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Original article

Metformin radiosensitizes OSCC in 2D and 3D models: possible involvement of CAF-1

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Summary

Objective. This study investigated metformin as a sensitizer for radiotherapy in oral squamous cell carcinoma (OSCC) to reduce the radiation intensity. It evaluated the drug's effect on Chromatin Assembly Factor-1 (CAF-1) expression, whose high levels correlate with worse prognosis of this cancer.

Methods. The effects of metformin, alone and with radiotherapy, were evaluated on CAL27 (HPV-) and SCC154 (HPV+) OSCC cells. The analyses were performed on cell monolayers by colony-forming assay, motility, and confocal microscopy. In spheroid 3D models, the sensitizing effect of metformin was assessed by measuring areas. CAF-1 expression affected by metformin was evaluated via Western blot, and its role was investigated by siRNAs.

Results. Metformin reduced the cells' ability to form colonies, migrate and invade, and promoted the acquisition of a less aggressive phenotype by increased E-cadherin and decreased N-cadherin expressions. Moreover, metformin lowered the IC50 of radiotherapy and showed strong effects on spheroid growth. Metformin downmodulated the expression of the major subunits of CAF-1, and the knockdown of this protein by siRNAs elicited a metformin-like effect on cell aggressiveness.

Conclusions. Metformin emerged as a promising adjuvant drug in OSCC because of its effects on cell aggressiveness and radiosensitizing action. These activities could be CAF-1-mediated.

Key words: OSCC, metformin, IR, CAF-1, spheroids

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Introduction

Oral squamous cell carcinoma (OSCC) is a subtype of head and neck squamous cell carcinoma (HNSCC) and develops from the mucosal epithelium of the oral cavity as result of a multifactorial process involving genetic alterations, epigenetic modifications and exposure to risk factors such as alcohol, tobacco and infection by human papillomavirus (HPV)[1]. Conventional treatments for this cancer encompass surgery, radiotherapy and chemotherapy, each of which can lead to severe repercussions for patients. Surgical resection of the area of interest may prejudice essential functions, like speech, swallowing and even respiration, making necessary a reconstructive intervention with postoperative problems[2]. Radiotherapy finds application in each stage of the tumor, from single treatment in early phase to palliative approaches in recurrent disease, not without obstacles, such as resistance[3] and complications, which mostly include mucositis, xerostomia, dysgeusia, and osteoradionecrosis[4].

Chemotherapy is based on cisplatin administration alone or in association with other drugs, mostly fluorouracil (5-FU) and docetaxel in the regimen known as TPF (docetaxel, cisplatin, fluorouracil), which can cause resistance[5] and toxicity reactions, like nausea, anemia, febrile neutropenia, mucositis, and gastrointestinal hemorrhage[6].

The complications and resistance to treatments, along with the late diagnosis, due to the absence or lack of recognition of the initial symptoms, the insurgence of local or distant metastasis, which mainly and respectively regard cervical lymph nodes and lung[7], and the frequent recurrences[8] are the causes of poor prognosis and an overall survival lower than 50%. This challenges researchers to find new biomarkers and therapeutic approaches to improve patients' quality and expectation of life.

In the context of new adjuvant approaches for cancer, metformin, well-known as anti-diabetic drug, has stood out for its antitumoral effects in several malignancies, like thyroid, prostate, and head and neck cancers[9]. This drug is described to have mild and reversible common side effects, represented by gastrointestinal reactions, with a favorable safety profile and anticancer action as a good candidate for adjuvant therapy[10]. Retrospective studies reported that metformin-using diabetic patients have a lower incidence of HNSCC compared to non-diabetic individuals even in presence of risk factors, and when affected from this cancer they presented fewer distant metastases and better survival outcomes[11]. Numerous *in vitro* studies, carried out on HPV- cell lines, demonstrated that metformin reduced cell proliferation and colony formation, arrested cell cycle, delayed migration and invasion processes. In addition, it slowed down the growth of xenograft tumors in *in vivo* models[12]. Moreover, this drug was shown to sensitize cells of nasopharyngeal carcinoma, another subtype of head and neck cancer, to radiation[13].

Chromatin Assembly Factor-1 (CAF-1) is a protein complex made up by three subunits, p150, p60 and p48, and fulfils the important role of histone chaperone of H3/H4 dimers during DNA replication and repair[14]. In various cancers, like glioma, melanomas, leukaemia, prostatic, breast and cervical, and squamous cell carcinoma it is upregulated[15]. In particular, the p60 subunit was detected more than doubled in peripheral blood of HNSCC patients and its high levels in tissue correlated with increased aggressiveness, metastasizing behavior, and worse prognosis[16].

Moreover, it was reported that the silencing of the major subunits of CAF-1 via RNA interference in OSCC cell lines led to an increased response to radiation, with the lowering of its IC50, due to a less efficient repair of double-strand breaks (DSBs) [17]. This evidence suggests that CAF-1 could be a potential target for adjuvant therapy, besides a new biomarker.

Our aim was to investigate whether metformin could sensitize OSCC cells to radiation and influence the expression of CAF-1.

Materials and methods

Cell cultures and treatments

The experiments were performed using two human immortalized cell lines derived from the squamous epithelium of the tongue: CAL27 (CRL-2095; American Type Culture Collection, Manassas, VA, USA) as model of HPV negative phenotype of oral cavity; SCC154 (CRL-3241; American Type Culture Collection, Manassas, VA, USA), characterized by positivity for HPV.

CAL27 were cultured in Dulbecco's modified Eagle's medium (DMEM; Euroclone s.p.a., Milan, Italy) with 10% ΔFBS and 1% penicillin-streptomycin (Euroclone s.p.a., Milan, Italy); whereas Minimum Essential Medium (MEM; Euroclone s.p.a., Milan, Italy) supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin-streptomycin was used for maintenance of SCC154. Cells were grown in an incubator at 37 °C in air-humidified 5% CO_2.

Metformin HCl (S1950; Selleckchem, Houston, TX, USA) was dissolved in sterile water, pre-warmed at 50 °C, at a concentration of 200 mM as stock solution and then stored at -80 °C. Upon use, the drug was diluted in medium at a final concentration of 10 mM for the experiments.

Radiotherapy was performed at University Hospital Federico II, Naples. The cells, as monolayer and spheroids, were irradiated with 0 Gy (control group), 2 Gy, 4 Gy and 8 Gy, using a linear accelerator (TrueBeam STx, Varian Medical Systems, Palo Alto, CA, USA) with 6 MV photon beams (same intensity used for patients). Plates were positioned on a 1.5 cm-depth block, placed on the radiotransparent table, at the proper source-axis distance (SAD) of 100 cm, to receive the 100% of radiation intensity.

