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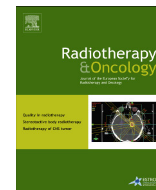

Original article

Regulation of the oxidative balance with coenzyme Q10 sensitizes human glioblastoma cells to radiation and temozolomide

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Objectives: To investigate how the modulation of the oxidative balance affects cytotoxic therapies in glioblastoma, *in vitro*.

Material and methods: Human glioblastoma U251 and T98 cells and normal astrocytes C8D1A were loaded with coenzyme Q10 (CoQ). Mitochondrial superoxide ion ($O_2^{\cdot-}$) and H_2O_2 were measured by fluorescence microscopy. OXPHOS performance was assessed in U251 cells with an oxytherm Clark-type electrode. Radio- and chemotherapy cytotoxicity was assessed by immunostaining of γH2AX (24 h), annexin V and nuclei morphology, at short (72 h) and long (15 d) time. Hif-1α, SOD1, SOD2 and NQO1 were determined by immunolabeling. Catalase activity was measured by classic enzymatic assay. Glutathione levels and total antioxidant capacity were quantified using commercial kits.

Results: CoQ did not affect oxygen consumption but reduced the level of $O_2^{\cdot-}$ and H_2O_2 while shifted to a pro-oxidant cell status mainly due to a decrease in catalase activity and SOD2 level. Hif-1α was dampened, echoed by a decrease lactate and several key metabolites involved in glutathione synthesis. CoQ-treated cells were twofold more sensitive than control to radiation-induced DNA damage and apoptosis in short and long-term clonogenic assays, potentiating TMZ-induced cytotoxicity, without affecting non-transformed astrocytes.

Conclusions: CoQ acts as sensitizer for cytotoxic therapies, disarming GBM cells, but not normal astrocytes, against further pro-oxidant injuries, being potentially useful in clinical practice for this fatal pathology.

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Grade IV astrocytoma or glioblastoma multiforme (GBM) is the most common type of malignant brain tumor in adults [1]. The current therapeutic protocol for this pathology includes maximal safe surgery, radiotherapy and chemotherapy with temozolomide (TMZ), an alkylating agent and radiosensitizer [2,3]. Nevertheless, even patients receiving the standard of care die early with a median survival of 14 months due to recurrence within the radiation field [1,4,5].

Several evidences link GBM radioresistance with the mitochondrial pathophysiology and the over-production of radical oxygen species (ROS) scavengers, which lately determine a shift to an antioxidative balance [6–10]. GBM cells are very glycolytic even in normoxia: the so called "Warburg effect" [11]. In this setting, a high level of $O_2^{\cdot-}$ is produced and rapidly dismutated to H_2O_2, which is translocated to the cytosol, orchestrating multiple mechanisms leading to radioresistance [12–14]. High levels of $O_2^{\cdot-}$ and H_2O_2 are toxic and thus, GBM cells increase their antioxidant

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defenses as a survival mechanism, i.e. the levels of catalase and mitochondrial superoxide dismutase (SOD2) are increased [6,7].

The cytotoxic effect of ionizing radiation is mediated by the induction of ROS, leading to DNA and RNA damage and genomic instability [14]. Thus, the naturally increased level of catalase and SOD2 enzymes ameliorates radiation-induced DNA damage, contributing to resistance to radiation-induced cell damage [6,15]. Moreover, the increased level of reduced glutathione (GSH) also contributes to cell protection against ROS and, thus, participates in the resistance to ionizing radiation [16]. Indeed, there is a negative correlation between the level of GSH and sensitivity to ionizing radiation in a series of brain tumors including GBM [17]. GSH level is also related to resistance to TMZ and mediates several mechanisms of resistance development [18]. Furthermore, high lactate levels produced by aerobic glycolysis are also interconnected with radioresistance. Actually, lactate behaves as an antioxidant [19], being positively correlated with resistance to ionizing radiation [14].

Instead of a single factor, radioresistance is orchestrated by several enzymes and small molecules that are linked to the increased generation of ROS. Therefore, the simultaneous modulation of these ROS scavengers may be useful in GBM therapies. In this work, we explore the role of coenzyme Q_{10} (CoQ) in the mechanisms evoking radioresistance using two human GBM cell lines, U251 and T98, compared to non-transformed astrocytes C8D1A. CoQ is a lipophilic antioxidant that crosses the blood–brain barrier [20]. Besides, it is a component of the mitochondrial electron transport chain (ETC) that dampens the $O_2^{\bullet-}$ generated by complex I in dystrophic mitochondria [12]. Our results show that treatment of human U251 GBM cells with CoQ did not affect OXPHOS, but slightly increased oxygen consumption associated with the mitochondrial complex I, which was paralleled by a decrease in the level of mitochondrial $O_2^{\bullet-}$ and cytosolic H_2O_2. Conversely, these reactive species were increased upon irradiation in GBM cells, but not in non-transformed astrocytes. Indeed, CoQ sensitized cells to radiation-induced DNA damage and apoptosis both in short- and long-term clonogenic assays. These effects are mediated by a shift in the oxidative balance toward pro-oxidant conditions, which relies on the reduction in catalase activity, a decrease in SOD2 level and a cut down in intracellular lactate and key metabolites involved in glutathione synthesis, which could be related to the reduced level of Hif-1α. On the other hand, neither SOD1 nor NQO1 levels were altered. These changes result in a reduction in the total antioxidant cellular capacity and a shift to a pro-oxidant cellular state that disarms GBM cells against further pro-oxidant injuries induced by standard therapies, without affecting non-transformed astrocytes. Our results indicate that CoQ combined with radiation and TMZ has a promising potential for improving the current therapeutic protocol for GBM.

Material and methods

Reagents

Coenzyme Q_{10} was provided by Kaneka Corporation. TMZ, Hoechst, crystal violet, EDTA, methanol, DMSO, dH_2O, Tris–HCl, DTT and other general reagents were acquired from Sigma–Aldrich.

