. The two dose response curves are not significantly different ( $p = 0.50$ ).

One tumor in the FLASH arm, treated with the highest dose of 60.2 Gy, displayed an unexpected growth after treatment, resembling the untreated control tumors (Supplementary Fig. 1). Both the entrance and mouse holder alanine doses showed correct beam delivery and no explanation could be found in the documentation of the experiment. Therefore, the tumor was included in the dataset, although a geographical miss is suspected as the mouse may have retracted its leg out of the field. This is leading to the data set in the FLASH arm not reaching 100% tumor control (Fig. 4A). Excluding the mouse from the analysis would lead to the TCD<sub>50</sub> for the FLASH arm changing to 51.1 (48.5–54.3) Gy and would not change the conclusion.



**Fig. 4.** Dose dependency of local tumor control and normal tissue toxicity. (A) Dose response curves of tumor control at either conventional dose rate (CONV) or FLASH dose rate. The 95% Confidence intervals are indicated at  $TDC_{50}$ . (B) Percentage of mice in each dose group with acute damage to the skin at either CONV or FLASH dose rate (Score 3.5). The dose response curve is fitted for FLASH treated mice, with 95% confidence intervals indicated at  $D_{50}$ . (C) Percentage of mice in each dose group which have developed radiation induced fibrosis at either CONV or FLASH dose rate. Only mice with tumor control were included in the analysis. 95% confidence intervals are indicated at  $D_{50}$ .

#### Acute skin toxicity

Acute skin toxicity was assessed in the tumor bearing mice after treatment, and the percentage of animals in each dose group showing moist desquamation at different levels of the treated foot, at any time between day 11 and 25 of mice in each dose group was calculated. Two mice from the FLASH arm were excluded from

the analysis, one was euthanized 9 days after treatment due to tumor growth, and one was euthanized 10 days after treatment due to general bad state. One mouse from the CONV arm was euthanized 9 days after treatment due to general bad state. Fig. 4B displays the result for score 3.5. As the experiment was designed to assess tumor control, the doses used were not optimal to obtain skin toxicity data. In the CONV arm, all mice in the four dose groups with highest doses (50.8–59.7 Gy) displayed skin toxicity, while 83–93% of the mice in the three dose groups with lowest doses (40.1–47.2 Gy) displayed skin toxicity at score 3.5 (Fig. 4B). For this reason, it was not possible to fit a dose response curve for acute skin toxicity for mice irradiated with CONV, but it could be concluded that the  $MDD_{50}$  value (dose to produce moist desquamation in 50% of mice) was below the lowest CONV dose of 40.1 Gy. The dose response curve for the FLASH group was fitted, resulting in an  $MDD_{50}$  value with 95% confidence interval of 52.3 (50.0–54.6) Gy. The dose modifying factor was thus at least  $52.3/40.1 \text{ Gy} = 1.30$ .

For the lower scores of acute damage, 99–100% of the CONV treated mice displayed skin toxicity. For the FLASH treated mice, across the dose levels, 100%, 90%, 77%, 66% and 47% displayed skin score 1.5, 2.0, 2.5, 3.0 and 3.5, respectively (Supplementary Fig. 2).

#### Radiation induced fibrosis (RIF)

RIF was assessed in the remaining animals from day 30 up to 24 weeks after radiation. The percentage of animals in each dose group with severe fibrosis of the treated foot, at any time during follow up is shown in Fig. 4C. Table 1 shows the number of animals in each dose group that were available after 24 weeks and thus included in the dataset. For late tissue damage, the  $FD_{50}$  values (dose to produce severe fibrosis in 50% of mice) with 95% confidence interval were 48.6 (43.2–50.8) Gy for CONV and 55.6 (52.5–60.1) Gy for FLASH, resulting in a dose modifying factor 1.14. The two dose response curves are significantly different ( $p = 0.0001$ ).

To determine if the dose modifying factor for fibrosis was significantly different from the dose modifying factor for acute skin toxicity, a previous published dataset for acute skin toxicity in the same model was used [5]. Here the dose modifying factor for acute skin toxicity, score 3.5, was found to be 1.50 (1.43–1.61), which is significantly different from the RIF dose modifying factor ( $p < 0.0005$ ).

Of the 15 mice which developed fibrosis in the FLASH arm, 13 had a score 3.5 for acute toxicity.

#### Discussion

The results showed that FLASH and CONV have similar effect on tumor control, while FLASH preferentially spared healthy tissue. The dose modifying factor of FLASH was found to be significantly lower for late compared to acute damages.