Individual silencing of the major subunits of CAF-1 was performed in cell lines using FlexiTube GeneSolution siRNA (Cat. No. 1027416; QIAGEN, Hilden, Germany) delivered with TransIT-X2 Mirus (MIR 6003; mirus bio, Madison, WI, USA) following the manufacturers' protocols. A dose-response curve was realized by analyzing the expression of p60 and p150 by Western blot of lysates derived from cells transfected for 24, 48, and 72 hours with 10, 20, and 40 nM of siRNAs. The best

conditions for protein knockdown were selected as follows: medium with 20 nM of siRNAs for p60 and 40 nM for p150 was placed in contact with CAL27 for 24 hours; while the concentration of 20 nM was shown to be effective in silencing both subunits within a time frame of 48 hours for SCC154. After the proper silencing of CAF-1 subunits, a time of 24 hours for functional assays (migration, invasion, and analysis of epithelial-mesenchymal transition markers) followed before stopping the experiments, with the knowledge that the knockdown was stable until 72 hours.

Spheroids generation and area analysis

Spheroids, used as three-dimensional (3D) model, were obtained by seeding CAL27 (2 x10^3 cells/well) and SCC154 (5 x 10^3 cells/well) in 96 well U-bottom plates (Corning Costar, New York, NY, USA), as previously described[18]. Eight days were needed for the growth of spheroids before treating them with metformin for 48 hours and subjected to radiotherapy (2, 4, 8 Gy). After 24 hours, metformin treatment for 72 hours was by changing the media. This time represented the first treatment time captured at microscope Axiocam 208 color ZEISS, 10x. Moreover, spheroids were photographed at 4, 7 and 12 days after radiotherapy. The dimensions of at least 20 spheroids for each cell line and experimental point (radiation intensity and time) were measured with ImageJ software (NIH, Bethesda, MD, USA) and the relative areas (area at experimental time/initial area at 8 days from seeding) of the spheroids were analyzed by comparing the treated versus control ones.

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay

CAL27 (5 x 10^3 cells/well) and SCC154 (5 x 10^3 cells/well) were seeded in 96-well plates and incubated at 37 °C with metformin at the final concentration of 2.5 mM – 5 mM – 10 mM – 15 mM – 30 mM for 24, 48 and 72 hours. At the end of experimental times, 25 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml, M5655; Sigma-Aldrich, Saint Louis, MO, USA) was added to each well and cells incubated for 3 hours. Then the media were removed, and the cells were lysed by adding 100 µl of DMSO (D8418; Sigma-Aldrich, Saint Louis, MO, USA) and shaking for 10 minutes, to solubilize formazan salts. A colorimetric assay was carried out by spectrophotometric reading of the plates at 550 nm using Multiskan Spectrum (Thermo Electron Corporation, Waltham, MA, USA).

Colony forming assay

CAL27 (6.5 x 10^3 cells/well) and SCC154 (1.5 x 10^5 cells/well) were seeded in 6-well plates and incubated at 37 °C with metformin 10 mM for 72 hours (eventually subjected to radiation after 48 hours) before changing the media. After one week of growth, cells were fixed with 1 ml di 4% v/v p-formaldehyde (Lonza, Basel, Swiss) for 10 minutes; dehydrated for 20 minutes with pure methanol (PanReac Applichem, Darmstadt, Germany); stained with 0.5% w/v crystalviolet in a 20% v/v methanol solution (Merck Chemicals, Darmstadt, Germany) for 30 minutes at room temperature (RT). The wells were washed with deionized water and photographed before dissolving crystals in 1% SDS and measuring the absorbance at 570 nm, as previously described [19]. When used to estimate radiosensitivity, optical density values were converted in percentages and the IC50 of radiations with or without metformin was calculated using the equations obtained from the curves.

Wound healing assay

CAL27 (2 x 10^5 cells/well) and SCC154 (3 x 10^5 cells/well) were seeded in 24-well plates. The following day, a wound was created in the confluent layer of cells with a sterile p10 pipette tip, the media was changed with or without 10 mM metformin and the wells were photographed at optical microscope EVOS, 10x (Life technologies Corporation, Carlsbad, CA, USA). Proliferation was blocked by the addition of mitomycin C (10 µg/ml, M5353; Sigma Aldrich, Saint Louis, MO, USA) to allow the exclusive study of migration. 24 hours later, the experiment was stopped by taking final pictures. The distance covered was calculated by subtracting the final wound width to the initial one.
For the analysis of the migration ability of the cells with knockdown of CAF-1, they were properly silenced in the selected times described above (*Cell cultures and treatments* Materials and methods), a wound was made, and the media refreshed.

Invasion transwell-assay

Cells (CAL27 1.5 x 10^4/well; SCC154 3 x 10^4/well) were added on a layer of matrigel (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in the upper chamber of a 8 µm-porous insert (Corning Incorporated, NY, USA), placed in a 24-well plate. Media with or without 10 mM metformin were put in the bottom of the wells. After 24 hours, cells which did not invade the matrix were washed away, while the invading ones were fixed with 1 ml di 4% v/v p-formaldehyde (Lonza, Basel, Swiss) for 10 minutes; dehydrated for 20 minutes with pure methanol (PanReac Applichem, Darmstadt, Germany); stained with 0.5% w/v crystalviolet in a 20% v/v methanol solution (Merck Chemicals, Darmstadt, Germany) for 30 minutes at RT. The excess dye was removed, and the inserts were washed in deionized water. Stained cells were photographed

with a optical microscope EVOS, 10x (Life Technologies Corporation, Carlsbad, CA, USA) and counted with ImageJ software.
For analysis of the invasion ability of cells with knockdown of CAF-1, they were firstly silenced, then trypsinized, counted and placed on the layer of matrigel with complete growth media in the lower chamber.

Confocal microscopy

Cells (5 x 10^4/well) seeded on coverslips placed in a 24-well plate were incubated with media with or without 10 mM metformin for 24, 48 and 72 hours (eventually subjected to radiation after 48 hours). At the end of experimental times (in the case of radiation, 3 hours after this treatment), cells were fixed using 4% v/v p-formaldehyde (Lonza, Basel, Swiss) for 10 minutes; permeabilized for 5 minutes with 0.5% v/v Triton X-100 (Lonza, Basel, Swiss) and nonspecific sites blocked with 20% v/v goat serum (Lonza, Basel, Swiss) for 30 minutes. The coverslips were incubated with the primary antibody against the protein of interest at the appropriate dilution, overnight at 4 °C: 1:100 E-cadherin (mouse, sc-8426; Santa Cruz, Dallas, TX, USA); 1:100 N-cadherin (rabbit, E-AB-64010; Elabscience, Houston, TX, USA); 1:150 γH2AX (mouse, sc-517348; Santa Cruz, Dallas, TX, USA). The following day, coverslips were incubated for 2 hours at room temperature with the dye 550-coniugated anti-mouse or -rabbit secondary antibodies (1:500) and DAPI (1:1000) preceding mounting ImGES were acquired with a Zeiss LSM 510 Laser Scanning microscope (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany) using 63x objective (in case of γH2AX zooming 3.7 for the best visualization of nuclei). Quantization was performed with ImageJ software.