Cell culture and irradiation

Human GBM U251 and T98 and non-transformed astrocytes C8D1A were obtained from ATCC and maintained under normoxia in DMEM (Sigma–Aldrich) at 37 °C and 5% CO_2. For irradiation purposes, cells were seeded in 24 well plates and pre-treated for 24 h with CoQ or vehicle (ethanol; control). Depending on the experiment, cells were treated with TMZ (50 μM) or vehicle (DMSO) and again 24 h later cells were irradiated in a Gulmay D 3150 (NCA) at doses ranging between 1 and16 Gy (1.211 Gy/min) at 100 kV tube potential. Different parameters were evaluated immediately after irradiation ($O_2^{\bullet-}$ and H_2O_2), at 24 h (DNA damage), at 96 h (apoptosis) or at 15d (colonies formation) of irradiation.

OXPHOS performance

Oxygen consumption in intact or digitonin-permeabilized cells was determined with an oxytherm Clark-type electrode (Hansatech Instruments, Norfolk, UK) as previously described [21]. Briefly, U251 cells were resuspended in culture medium or respiration buffer (10 mM $MgCl_2$, 250 mM Sucrose, 20 mM HEPES pH 7.4, 1 mM ADP, 2 mM KH_2PO_4), respectively. Oxygen consumption was recorded at 37 °C with stirring. 7.5 μM carbonyl cyanide-4-(trifluor omethoxy)phenylhydrazone (FCCP) was used to uncouple respiration in intact cells. For polarographic measurements, cells were permeabilized with 1% digitonin (1.2 ul/10^6 cells) for 10 min at 37 °C and oxygen consumption was recorded after the addition of substrates and inhibitors for Complex I (5 mM Glutamate plus 5 mM Malate, and 2 μM Rotenone), Complex II + III (5 mM Succinate plus 5 mM Gliceraldehide-3-P, and 0.1 μM Antimycin A) and Complex IV (1.2 mM TMPD, and 6 mM KCN). Reactions were terminated by the addition of 6 mM KCN to assess any O_2 consumption not derived from the respiratory chain.

Single-cell determination of $O_2^{\bullet-}$ and H_2O_2

Mitochondrial $O_2^{\bullet-}$ and total H_2O_2 levels were determined with MitoSOX and H_2DCFDA probes, respectively (Life Technologies). Cells were seeded in 96 well plates and incubated for 24 h with vehicle (control) or CoQ (2.5–10 μM) and then irradiated as indicated above. Then, cells where immediately loaded for 30 min with 1 μM MitoSOX or H_2DCFDA, washed in fresh medium and imaged in a Nikon TiU microscope (20× objective). Images were analyzed and processed with ImageJ software (NIH). Results are expressed as the percentage of cell signal *vs.* control (minimum 50 cells, $n = 3$).

Determination of total antioxidant capacity

Cells were seeded in 96 well plates and incubated for 24 h with vehicle (control) or CoQ (5 μM). Total antioxidant capacity was determined in cell lysates and in culture media using a commercial kit, following the manufacturer's instructions (MAK187, Sigma–Aldrich). Results were expressed as nmol/μg of protein ($n = 4$).

Determination of DNA damage, apoptosis and viability

Cells treated with CoQ and irradiated, after 24 and 72 h for DNA damage and apoptosis and viability assays, respectively, were fixed for 2 min in 4% para-formaldehyde (PFA), stained with 1 μg/ml Hoechst and immunolabeled for annexin V (1:500; BD Pharmingen) and γH2AX antibody (1:500; Santa Cruz Biotechnology). For γH2AX staining, cells were permeabilized for 2 min in ice-cold methanol after PFA fixation. Binding of primary antibodies was detected with fluorescence-labeled secondary antibodies conjugated with Alexa-594 (1:2000; Life Technologies). Apoptotic nuclei were determined from Hoechst-stained nuclei images according to morphometric criteria with ImageJ. Those cells exhibiting normal, non-apoptotic, nuclei morphology were considered as viable. Results are shown as percentage of apoptotic *vs.* total cells (at least 100 cells, $n = 4$). DNA damage was determined and represented as the number of γH2AX foci per cell (at least 100 cells, $n = 4$). Apoptotic cells were determined according to annexin V level. Results

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were represented as the average fluorescent signal per cell (at least 100 cells, $n = 4$).

Clonogenic assays

Cells, seeded at 200 cells/well in 24 well plates, were treated as indicated in paragraph above. At 15 days from irradiation, cells were fixed for 2 min in cold methanol and stained with 0.01% (w/v) crystal violet for 30 min. Dishes were dried and colonies containing more than 50 individual cells determined using bright field microscopy with a Nikon TiU microscope (2× objective) ($n = 3$).

Sensitivity to exogenous H_2O_2

Cells were seeded to subconfluence in 96 well plates and incubated for 24 h with vehicle (ethanol; control) or 5 µM CoQ. Then, cells were treated with increasing concentrations of H_2O_2 (12.5–100 µM) and 8 h later cells were fixed and permeabilized for 2 min in ice-cold methanol and stained with 1 µg/ml Hoechst. The number of adhered cells was counted from fluorescence microscopy images. Results were computed as the percentage of cells *vs.* control (at least 300 cells, $n = 5$).

Catalase activity

Cells were seeded in 6 well plates and incubated for 24 h with vehicle (ethanol; control) or 5 µM CoQ. Proteins were extracted with a lysis buffer (Tris–HCl 10 mM, EDTA 1 mM & DTT 0.1 mM). Catalase activity was quantified by spectrophotometric assays as previously described elsewhere [22] and the fold change in catalase activity *vs.* control conditions ($n = 3$) computed.

Quantification of SOD1, SOD2, NQO1 and Hif-1α

Cells were seeded in 6 well plates and incubated for 48 h with vehicle (ethanol; control) or 5 µM CoQ. Proteins were extracted with SDS–DTT buffer. The level of SOD2, SOD1, NQO1 and Hif-1α (1:1000 dilution, Santa Cruz Biotechnology) was quantified by western blot as described before [23]. Results are expressed as the mean band intensity *vs.* actin and normalized to control condition ($n = 4$). The level of Hif-1α was also measured by quantitative immunocytochemistry and epifluorescence and the results expressed as the mean cell-fluorescence intensity normalized to control (at least 50 cells, $n = 4$).