For FLASH to be used broadly in the clinic, there are still a number of issues to be solved. One of them is determining the effect of FLASH on tumor response. A number of studies have addressed tumor response in tumor growth delay assays (TGD), and demonstrated that FLASH irradiated tumors are responding similarly, or better, than tumors irradiated with conventional dose rates (reviewed in [18]). However, TGD is not necessarily a good surrogate for tumor control. From a biological point of view, TGD and tumor control are two very different endpoints [28]. In a TGD assay, the radiation effects on the bulk tumor cells are assessed, but not the inactivation of the clonogenic tumor cells, cancer stem cells (CSC) [28,29]. In contrast, permanent tumor control after RT depends, amongst others, on the inactivation of all CSC in the tumor, and

therefore tumor control assays functionally assess the effect of radiation on the inactivation of CSC [30]. Preclinical and clinical data have indicated that CSC are protected against radiation effects by hypoxia, and tumor stem cells are hypothesized to reside in hypoxic niches [30,31]. As oxygen levels have been demonstrated to play a role in the FLASH mechanism [3,32], assessing the radiation effect on the CSC is an especially important point for understanding the FLASH effect. Furthermore, it can be argued that from a clinical point of view, tumor control is the relevant endpoint, not growth delay. In the current study, we have assessed the effect of FLASH therapy on tumor control in a syngeneic mouse mammary carcinoma model and demonstrated by full dose response curves that FLASH dose rate and CONV dose rate lead to the same level of tumor control. This was done in the same setup and mouse model as previously used to demonstrate the normal tissue sparing effect of FLASH on acute skin toxicity [5].

The normal tissue toxicity was a secondary endpoint in the current study. Acute skin damage was assessed up to 25 days after irradiation in the tumor bearing mice. Acute skin toxicity is a well-defined endpoint in classical radiobiology, with well described biology of skin erythema and dose dependent intensity [33]. The dominating process behind the observed moist desquamation is epidermal cell death and the consequent reduction in epidermal cellularity and reduced number of cell layers, leading to reduced epidermal thickness. The main erythema wave begins about the tenth day after treatment, and it increases to reach a peak around day 14 [33]. Acute skin damage assays have been used in a large range of studies for demonstrating changes in radiosensitivity between different modalities and combination of treatments. The current study was designed with tumor control as primary endpoint, and the used doses were too high to reveal the dose-response curve for skin toxicity for the mice treated with CONV dose rates. The MDD<sub>50</sub> value of 51.3 (50.0–54.6) Gy found for FLASH in the current study is in excellent agreement with a previous study [5] that found an MDD<sub>50</sub> of 52.2 (50.9–55.0) Gy for the same endpoint for the FLASH arm. The MDD<sub>50</sub> for CONV for the same endpoint was established to be 34.8 (34.1–35.6) Gy in the previous study [5], which is more than 10 Gy below the lowest dose applied in the current study. The high fraction of animals with skin toxicity with CONV is supporting this.

RIF was assessed for the mice that obtained tumor control, using a leg contracture assay. This is a method previously described by Stone [22], and is a functional assay simulating a clinical endpoint of radiation induced fibrosis, which is demonstrating quantifiable dose dependency of fibrosis. Another way to assess fibrosis is immunohistochemical staining of tissue sections with eg Masson's Trichrome, which is staining the collagen deposition, but this was not performed in the current study. With the functional assay, RIF was assessed up to six months after irradiation, and a normal tissue sparing effect of FLASH was found, with a dose modifying factor of 1.14.

In the current study, three individual endpoints were assessed in the same animals, highlighting the differential effect between tumor and normal tissue response after FLASH treatment. A limitation to the data is the relatively low number of animals that had tumor control and thus were available for fibrosis analysis. Furthermore, the number of animals for fibrosis scoring was very unevenly distributed between low dose groups and high dose groups with a strong bias towards high doses (Table 1). In a study by Diffenderfer and colleagues [18], the effect of proton FLASH was assessed in the small intestine of mice. Both crypt proliferation, an early endpoint, and fibrosis, a late effect, were evaluated, and the data indicated a more prominent FLASH effect in the late endpoint than the early endpoint, which is opposite to the findings in the current study. This highlights the need for further studies dedicated to establish the normal tissue response in late reacting tis-

ues, and to elucidate which factors are influencing the FLASH effect. Furthermore, in depth analysis of the molecular pathways could shed some light on the differential effects of FLASH on the underlying molecular mechanisms leading to the different endpoints.

The current study was carried out in the entrance region of the proton beam, where a relative low LET is found. In a clinical setting, a clear advantage would be to use a Spread Out Bragg Peak, SOBP, in order to benefit from the physical properties of protons with the sharp dose gradient behind the Bragg Peak. Irradiation in the SOBP, and especially in the distal edge, leads to a higher LET and a higher RBE, Relative Biological Effectiveness. How an increased RBE influences the FLASH effect is yet to be established.

In conclusion, FLASH dose rate resulted in tumor control equal to CONV dose rate but led to reduced normal tissue damage assessed as acute skin damage and radiation induced fibrosis.

### Conflict of interest statement

This study presented in this manuscript is partly funded by Varian. Two co-authors are co-inventors on a patent-application filed with application number 63257211 and EFS ID: 44064136, which contains parts of the data included in the manuscript.

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### Disclosure statement

This study was partly funded by Varian, a Siemens Healthineers Company.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.radonc.2022.05.014>.

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