Western blotting (WB)

Whole lysates derived from cell cultures were resolved by Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) using Bio-Rad equipment (Hercules, CA, USA). In detail, at the end of experimental times, cells were harvested and lysed with homemade Laemmli buffer 1X diluted from 4X stock (40% v/v glycerol, 20% v/v 2-mercaptoethanol, 8% w/v SDS, 0.25M Tris-HCl pH 6.8, 6 mM Bromophenol blue). Primary antibodies anti-CHAF1B (rabbit polyclonal HPA021679; Sigma-Aldrich, St. Louis, MO, USA); CAF-1 p150 (mouse monoclonal sc-32742; Santa Cruz, Dallas, TX, USA); GAPDH (rabbit monoclonal, (14C10) #2118; Cell Signaling Technology, Danvers, MA, USA) were used overnight at 4°C. Finally, secondary antibodies peroxidase-conjugated (115-035-003/111-035-003 Jackson ImmunoResearch, West Grove, PA, USA) allowed the detection of signals using a CCD camera ImageQuant LAS 4000 (GE Healthcare, Life Sciences, Chicago, IL, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 or Excel software. All data are presented as mean ± standard deviation (SD) from at least three independent experiments. Significance was estimated through Student's t test or one-way ANOVA with Dunnett's or Tukey's post-hoc depending on the case, considering $p \leq 0.05$ as reference value to reject the null hypothesis.

Results

Metformin induced significant reduction of colony formation, motility and aggressive behavior of HPV- and HPV+ OSCC cell lines.
In the first instance, we wanted to confirm the antitumoral effect of metformin, already reported in the literature[12], on two OSCC cell lines: CAL27, representative of HPV- phenotype, and SCC154, characterized by HPV positivity.
We treated cells at different times with metformin at a concentration of 10 mM, which was selected based on the MTT assay (Fig. Suppl. 1).
As shown in Figures 1A and 1B, metformin notably reduced the growth of colonies of these cells in a time-dependent manner, with the maximum effect at 72 hours of drug administration.
Moreover, metformin hampered cellular motility in both cells, in terms of speed of migration (Fig. 1C, 1E) and invasion (Fig. 1D, 1F), respectively evaluated by wound healing and transwell assays.
Cells treated with metformin showed a reversion of the expression of two proteins belonging to the family of cadherins, E- and N-cadherins, considered as epithelial-mesenchymal transition (EMT) markers. The analysis through confocal microscopy of CAL27 and SCC154 under the effect of metformin from 24 to 72 hours demonstrated a reduction of the level of N-cadherin, a mesenchymal marker and an increase of E-cadherin, indicator of epithelial phenotype (Fig. 1G). Thus, metformin was confirmed to reduce colony formation and cell motility and induced a reversion of the expression of epithelial-mesenchymal markers.

OSCC cell lines were more sensitive to irradiation when treated with metformin

Among the approaches employed in the therapeutic protocols of OSCC, radiotherapy was selected to be investigated insofar as the first-line treatment after surgery, besides finding application in each stage of this cancer.

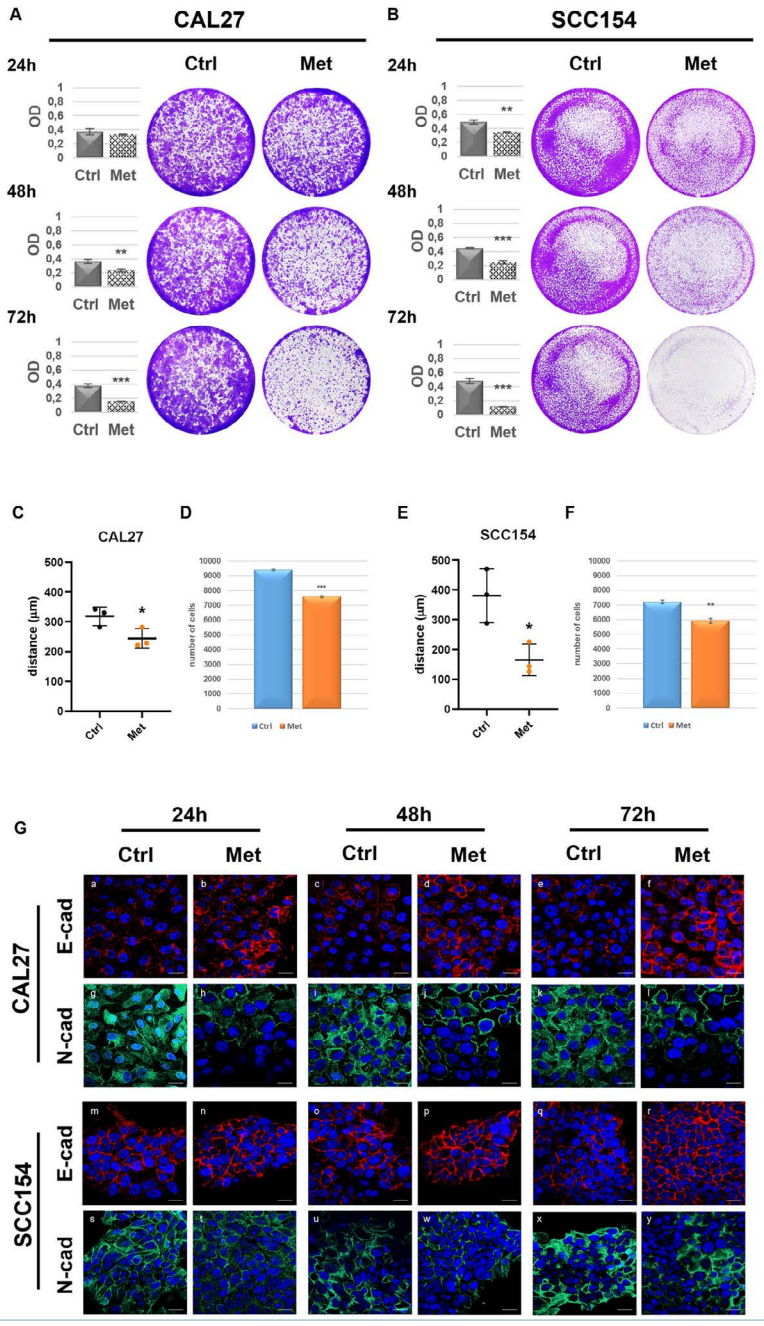

Figure 1. Effects of metformin on cell aggressiveness. (A) Representative images of colony formation of CAL27 treated with metformin for 24, 48, 72 hours and dissolution of crystal violet with SDS (histograms). (B) Representative images of colony formation of SCC154 treated with metformin for 24, 48, 72 hours and dissolution of crystal violet with SDS (histograms). (C) Wound healing assay of CAL27 under metformin treatment. (D) Invasion transwell-assay of CAL27 with metformin. (E) Wound healing assay of SCC154 under metformin treatment. (F) Invasion transwell-assay of SCC154 with metformin. (G) Immunofluorescence of E- and N-cadherin of CAL27 (a-l) and SCC154 (m-y) with 24, 48, 72 hours of metformin. Scale bar = 20 µm. Significant differences from control conditions are indicated by asterisks (* $p \leq 0,05$; ** $p \leq 0,01$; *** $p \leq 0,001$).