Determination of metabolites by Nuclear Magnetic Resonance (NMR)

NMR experiments were carried out on a Varian VNMRS-400 NMR system using a nanoprobe with rotors of 40 µL. 1D and 2D NMR experiments were recorded for peak identification. Chenomx Profiler (Chenomx Inc.) was used to assist in peak identification. TSP was used as internal standard for quantification of metabolites. The NMR spectra were processed with Mestrenova (Mestrelab Research). Cells were grown onto 25 cm2 flasks and treated for 24 h with vehicle (ethanol; control) or 5 µM CoQ. The level of lactate, glutamate, glycine, serine and glutamine was assessed as described before [24].

Glutathione determination

Cells were grown in 25 cm^2 flasks and incubated for 24 h with vehicle (ethanol; control) or 5 µM CoQ. Reduced (GSH) and oxidized (GSSG) glutathione were determined as described before [25]. Both absolute GSH and GSSG values and the GSSG/GSH ratio ($n = 4$) were computed.

Radiobiological parameter in the dose–response curves

Dose–response curves for cell viability were fit using a Lineal-quadratic model for the surviving fraction SF(D) = exp(-αD-βD^2). α and β were fit using least squares methods from the data on response to intermediate doses considering realistic values for α/β.

Statistical analysis

Data are expressed as mean ± S.E.M. obtained from, at least, three independent experiments. Statistical analysis was carried out with GraphPad Prism 6, using Student's t-test or a one-way ANOVA (Kruskal–Wallis' test) followed by a statistical test for multiple comparisons (Dunn's test). Differences were considered significant at $p < 0.05$. Results were analyzed for synergism using the combination index method calculated by CompuSyn V 1.0, obtaining the values for the coefficient of interaction (CI; 0.1–0.3, strong synergism; 0.3–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.9, slight synergism and 0.9–1, nearly additive) and the fractional effect (FE).

Results

CoQ potentiates radiation-induced rise in $O_2^{\cdot-}$ and H_2O_2 levels and decreases the total antioxidant cell capacity in GBM cells

We first explored the effect of the incubation of human U251 and T98 cells and non-transformed astrocytes C8D1A with 5 µM CoQ on the levels of mitochondrial $O_2^{\cdot-}$ and H_2O_2 both under basal conditions and upon irradiation. Our results show that a 24 h pretreatment with CoQ reduced the levels of both reactive species by 45% and 35%, respectively, in non-irradiated U251 cells (Fig. 1A). A similar albeit lower reduction (of about 15% and 14% respectively) was observed in T98 cells (Fig. 2B), whereas no significant decrease was detected in non-transformed astrocytes C8D1A (Fig. 1C). However, U251 cells treated with CoQ showed significantly higher levels of $O_2^{\cdot-}$ and H_2O_2 than untreated cells after irradiation. In this setting, mitochondrial $O_2^{\cdot-}$ was increased by 1.2 and 1.4-fold for 2 and 4 Gy, respectively, in CoQ-treated *vs.* non-treated cells (Fig. 1A and Supplementary Fig. 1). Similar results were obtained for H_2O_2. In CoQ-treated cells, H_2O_2 levels increased 1.35-fold at 2 Gy and 1.3-fold at 4 Gy *vs.* non-treated cells (Fig. 1A). Comparable results were obtained for T98 cells, being significant only at 4 Gy dose for $O_2^{\cdot-}$ (Fig. 1B). Opposingly, levels of both $O_2^{\cdot-}$ and H_2O_2 were significantly reduced after irradiation in CoQ-treated non-transformed astrocytes (Fig. 1C). This dual effect of CoQ observed in GBM cells was due to the modulation of total antioxidant cell capacity. Indeed, U251 and T98 cells treated with CoQ showed a 15% decrease in the total antioxidant capacity *vs.* non-treated cells, which reflects a shift to a pro-oxidant cell state, whereas no change was detected in the culture media (Fig. 1D and E).

CoQ enhances radiation-induced DNA damage and apoptosis

Since incubation of GBM cells with CoQ increased the level of radiation-induced $O_2^{\cdot-}$ and H_2O_2, we next studied the effect of this compound on DNA damage in control and CoQ-treated U251 cells, by measuring the number of γ-H2AX foci per cell after irradiation. We observed an average of 3.5 positive foci/cell in irradiated control cells, and preincubation with 5 µM CoQ for 24 h increased by 2.7-fold the number of positive γ-H2AX foci at 24 h from irradiation (Supplementary Fig. 2A). This exacerbation of DNA damage was translated into an increased pro-apoptotic effect of radiation, measured as annexin V exposure at the external hemi-layer of the plasma membrane. Radiation induced an average of 7.94 relative fluorescence units (RFU) in irradiated control cells and this value was increased by 13.6-fold in cells that had been preincu-

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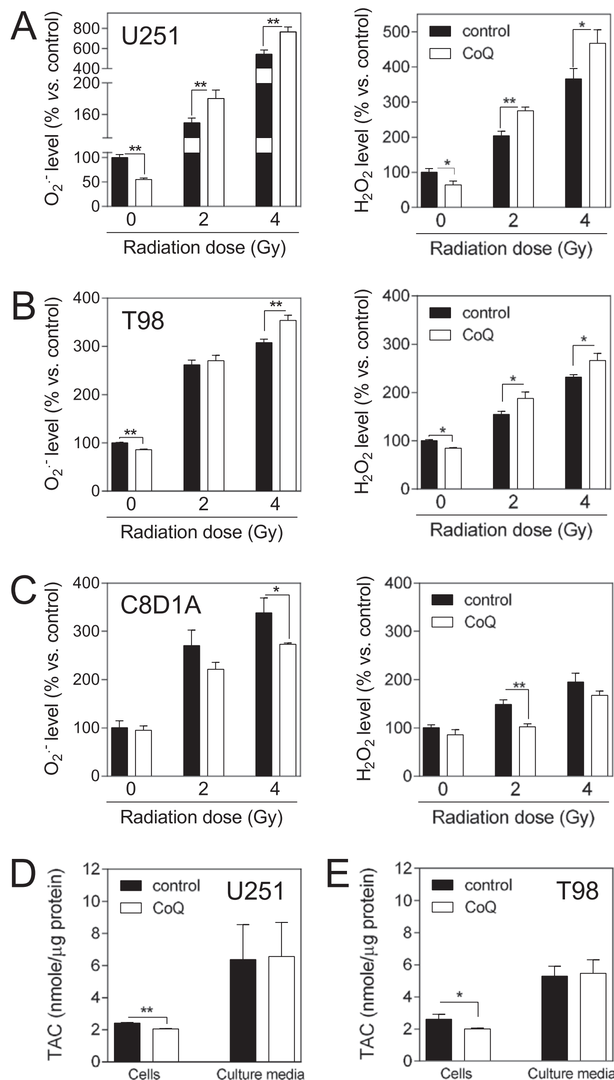

Fig. 1. Effect of exogenous CoQ on oxidative stress and radiation-induced cell damage in human GBM cells and non-transformed astrocytes, *in vitro*. Cells were incubated with 5 µM CoQ for 24 h and then irradiated (0–4 Gy). O_2.– (A–C, left graphs) and H_2O_2 (A–C, right graphs) levels were quantified by fluorescence microscopy with the probes MitoSOX and H_2DCFDA, respectively. Results are expressed as the percentage of cell signal *vs.* control, 0 Gy, non-treated and non-irradiated for GBM U251 and T98 cells (A and B) and non-transformed astrocytes C8D1A (C). min = 50 cells, $n = 3$, * $p < 0.05$; ** $p < 0.005$. Total antioxidant cell and supernatant capacities, TAC, was determined by colorimetry in GBM U251 and T98 cells (D and E). Results are expressed as nmol normalized to protein concentration, quantified by BCA. $n = 4$, ** $p < 0.005$.

bated for 24 h with 5 µM CoQ and measured 96 h after irradiation (Supplementary Fig. 2B). Indeed, this increase in annexin V staining and apoptosis was translated into a decrease in cell viability (Fig. 2A, left graph). Radiation alone reduced GBM cell viability by 35% and 50% at 2 and 4 Gy, respectively, and preincubation with CoQ diminished cell viability by 60% and 70% for the same radiation doses (Fig. 2A, left graph). Indeed, the IC_{50} was reduced from 2.5 in control conditions to 1.3 Gy in cells preincubated with CoQ (Fig. 1F). Thus, CoQ almost doubled the cytotoxic effect of radiation measured after 96 h. The radiobiological response parameters for both curves were $\alpha = 0.1100$ and $\beta = 0.0120$ (CoQ-treated) and $\alpha = 0.02$ and $\beta = 0.12$ (control) suggesting much weaker single-strand break repair mechanisms mediated by CoQ (term linear in the dose D). The data were very well described with the standard Linear-quadratic model for the surviving fraction. This reduction in cell viability exerted by CoQ upon radiation was reproduced in T98 cells (Fig. 2A, middle graph), whereas no effect was shown in non-transformed C8D1A astrocytes (Fig. 2A, right graph).

CoQ improves the long-term cytotoxic effect of radiation

To check the remaining viable cells' ability to regrow, we performed clonogenic assays. Human GBM cells were preincubated with vehicle or CoQ 24 h before irradiation and were then irradiated with a single dose of 1, 4 or 8 Gy. Cells were cultured for 15 days and then stained to count the number of clones in each well. In control U251 and T98 cells, radiation alone reduced the surviving fraction by 15% and 35% at 4 Gy and by 60% and 68% at 8 Gy, respectively (Fig. 2B, left and middle graphs and Supplementary Fig. 3A and B). Preincubation with CoQ reduced the surviving fraction by 48% and 55% for 4 Gy and by 81% and 76% at 8 Gy, respectively, thus triplicating the effect obtained for control cells at 4 Gy (Fig. 2B, left and middle graphs). On the other hand, CoQ did not affect the surviving fraction in non-transformed C8D1A astrocytes, but even induced a slight, although statistically not significant, protective effect (Fig. 2B, left and middle graphs and Supplementary Fig. 3C). Our results show that the CoQ-mediated radiosensitization of GBM cells involves an exacerbation of lethal DNA damage that cannot be repaired by these tumor cells.

CoQ does not affect oxygen consumption but reduces lactate and Hif-1α levels

As complex I in tumor cells dystrophic mitochondria generates high amounts of O_2^{-} radicals [12] and our results have shown that CoQ diminishes the level of this radical species (Fig. 1A and B), we wondered if mitochondrial respiration was affected upon addition of the antioxidant. Incubation of GBM U251 cells with 5 µM CoQ did not modify neither endogenous nor uncoupled oxygen consumption (Fig. 3A), though it slightly increased oxygen consumption associated with complex I from 0.59 to 0.96 fmol/min/cell (Fig. 3B).

Considering that GBM are highly glycolytic and intracellular lactate level is involved in radioresistance [11,14], we then measured the level of intracellular lactate in U251 cells treated for 24 h with vehicle (control) or 5 µM CoQ showing a decrease in lactate by 46% *vs.* control (Fig. 3C). This decrease was paralleled by a 40 and 25%-reduction in U251 and T98 cells, respectively, in the level of the transcription factor Hif-1α, the main inductor of glycolytic metabolism upon CoQ pretreatment (Fig. 3D and E). On the other hand, the level of Hif-1α was not affected by CoQ treatment in non-transformed astrocytes C8D1A (Fig. 3F).

CoQ reduces catalase activity and the level of SOD2 but does not modify the levels of SOD1 or NQO1

Exacerbated ROS levels lead to an increase in the level of antioxidant phase II enzymes, including catalase and SOD2 [6,7]. Since CoQ reduced the total antioxidant cell capacity and increased the level of both O_2^{-} and H_2O_2 in GBM cells after irradiation, we wondered if these phase-II antioxidant enzymes were downregulated. Incubation of human GBM cells with CoQ ranging from 2.5 to 10 µM reduced catalase activity between 35–50% and 25–50% in U251 and T98 cells, respectively, which was not affected in non-transformed astrocytes (Fig. 4A). Also, human GBM cells preincubated with 5 µM CoQ were more sensitive than control cells to cytotoxicity of exogenous H_2O_2, obtaining IC_{50} values of 3 and 8 µM in U251 and 12.7 and 25.3 µM in T98 for CoQ-treated or control cells, respectively, whereas no difference was found for non-transformed astrocytes C8D1A (Fig. 4B). Similarly, the level of SOD2 polypeptide was reduced by 40% after CoQ treatment in