To determine if metformin may potentiate the effect of radiotherapy, a clonogenic assay, the gold-standard technique to evaluate radiosensitivity *in vitro*, was performed with CAL27 and SCC154 subjected to ionizing radiation (IR) following treatment with the drug.
Colonies appeared significantly lower in number and dimension when exposed to metformin and radiation in direct dependence with the intensity of this latter (Fig. 2A, 2D). The spectrophotometric reading of crystal violet dissolved with SDS (Fig. 2B, 2E) and the conversion of the absorbance values in percentages, revealed a notable reduction of the IC50 of radiation for these cells (Fig. 2C, 2F), apparently more remarkable for CAL27 than for SCC154. In detail, for CAL27 the IC50 decreased from 3 to 1.9 Gy and for SCC154 from 3.9 to 3.3 Gy.
The minor capability of cells to form colonies demonstrated that the treatment with metformin made them more sensitive to IR, lowering its IC50.

Radiation-induced γH2AX foci formation was significantly enhanced by metformin in OSCC cell lines

Exposure to ionizing radiation induces DSBs in the genome of irradiated cells, and phosphorylation of histone H2AX (γH2AX) at the site of damage is a constant and early event in damage detection and repair mechanisms[20]. The count of γH2AX foci was evaluated as a measure of DNA damage response for an IR dose range of a maximum of 4 Gy, since cells appeared unhealthy at higher doses of IR.
As shown in Figure 3, the number of γH2AX foci increased with the dose of IR (Fig. 3A panels a, c, e, and Fig. 3C panels a, c, e) and even further when com-

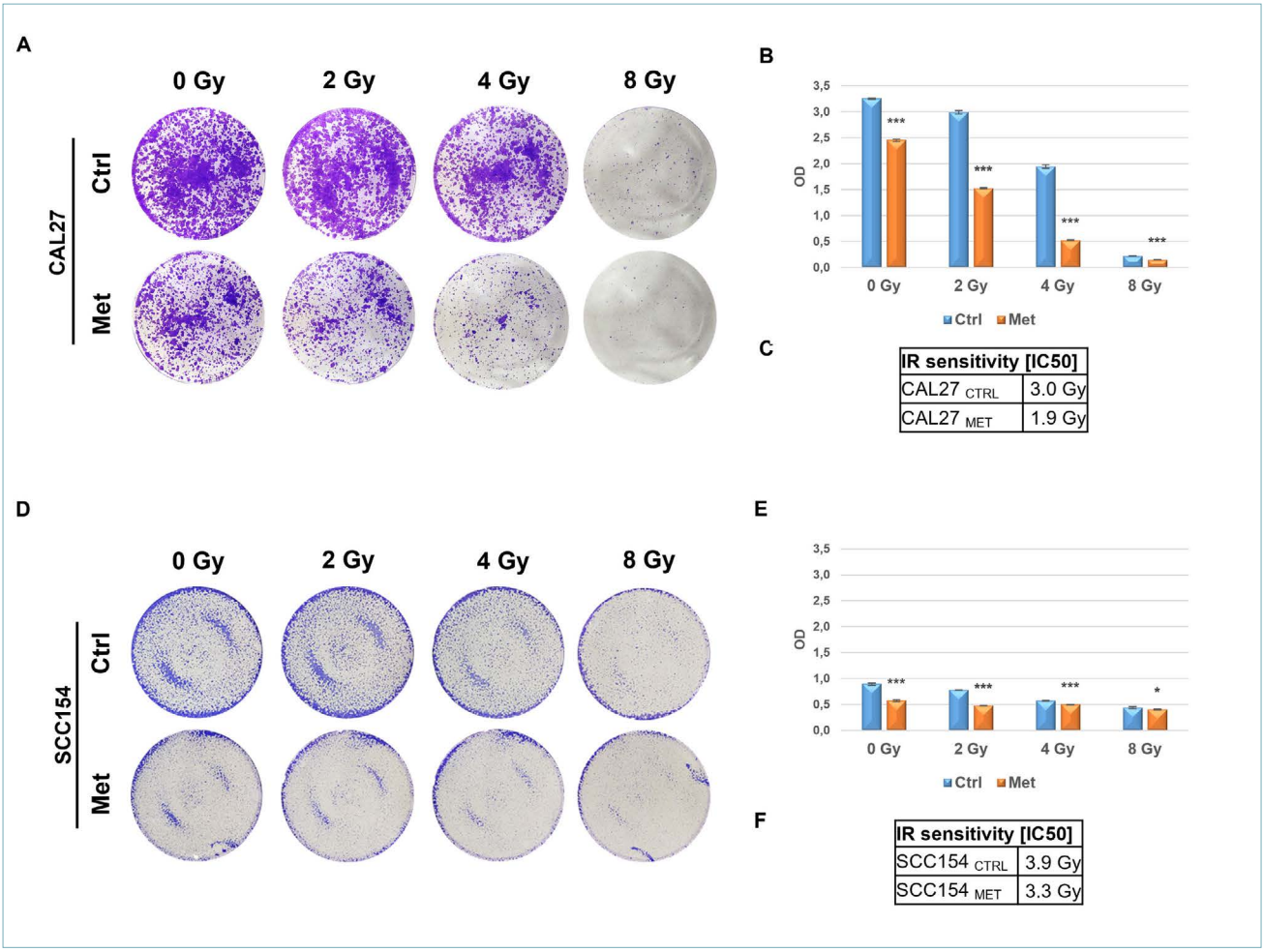

Figure 2. Radiosensitizing action of metformin. (A) Colony formation assay of CAL27 irradiated with 0, 2, 4, 8 Gy with or without 72 hours of metformin treatment. (B) OD values by dissolution of crystal violet used for staining of colonies of CAL27. (C) IC50 of IR of CAL27 +/- Met. (D) Colony formation assay of SCC154 irradiated with 0, 2, 4, 8 Gy with or without 72 hours of metformin treatment. (E) Optical density values by dissolution of crystal violet used for staining of colonies of SCC154. (F) IC50 of IR of SCC154 +/- Met. Significant differences from control conditions are indicated by asterisks (* $p \leq 0,05$; *** $p \leq 0,001$).