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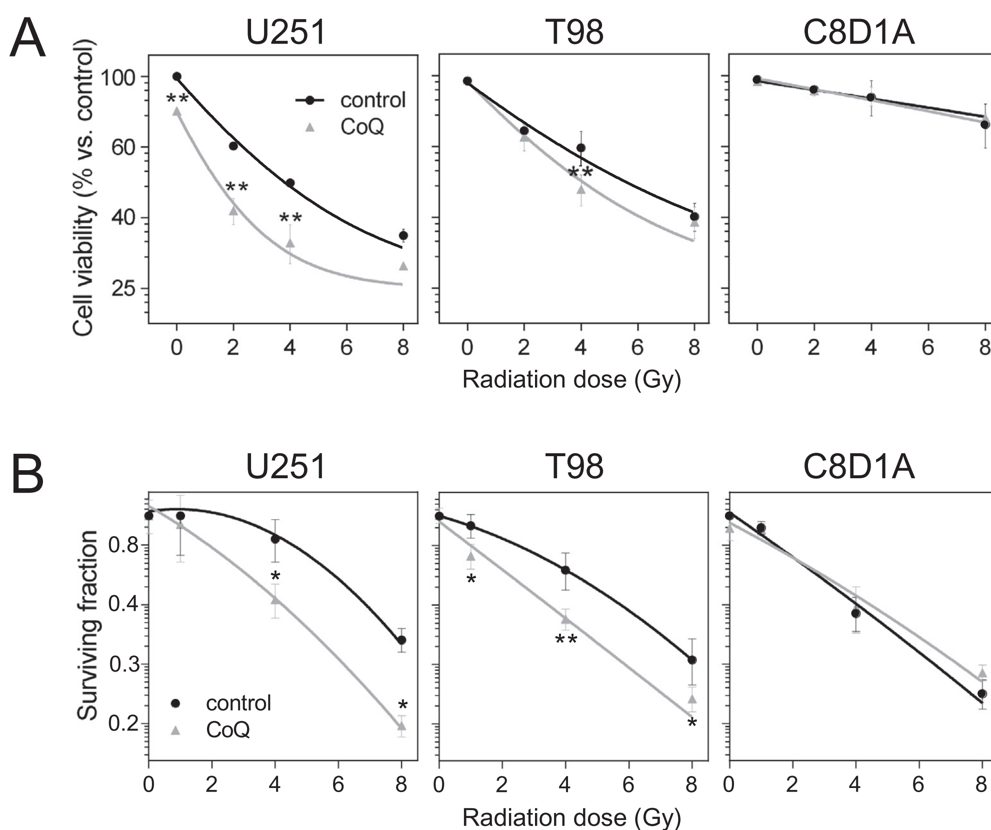

Fig. 2. CoQ reduces radiation-induced cell viability at short and long term in human GBM U251 and T98 cells but not in non-transformed astrocytes C8D1A, *in vitro*. Cells were incubated with 5 μM CoQ for 24 h and then irradiated (0–8 Gy). (A) Cell viability was calculated from fluorescence microscopy images, attending cell nuclei morphology. Results are expressed as percentage of decrease *vs.* control, non-treated and non-irradiated for GBM U251 (A, left) and T98 cells (A, middle) and non-transformed astrocytes C8D1A (A, right). min = 100 cells, $n = 4$, $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$. (G) For clonogenic assays, cells were incubated with 5 μM CoQ for 24 h and then irradiated (0–8 Gy). The number of clones was determined at 15d from irradiation, by staining with crystal violet and counting. Results are expressed as the surviving fraction *vs.* control non-treated and non-irradiated for GBM U251 (B, left) and T98 cells (B, middle) and non-transformed astrocytes C8D1A (B, right). $n = 4$, $^{*}p < 0.05$; $^{**}p < 0.005$.

U251 and T98 cells, whereas it was not affected in C8D1A cells (Fig. 4C–E). Neither SOD1 nor NQO1 was altered in U251 cells (Fig. 4C).

CoQ regulates the synthesis of glutathione

Glutathione is one of the main cellular antioxidants, being its synthesis upregulated during oxidative stress [16]. An increased level of oxidants such as H_2O_2 causes short-term falls in intracellular GSH, which is associated with higher levels of GSSG [16]. Our results in human U251 cells show that the level of GSH was neither altered by treatment with vehicle (control) nor with CoQ (Supplementary Fig. 4A, left). However, CoQ significantly decreased the level of GSSG (Supplementary Fig. 4A, middle), which resulted in a decrease in GSSG/GSH ratio (Supplementary Fig. 4A, right) and in a smaller glutathione-antioxidant capacity of CoQ-treated cells. This change in the whole glutathione pool could be due to the lowering of critical amino acids essential for the synthesis of the antioxidant, glutamate, glycine, serine and glutamine, all of them reduced by 42%, 52%, 50% and 40%, respectively, in CoQ-treated *vs.* control cells (Supplementary Fig. 4B).

CoQ potentiates TMZ in the radiosensitization of human GBM cells

TMZ, the standard chemotherapeutical in the clinical management of GBM, is known to have also a radiosensitizing effect [2]. We have analyzed the interaction between TMZ and CoQ on radiation-induced cytotoxicity in U251 and T98 cells compared to non-transformed C8D1A astrocytes. In U251 cells, CoQ or TMZ alone increased radiation-induced apoptosis by 13% and 17%, respectively, whereas the combined treatment with CoQ and TMZ increased radiation-induced apoptosis in 28% *vs.* cells treated with vehicle, at 4 Gy with a fractional effect of 0.58 and CI of 0.22 showing strong synergism (Supplementary Fig. 5, left and Supplementary Table 1). In T98 cells, CoQ or TMZ alone increased radiation-induced apoptosis by 12% and 6%, being CoQ + RT-mediated effect more effective than TMZ + RT. Combined treatment with CoQ and TMZ increased radiation-induced apoptosis in 17% *vs.* cells treated with vehicle, at 4 Gy with a fractional effect of 0.51 and CI of 0.68 showing moderate synergism (Supplementary Fig. 5, middle and Supplementary Table 1). No difference was found between treatments for non-transformed C8D1A astrocytes (Supplementary Fig. 5, right).

Discussion

Radioresistance is a hallmark of GBM [6–10]. Even with the current treatment that encompasses maximal function-preserving surgery followed by TMZ concomitant with radiotherapy (Stupp's protocol), the tumor regrows within the radiation field [1,3–5]. Radioresistance is orchestrated by multiple and interconnected factors, including the recruitment of different antioxidant systems due to the overproduction of high levels of O_2^- and the glycolytic metabolism and [1,6,7]. Here we describe a novel combined treatment for GBM, *in vitro*, using CoQ as radiosensitizer, targeting mitochondrial O_2^- and H_2O_2, diminishing the total antioxidant cell capacity by reducing the level of catalase, SOD2, glutathione and

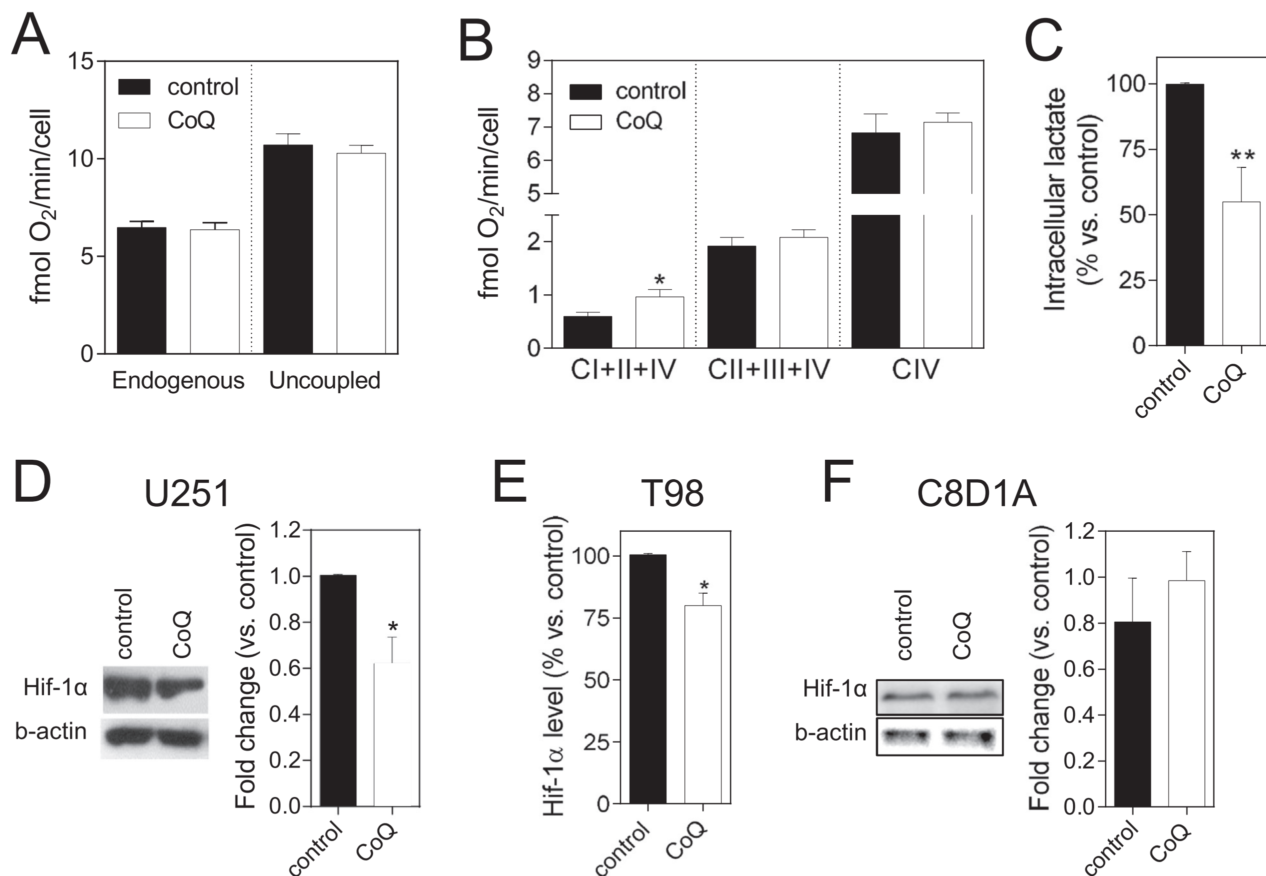

Fig. 3. CoQ does not affect OXPHOS but reduces the level of intracellular lactate and Hif-1α in GBM cells, *in vitro*. Cells were incubated with 5 μM CoQ for 24 h. Oxygen consumption in intact or digitonin-permeabilized cells was determined with an oxytherm Clark-type electrode. (A) FCCP was used to uncouple respiration in intact cells. (B) For polarographic measurements, cells were permeabilized with 1% digitonin and oxygen consumption was recorded after the addition of substrates and inhibitors for Complex I (5 mM Glutamate plus 5 mM Malate, and 2 μM Rotenone), Complex II + III (5 mM Succinate plus 5 mM Gliceraldehide-3-P, and 0.1 μM Antimycin) and Complex IV (1.2 mM TMPD, and 6 mM KCN). Results are expressed as fmol O_2/min/cell. *n* = 4, * *p* < 0.05. (C) Intracellular lactate level was quantified by Nuclear Magnetic Resonance (NMR) in U251 cells. Results are expressed as % decrease *vs.* control. *n* = 4, ** *p* < 0.005. (D) The level of Hif-1α was quantified in U251 and T98 GBM cells and in C8D1A non-transformed astrocytes by immuno-techniques with an anti- Hif-1α antibody. *n* = 4, ** *p* < 0.05.

lactate, by mechanisms involving a reduction in the level of Hif-1α, improving the overall response to radiotherapy and TMZ *in vitro*, which targets specifically tumor cells without affecting non-transformed astrocytes.

CoQ is a lipophilic antioxidant that crosses the blood–brain barrier [20,26]. Exogenous CoQ is first accumulated in the endo-lysosomal compartment, and then rapidly becomes incorporated to mitochondria-associated membranes and mitochondria [26]. In this organelle, CoQ participates in oxidative phosphorylation under normal conditions, whereas under pathological conditions, as in tumor cells, it diminishes the high levels of $O_2^{\cdot-}$ generated by complex I [12]. In this context, our results show that the preincubation of GBM cells with CoQ diminished by 50% the level of mitochondrial $O_2^{\cdot-}$ and, in parallel, the level of H_2O_2 (Fig. 1A and B), increasing oxygen consumption associated with mitochondrial complex I, without affecting the overall respiration rate (Fig. 3A and B). Since complex I is considered to be one of the main cellular sources of ROS production and both the increase and the decrease in its enzymatic activity have been previously related to this process [27], our results could agree with a model that is associated with the reduction in oxidative stress by favoring the non-accumulation of reducing power.