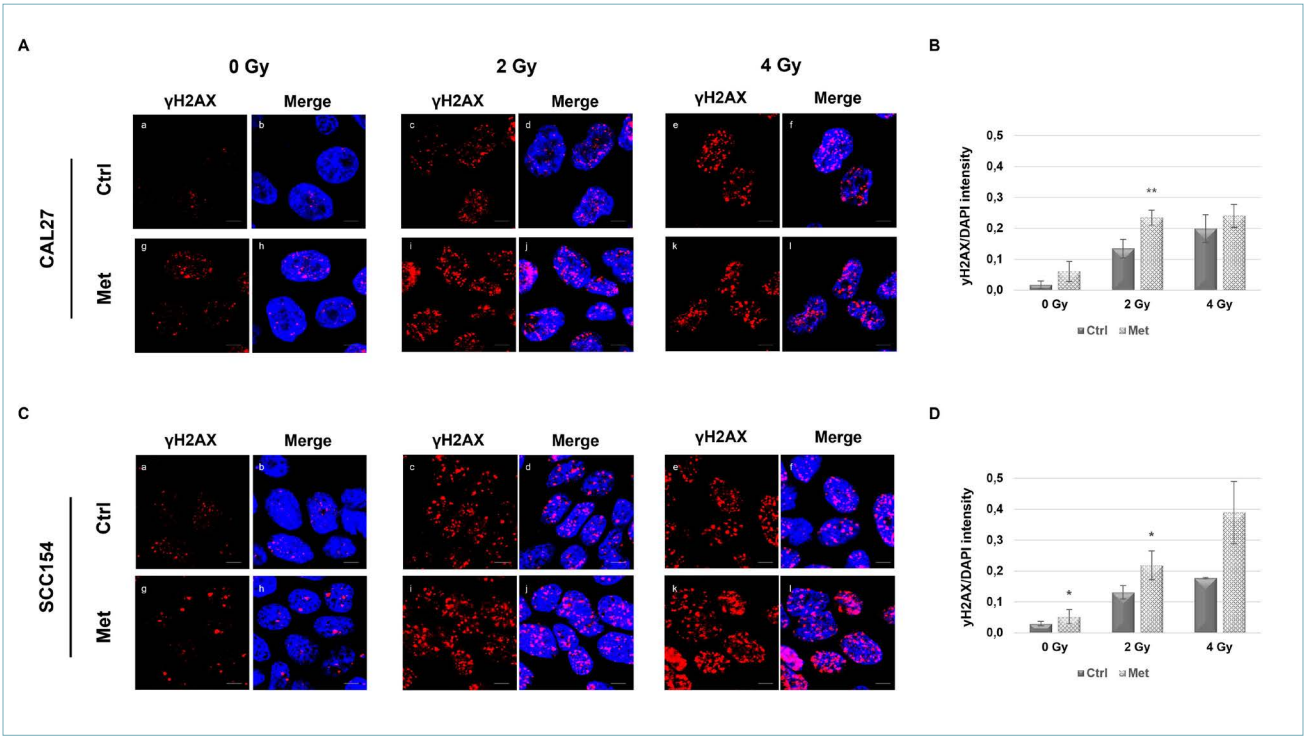

Figure 3. Effects of metformin on irradiation-induced γH2AX foci formation. (A) Immunofluorescence of γH2AX of CAL27 treated with Met, IR and their combination. (B) γH2AX/DAPI intensity in CAL27. (C) Immunofluorescence of γH2AX of SCC154 irradiated with 0, 2, 4 Gy with or without metformin treatment. (D) γH2AX/DAPI intensity in SCC154. Scale bar = 5 μm. Significant differences from control conditions are indicated by asterisks (* $p \leq 0,05$; ** $p \leq 0,01$).

bined with metformin (Fig. 3A panels g, i, k, and Fig. 3C panels g, i, k), indicating an enhanced action of radiation due to treatment with the drug.

The immunofluorescence of this marker, in addition to showing this effect in both cell lines, demonstrated metformin's efficacy as a sensitizer to radiation also in SCC154, which was less detectable by clonogenic assay.

These data confirmed metformin's sensitizing action to radiation, in accordance with the lowering of its IC50.

Metformin alone and in combination with radiation hampered OSCC spheroid growth

To study the impact of metformin treatment on spheroids, a more complex system and closer to *in vivo* models obtained with OSCC cell lines were set up and used as a 3D model. This mimicked the typical spatial structure of solid tumors, characterized by a necrotic core and a proliferative external layer[18].

First, we observed that spheroids formed by CAL27 and SCC154 differed in aspect and dimension, although they were seeded in appropriate number to counterbalance their dissimilar proliferation rate. In detail, CAL27 made compact spheroids, characterized by wider area and budding of several smaller cellular organizations in the time, in comparison with SCC154 which produced smaller spheroids more likely to aggregate and surrounded by some loose cells, as reported in literature[21].

Following the same experimental timing for the administration of metformin and radiation previously applied to the bidimensional (2D) model, the growth of spheroids was subsequently observed for 12 days after exposure to IR.

From the analysis of their dimension changes in the time, both metformin and radiation hampered the growth of CAL27 spheroids when administered alone and strongly when combined, more evident with increasing dose of irradiation (Fig. 4, Suppl. 2A). On the other hand, SCC154 spheroids dimensions got smaller in proportional way with the intensity of IR alone and more significantly with metformin independently of the association with radiation (Fig. 5, Suppl. 2B), possibly for the higher sensitivity to drug treatment alone.