Elevated $O_2^{\cdot-}$ and H_2O_2 levels are toxic to normal cells [28]. However, cancer cells overcome high levels of ROS by turning on multiple antioxidant systems that also protect cells from further pro-oxidant stimuli, as radiation [28]. Cancer cells take advantage of the situation since $O_2^{\cdot-}$ and H_2O_2 can also sustain proliferation [29]. Our results indicate that CoQ can deactivate several of these antioxidant systems, reflected by a decrease in the total cell antioxidant capacity.

We have shown that CoQ reduces by almost 50% the level of intracellular lactate (Fig. 3C), possibly regulating the Warburg effect. Intracellular lactate has an antioxidant role by dampening free oxygen radicals induced by radiation [14,19]. Thus, CoQ-mediated lactate decrease seems to contribute to the elevated $O_2^{\cdot-}$ and H_2O_2 levels induced by radiotherapy (Fig. 1A and B), leading to an enhanced DNA damage and apoptosis (Supplementary Fig. 2). Moreover, lactate is one of the main energy sources of GBM cells [14,30]. Thus, the CoQ-mediated lowering of the lactate levels may be associated with the small but significant decrease in cell viability in non-irradiated *vs.* non-treated cells (Fig. 2A, left). This effect could be related to the decrease observed in Hif-1α level after incubation with CoQ (Fig. 3D and E). Multiple evidences pointed toward Hif-1α as the main driver for glycolytic metabolism and radioresistance [31–33]. Hif-1α changes glucose metabolism toward a glycolytic phenotype. Indeed, the inhibition of Hif-1α results in a decreased rate in glucose uptake, lactate production and an increase in oxygen consumption, which denotes an enhanced mitochondrial oxidation [34,35]. In this sense, our results indicate that CoQ is reducing the level of Hif-1α (Fig. 3D

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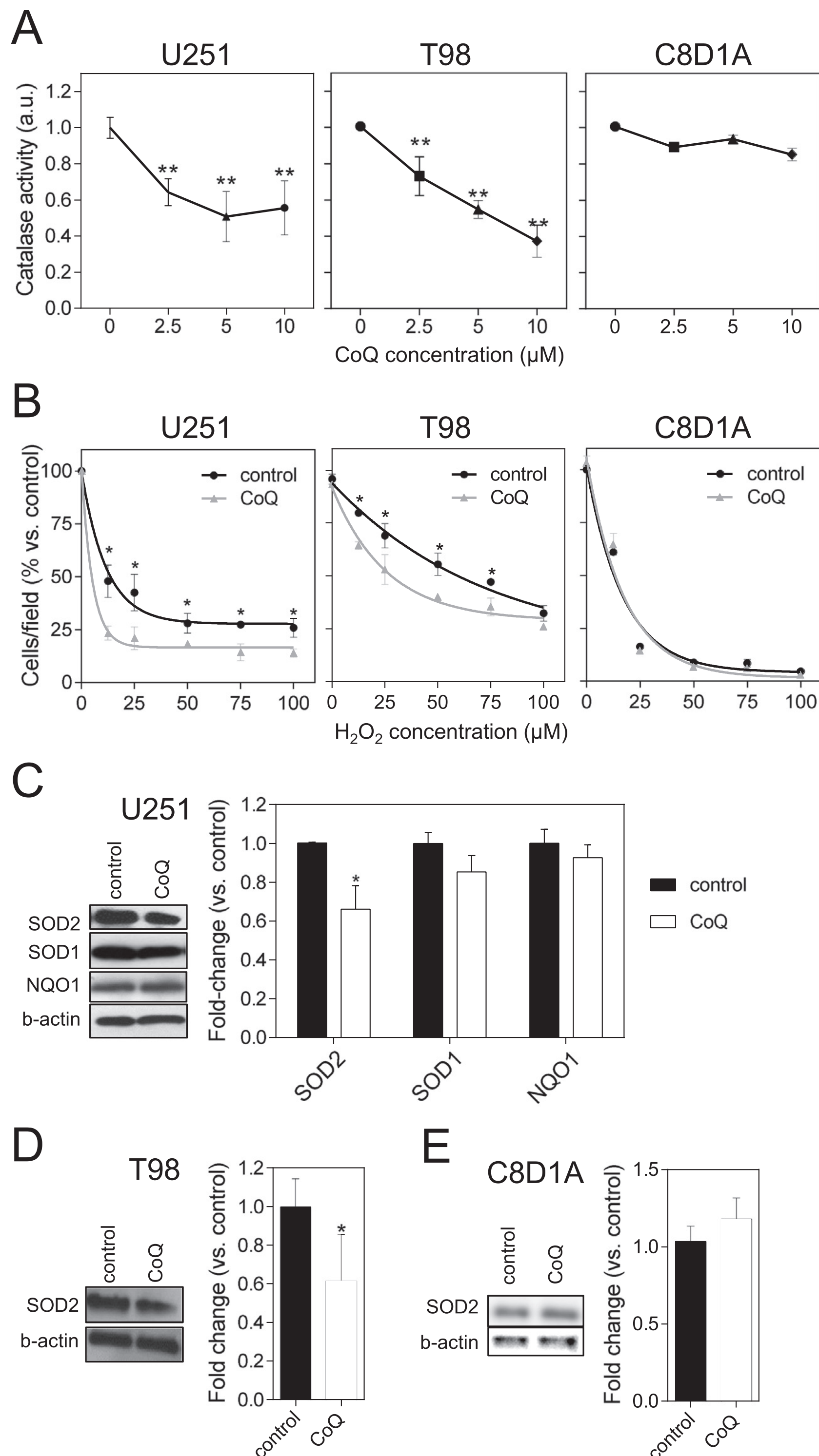