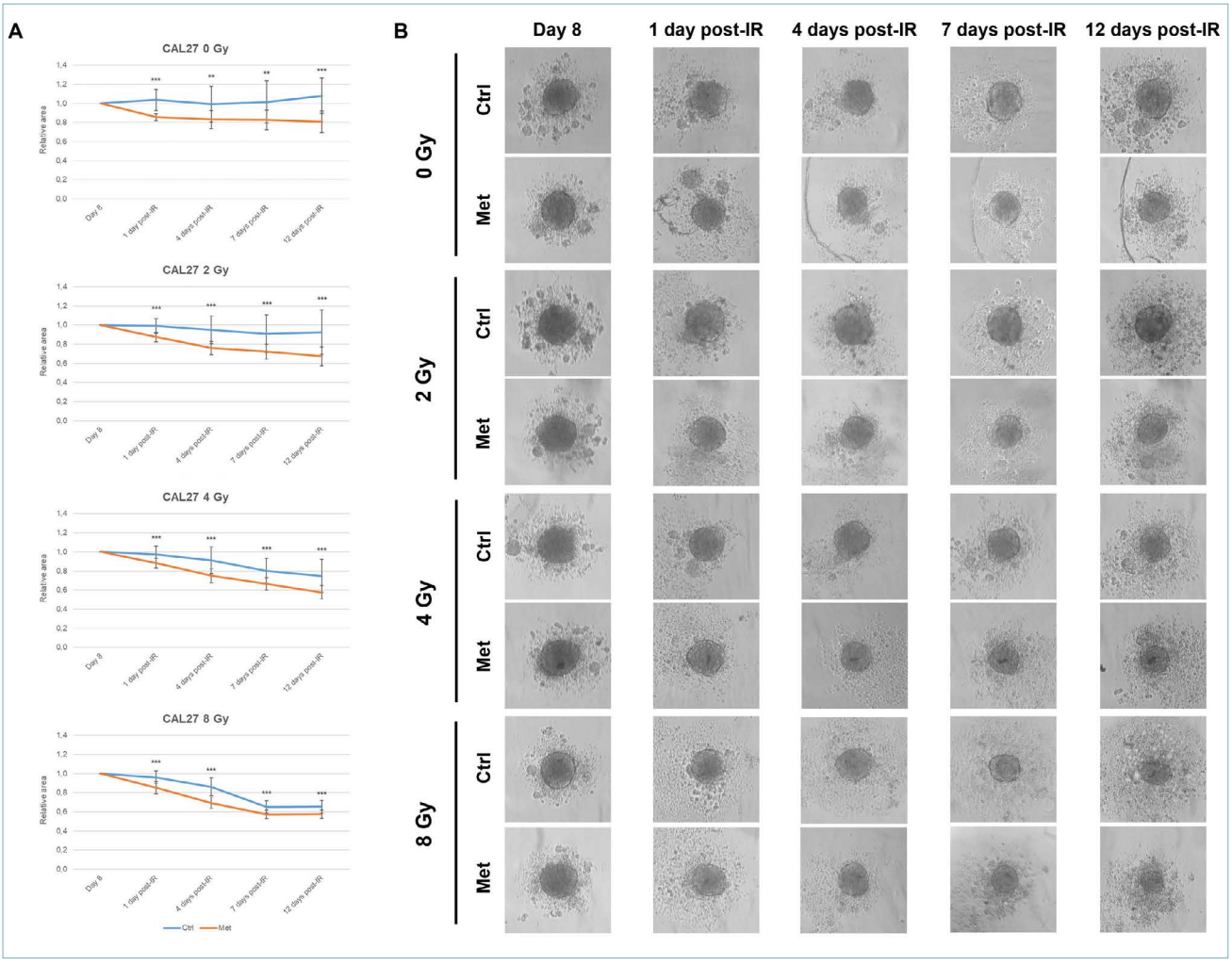

Figure 4. Impact of metformin on CAL27 spheroid growth. (A) Blue line: spheroids irradiated with 0, 2, 4, 8 Gy; orange lines: metformin alone (0 Gy) and in combination with IR. (B) Spheroids were photographed and measured at 1-, 4-, 7- and 12-days post-IR, and at 8 days after seeding. Each picture is representative of at least 10 spheroids. Significant differences from control conditions are indicated by asterisks (** $p \leq 0,01$; *** $p \leq 0,001$).

CAF-1 expression was downregulated with metformin and its modulation with siRNAs induced metformin-like effects on cell aggressiveness

Since CAF-1 was recently identified as new potential biomarker of HNSCC[16] and its downregulation by shRNAs made cells more sensitive to radiation[17], we investigated whether metformin was able to negatively affect the expression of the major subunits of this complex, p150 and p60, clarifying the mechanism of its radiosensitizing effect.

Western blots of CAL27 and SCC154 revealed that metformin reduced the levels of these proteins in a time-dependent manner (Fig. 6A, 6F).

To demonstrate the involvement of this complex, the knockdown of its major subunits was realized with siRNAs (Fig. 6B, 6G) and the characteristics of OSCC cell lines were observed in terms of cell motility and phenotype.

CAF-1 silenced cells showed significantly less capability to migrate (Fig. 6C, 6H) and invade (Fig. 6D, 6I), in line with the acquisition of a different phenotype, characterized by an increased expression of epithelial marker E-cadherin and lower levels of the mesenchymal one, N-cadherin, in comparison with untreated cells. These effects were similar to those induced by metformin administration (Fig. 1).

Metformin downmodulated the expression of CAF-1, an event that, when occurring through RNA interfer-

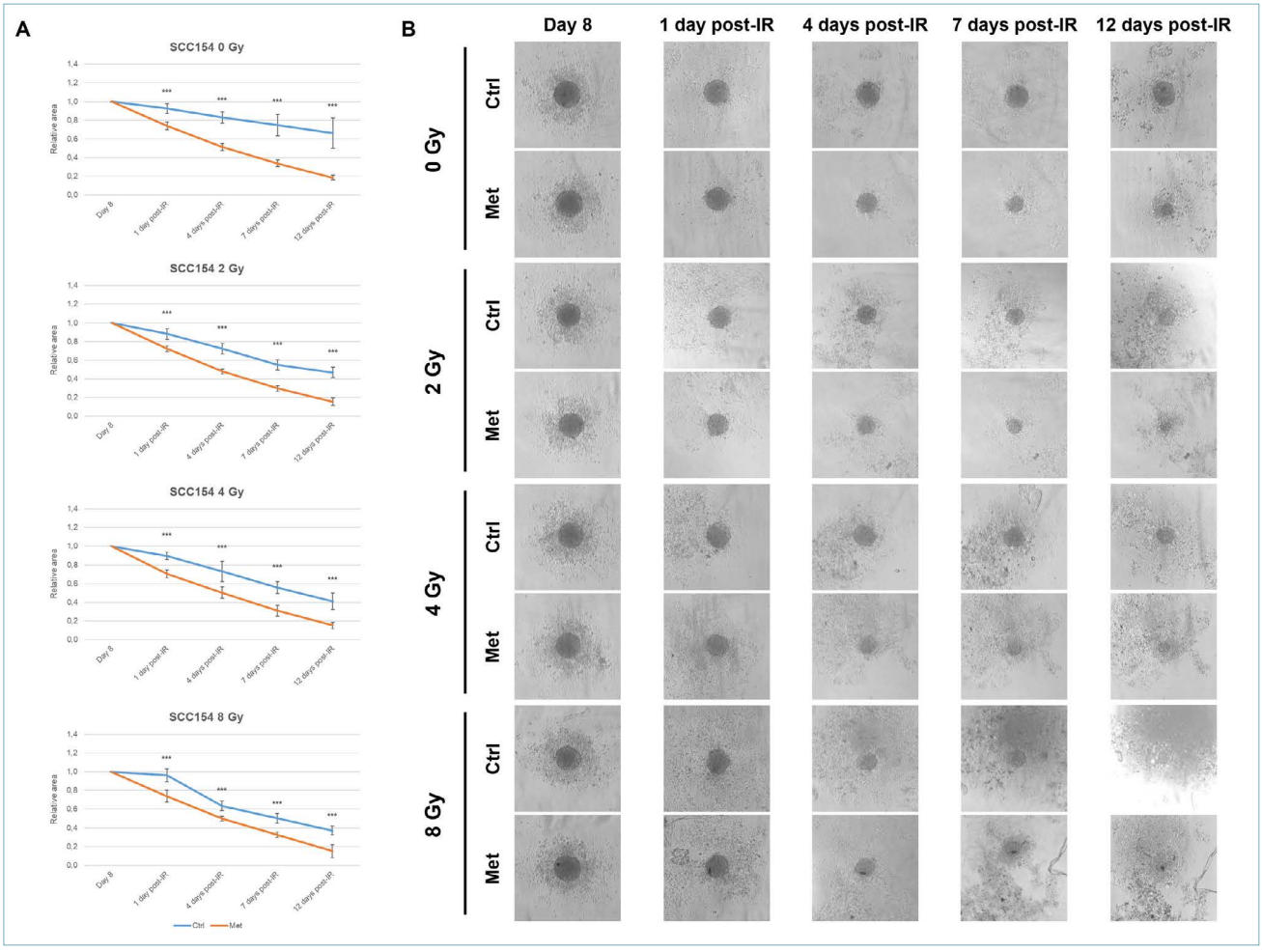

Figure 5. Impact of metformin on SCC154 spheroid growth. (A) Blue lines: spheroids irradiated with 0, 2, 4, 8 Gy; orange lines: metformin alone (0 Gy) and in combination with IR. (B) Spheroids were photographed and measured at 1-, 4-, 7- and 12-days post-IR, and at 8 days after seeding. Each picture is representative of at least 10 spheroids. Significant differences from control conditions are indicated by asterisks (*** $p \leq 0{,}001$).

ence, induced a decrease in cell aggressiveness in metformin-dependent manner.

Discussion

OSCC is an aggressive cancer of the oral cavity that challenges researchers to uncover new biomarkers, targets and approaches to anticipate diagnosis, increase response to therapies and improve survival and quality of life, as well as minimize complications due to treatments, beyond that of the cancer itself.
In this context, one of the main interests of health professionals is establishing adjuvant approaches to enhance conventional therapies, reducing doses and times of treatments.

Other two important aspects that are worth considering concern the high costs of traditional chemotherapy for the healthcare system and the low compliance of this therapeutic approach for patients, due to parenteral administration, in addition to adverse reactions. In contrast, metformin is an oral drug, easily accessible, and used as first line treatment for type 2 diabetes, but also has promising off-label antitumor effects, mainly regarding the inhibition of proliferation and motility in several malignancies, such as thyroid and prostate, besides head and neck[9]. Another important benefit concerns the safety profile of metformin, whose common side effects are represented by gastrointestinal reactions, in contrast to the severe toxicity associated with chemotherapy[6]. Additionally, some clinical retrospective studies found that diabetic patients treated

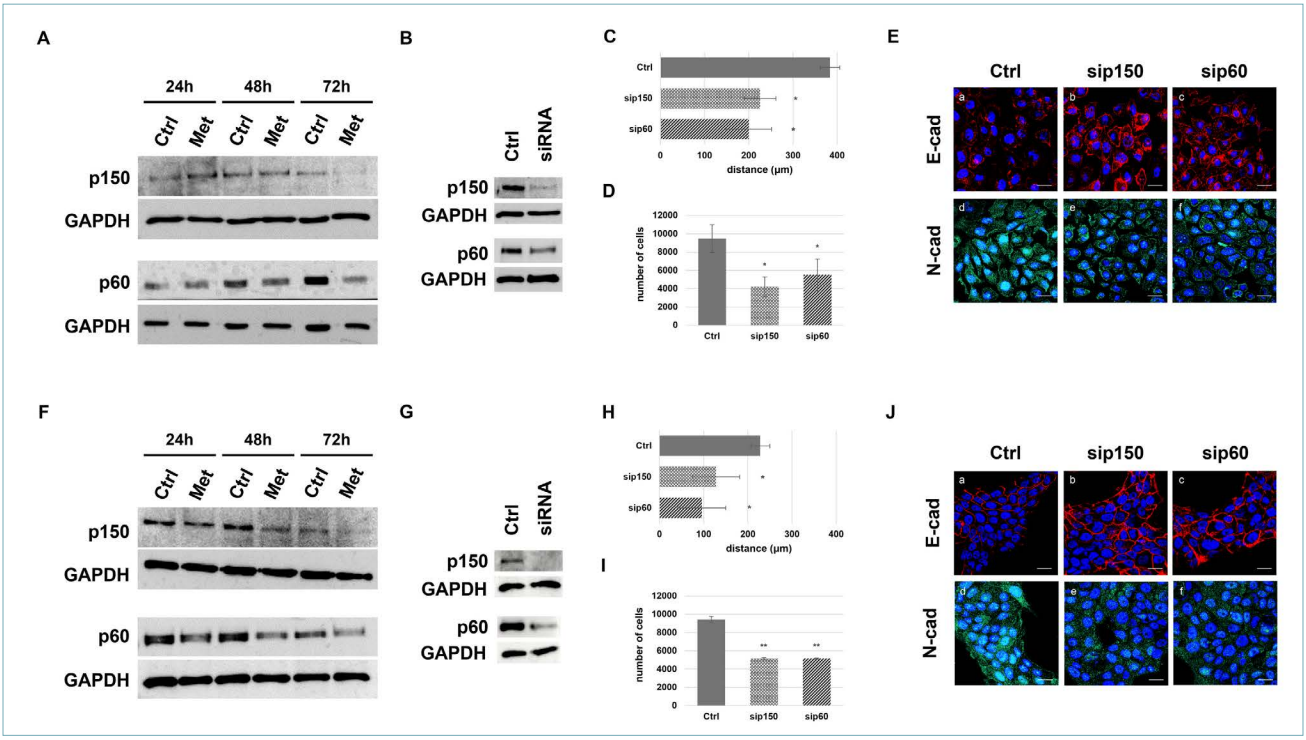

Figure 6. Metformin and CAF-1. (A) Western blots for CAF-1 major subunits, p150 and p60, of CAL27 treated with metformin for 24, 48 and 72 hours. (B) CAF-1 silencing by siRNAs in CAL27 assessed via WB. (C) Wound healing assay of CAL27 silenced for CAF-1/p150 and CAF-1/p60. (D) Invasion assay of CAF-1 silenced CAL27. (E) Immunofluorescence of E- and N-cadherin of CAL27 silenced for CAF-1 major subunits (scale bar = 20 μm). (F) Western blots for CAF-1 major subunits, p150 and p60, of SCC154 treated with metformin for 24, 48 and 72 hours. (G) CAF-1 silencing by siRNAs in SCC154 assessed via WB. (H) Wound healing assay of SCC154 silenced for CAF-1/p150 and CAF-1/p60. (I) Invasion assay of CAF-1 silenced SCC154. (J) Immunofluorescence of E- and N-cadherin of SCC154 silenced for CAF-1 major subunits (scale bar = 20 μm). Significant differences from control conditions are indicated by asterisks (* $p \leq 0,05$; ** $p \leq 0,01$).

with this drug have a lower incidence of HNSCC, and if affected, fewer metastases and better survival[11]. For these reasons, metformin is increasingly proposed as an adjuvant drug in cancer treatment.

First, our results demonstrated through *in vitro* experiments that metformin negatively affected OSCC aggressiveness, acting on proliferation, motility and cell phenotype. Although there are several reports of its antitumor effects in this cancer, they were mainly obtained on HPV- cell lines[12], and thus our data add new interesting observations to the few studies which also encompass HPV+ models. Our experiments showed for the first time in OSCC cell lines the reversion of the expression of EMT markers through immunofluorescence, with a decrease of the levels of N-cadherin, a protein more expressed by mesenchymal cells and an increase of E-cadherin, a protein which mediates cell-cell adhesion. To our knowledge, E-cadherin levels under the action of metformin were previously analyzed only in CAL27 by RTqPCR[22], while there are no reports of its expression and localization by confocal microscopy in other OSCC lines. Furthermore, our study included the assessment of N-cadherin expression, since it was described as counterpart of E-cadherin in the process known as "cadherin switching", which represents one of the key steps of cancer progression, promoting EMT and metastasis. Coherently with its function, N-cadherin is found overexpressed and related to worse 3-year survival in head and neck cancers[23], and is thus worthy of evaluation. In the light of these considerations, the phenotypic change induced by metformin, along with the reduction of proliferation and motility, acquire important biological meaning to an expected decrease of tumor aggressiveness in *in vivo* models and patients.