Fig. 4. CoQ reduces catalase activity and SOD2 polypeptide sensitizing human GBM cells but not non-transformed astrocytes to H_2O_2-induced cell death, *in vitro*. Cells were incubated with 5 μM CoQ for 24 h. Catalase was determined following spectrophotometric procedures and expressed as % *vs.* control, non-treated, in GBM U251 (A, left) and T98 (A, middle) cells and in non-transformed astrocytes C8D1A (A, right). Control and CoQ-treated cells were incubated for 8 h with increasing H_2O_2 concentrations (12.5–100 μM) and then, washed and fixed. Results show the % of cells attached to the culture dish *vs.* control in U251 (B, left) and T98 (B, middle) cells and in non-transformed astrocytes C8D1A (B, right). Western blot and densitometric analysis showed a decrease in SOD2 and no variation in SOD1 and NQO1 in U251 cells (C). SOD2 level is also decreased in T98 cells (D) but unaffected in non-transformed C8D1A astrocytes (E). $n = 4$, * $p < 0.05$; ** $p < 0.005$.

and E), which agrees with the reduction observed in the level of lactate (Fig. 3C), probably also related to the slight increase in oxygen consumption associated with the mitochondrial complex I (Fig. 3A). Furthermore, H_2O_2 is sufficient to stabilize HIF-1α protein under normoxia [36,37] and preincubation of U251 and T98 cells with CoQ reduced the levels of H_2O_2. As a whole, CoQ contributes to radiosensitize GBM cells through modulation of Hif-1α, lactate level and probably mitochondrial respiration.

A search in the TCGA and Murat series included within the *Oncomine* database (https://www.oncomine.org/resource/ login.html) shows that catalase and SOD2 levels are increased in glioblastoma *vs.* normal brain (Supplementary Figs. 6 and 7) whereas SOD1 and NQO1 levels are not altered (Supplementary Figs. 8 and 9). Our results show a decrease in catalase activity and 44% reduction in SOD2 polypeptide level in human GBM cells but not in non-transformed astrocytes (Fig. 4C–E). Conversely, CoQ neither alters the levels of SOD1 nor NQO1 (Fig. 4C). CoQ has been shown to inhibit deregulated catalase activity and SOD2 levels in a model of DMBA induced breast cancer [38] as well as in a model of diabetic rats, a non-tumor disease with a strong oxidative component [39]. To our knowledge, there was no previous evidence that CoQ reduces catalase and SOD2 levels in GBM cells. It is known that an increase in catalase and SOD2 activities is linked to radioresistance in human GBM U251 cells [6], and that the inhibition of the enzymes sensitizes rat glioma cells to apoptosis induced by pro-oxidant injuries [40]. Lessening of catalase and SOD2 by CoQ combined with the reduced intracellular lactate level could be related to the radiosensitization and potentiation of both, short and long-term radiation-induced apoptosis reported here (Fig. 2A and B).

GSH is the most abundant non-enzymatic antioxidant molecule in the cell, essential for cell survival and redox homeostasis [28]. It protects the cell against O_2^- and H_2O_2-induced oxidation, but it is likewise involved in cancer cell-resistance to ionizing radiation [16]. Indeed, the level of GSH is negatively correlated to the sensitivity of brain tumors to radiation [17]. There is no previous evidence of GSH regulation by CoQ in tumor pathologies. However, CoQ reduced the level of hepatic GSH triggered by ischemia-reperfusion in a rat model of the disease [41]. Our results show that CoQ clearly diminished the oxidized form of glutathione, GSSG, and the ratio GSSG/GSH (Fig. 3G). Thus, CoQ decreased the total and effective amounts of glutathione, probably through inhibition of glutamate, glycine, serine and glutamine levels (Supplementary Fig. 4), all of them being key precursor metabolites of glutathione synthesis [28]. Our results show that the reduction in total glutathione level could be involved in the sensitization of GBM cells to pro-oxidant stimuli, as radiation (Fig. 1). Glutathione is also related to the TMZ-induced bystander effect described in GBM previously [18]. TMZ is the standard chemotherapeutic for the treatment of GBM, concomitant with surgical resection and/or radiotherapy [2,3]. While many mechanisms contribute to the development of resistances, it is known that small fractions of resistant cells can induce a general resistance to temozolomide by mechanisms that involve an increase in the antioxidant machinery [18]. This effect can be reversed by glutathione-depleting agents. Our results show that CoQ reduces the effective amount of glutathione, sensitizing, in parallel, GBM cells, but not astrocytes, to TMZ in non-irradiated cells (Supplementary Fig. 5), which suggests that this action could be due to the mechanism reported hitherto [15]. It is interesting to note that computer simulations of mathematical models of GBM growth have predicted that the effect of an antioxidant therapy with the properties reported here may have a major effect on the evolution of the disease when other factors are taken into account [42].

Our results reported herein provide the first evidence, *in vitro*, that CoQ radiosensitizes and potentiates TMZ cytotoxicity, inducing apoptosis in human GBM cells, without affecting normal astrocytes, by regulating, simultaneously, intracellular lactate and Hif-1α, catalase activity and SOD2 and glutathione levels. Thus CoQ, known as antioxidant, can diminish the total antioxidant cell capacity deactivating the cell antioxidant machinery. CoQ emerges as an attractive molecule for combined therapies with radiation and TMZ aimed to improve the prognosis of patients suffering this, nowadays, fatal pathology.

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Authors' contributions

JF-R performed O_2^- and H_2O_2 measurements, cell viability, clonogenic assays, cell-sensitivity to exogenous H_2O_2 and western blot and performed statistical analysis. RS-M quantified DNA damage and apoptosis and performed statistical analysis. CMN-V determined the total antioxidant cell capacity. G-F analyzed oxygen consumption. AM-G and VMP-G fitted the curves for cell viability and participated in drafting the manuscript. MV-G and MM identified and quantified intracellular metabolites by NMR. JA analyzed NQO1 by western blot. EL, JA-G and AG-A performed cells irradiation. MP quantified glutathione levels. JRP determined catalase activity. LPR participated in experimental design and data analysis. JMV participated in data analysis and drafting the manuscript. FJA participated in experimental design, data analysis and drafting the manuscript. MD-P conceived the study, designed the experiments, analyzed the data and wrote the manuscript.

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Conflict of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.radonc.2018.04.033.

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