In addition, two main findings emerged from our study: one is the radiosensitization induced by metformin in 2D and 3D models of OSCC; the other is

that this drug downmodulated the expression of CAF-1, a histone-chaperone involved in DNA replication and repair, and which could be part of the mechanism through which metformin acts.

Regarding the first point, surprisingly we found that OSCC cells treated with metformin were more sensitive to IR, with an increase in DNA damage, as demonstrated by fluorescence of γH2AX, a marker for DSBs. This sensitization is translated into lowering of the IC50 of IR in the bidimensional model, which could allow a reduction in IR intensity used for patients, with potentially fewer complications.

The process of drug discovery and development that leads to define a new therapeutic approach in patients passes through a long pre-clinical stage, which includes *in vitro* and *in vivo* experiments. Before approaching animals, with related ethical and technical problems, the scientific community is progressively accepting the use of 3D models, such as spheroids and organoids, as an intermediate step between the two kinds of experimentation. In vitro systems are easy to manage, but characterized by similar aspects to *in vivo* tumors, and in the case of organoids, directly deriving from patients' cancer, consenting the evaluation and selection of a tailored therapy.

The simplest 3D model is represented by spheroids. Those are generally composed of only one cellular type and used to mimic the spatial structure of solid tumors, with a proliferative external layer and a necrotic inner core[18,21].

By the analysis at the final experimental time of 12 days of growth of OSCC spheroids subjected to IR alone and in combination with metformin, our work revealed two different effects on their growth based on the original cell line.

For CAL27, HPV- cells, we observed a reduction of the areas of spheroids with increasing radiation intensity. Metformin induced a more notable shrinking of their dimensions, but with a less significant difference comparing with the control groups with the increase of IR, meaning that the more intense the irradiation was, the less evident the metformin contribution became. Moreover, the most interesting result is that the combination of metformin and 2 Gy of IR produced a comparable effect to 4 Gy on spheroid growth, with similar areas when treated with the combination of metformin plus 4 Gy of IR and 8 Gy of IR alone. The 3D model confirmed the sensitizing effect of metformin to radiation previously seen in cell monolayers and suggest that, if treated with metformin, patients may need lower doses of radiation to obtain the same biological effect than high IR alone, with fewer complications

On the other hand, for SCC154, HPV+ cells, there was a progressive reduction of the areas of spheroids with the intensity of radiation alone, analogously to CAL27, and metformin strongly influenced their growth, independently of the association with IR. Each group of SCC154 spheroids treated with metformin, even not irradiated, showed significant smaller dimensions compared to any group of irradiated spheroids.

This discrepancy between the impact on growth on the two kinds of spheroids following the administration of metformin plus radiation may be explained by the higher sensitivity to treatments associated to HPV [24], which could prevent the detection of any eventual additivity of the combination, due to the major effect of the drug alone on SCC154 spheroids against CAL27 ones. Nonetheless, this data shows the efficacy of metformin on tumor growth and of enhancement to radiation, especially of HPV-OSCC, which is notoriously more aggressive and harder to treat[24].

The mechanism of action of metformin in cancer implies numerous cellular actors, such as AMPK, LKB1, p53 and many others, in a complex synergism not completely clarified that still leaves room for further investigations [9,12]. Furthermore, this drug is reported to be involved in epigenetic modifications, responsible for chromatin structure and gene expression, with potential clinical implications in cancer and other diseases[25,26]. There are many studies in the literature that support the importance of chromatin structure and its remodelers and histone modifiers in regulating transcriptional patterns of genes involved in tumor development and progression[27,28]. In this context, histone chaperones play a coordinating role in these processes. CAF-1 is a histone-chaperone, with a crucial role in regulating heterochromatin organization and in modulating epigenetic factors of chromatin. This complex is also essential for DNA replication and repair and was designed as new diagnostic and prognostic marker in OSCC, correlating with worse prognosis since it is overexpressed in patients' tissues and peripheral blood[16]. Interestingly, the silencing of p60 and p150 subunits of CAF-1 induced an increase sensitivity to radiation with a marked reduction of IC50, demonstrated *in vitro* by colony forming assay and caused by the impairment of repair of DSBs[17]. In the present paper, based on (i) the important role of CAF-1 in both epigenetic modifications and DSB repair, (ii) its downmodulation associated with radiosensitization, (iii) the involvement of metformin in epigenetic modifications and its ability to radiosensitize by inducing increased DNA damage, we investigated if CAF-1 may be responsible for the action of metformin, and if its expression was affected by the drug. This led us to the second finding of our research: unexpectedly metformin reduced the levels of the major subunits of CAF-1, p150 and p60, in OSCC cell lines in a time-dependent

manner. To date, no drug has been reported to be able to suppress the expression of this protein, which is upregulated in several malignancies and considered to be a marker of aggressiveness. The identification of metformin action on CAF-1 would allow avoiding the long process of drug development required for a new molecule, leading to fast clinical access

Subsequent to the silencing of CAF-1 by siRNAs, we observed the acquisition of a less aggressive behavior, by slowing down migration and invasion, reverting the expression of cadherins, markers of EMT, thereby inducing a phenotype with characteristics closer to epithelial cells and more distant to mesenchymal ones, and tehrefore less prone to metastasis and tumor progression.

The similarity of the impact on cell aggressiveness and radiosensitization[17] due to CAF-1 silencing and metformin administration, and the capability of this latter to downmodulate CAF-1 expression, demonstrates the complexity of mechanisms trigged by this drug and responsible of its antitumor effects, probably attributable to the pivotal role of CAF-1 in genomic replication and repair of DNA damage induced by radiation.

Conclusions

In our 2D and 3D models, metformin reduced cell aggressiveness and sensitized to IR, especially in HPV-OSCC. We believe that this action can be also attributable to CAF-1 downmodulation, since when induced by siRNAs produced metformin-like effects on tumor characteristics.

Our results add to the evidence that metformin is a safe and easily accessible drug traditionally used for T2DM, but also has antineoplastic effects. In addition, our work introduces it as a potential candidate for combined therapy with radiation and suggests that CAF-1 downmodulation potentially represents a new therapeutic strategy in OSCC for the consequences of its silencing on tumor aggressiveness. Finally, our findings pave the way for further investigations on the complex mechanism by which metformin acts and affects CAF-1 expression

Conflicts of interest statement

The authors declare no conflict of interests.

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Authors' contributions

Data curation, M.P., N.N., R.B.; Formal analysis, M.P., N.N., R.B.; Funding acquisition, A.P.; Investigation, M.P., N.N., R.B.; Project administration, S.S., A.P.; Resources, C.O., R.P., F.M., S.S.; Supervision, N.N., R.B., A.P., R.P., F.M., S.S.; Writing—original draft, M.P., N.N., R.B.; Writing—review & editing, F.M., R.P., A.P. All authors have read and agreed to the published version of the manuscript.

Ethical consideration

Not applicable.

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