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Title: ^{18}F -HX4 Hypoxia PET Holds Promise as a Prognostic and Predictive Imaging Biomarker in a Lung Cancer Xenograft Model Treated with Metformin and Radiotherapy

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ABSTRACT

Metformin may improve tumor oxygenation and thus radiotherapy response, but imaging biomarkers for selection of suitable patients are still under investigation. First, we assessed the effect of acute metformin administration on non-small cell lung cancer (NSCLC) xenograft tumor hypoxia using positron emission tomography (PET) imaging with the hypoxia tracer ^{18}F -flortanidazole (^{18}F -HX4). Second, we verified the effect of a single dose of metformin prior to radiotherapy on long-term treatment outcome. Third, we examined the potential of baseline ^{18}F -HX4 as a prognostic and/or predictive biomarker for treatment response. **Methods:** A549 tumor-bearing mice underwent a ^{18}F -HX4 PET/computed tomography (CT) scan to determine baseline tumor hypoxia. The next day, mice received an intravenous (IV) injection of 100 mg/kg metformin. ^{18}F -HX4 was administered IV 30 min later and a second PET/CT scan was performed to assess changes in tumor hypoxia. Two days later, mice were divided into three therapy groups: controls (group 1), radiotherapy (group 2), and metformin+radiotherapy (group 3). Animals received saline (groups 1-2) or 100 mg/kg metformin (group 3) IV, followed by a single radiotherapy dose of 10 Gy (groups 2-3) or sham irradiation (group 1) 30 min later. Tumor growth was monitored triweekly by caliper measurement and relative tumor volumes ($\text{RTV} = V_{\text{time}} \times V_{\text{baseline}}$) were calculated. The tumor doubling time (TDT), i.e. the time to reach 2× the pre-irradiation tumor volume, was defined as the endpoint. **Results:** Thirty min post-metformin treatment, ^{18}F -HX4 demonstrated a significant change in tumor hypoxia with a mean intratumoral reduction in ^{18}F -HX4 tumor-to-background ratio (TBR) from 3.21 ± 0.13 to 2.87 ± 0.13 ($p=0.0001$). Overall, RTV over time differed across treatment groups ($p<0.0001$). Similarly, the median TDT was 19, 34 and 52 days in controls, the radiotherapy-group and the metformin+radiotherapy-group, respectively (log-rank $p<0.0001$). Both baseline ^{18}F -HX4 TBR (HR 2.0; $p=0.0004$) and change from baseline TBR (HR 0.39; $p=0.04$) were prognostic biomarkers for TDT irrespective of treatment, and baseline TBR predicted metformin-specific treatment effects which were dependent on baseline tumor hypoxia.

Conclusion: Using ^{18}F -HX4 PET imaging in a NSCLC xenograft model, we showed that metformin may act as radiosensitizer by increasing tumor oxygenation and that baseline ^{18}F -HX4 shows promise as an imaging biomarker.

KEY WORDS

^{18}F -HX4 PET, tumor hypoxia, metformin, radiotherapy, imaging biomarkers

INTRODUCTION

Tumor hypoxia is a negative prognostic factor for radiotherapy-treated tumors, including non-small cell lung cancer (NSCLC) (1). Hypoxia is often described as chronic (i.e. the result of diffusion limitations) or acute (i.e. the results of transient fluctuations in blood flow), although more granular classifications have been proposed (2). Hypoxia causes radiotherapy resistance primarily by limiting oxygen fixation, the crucial step for radiation to effect DNA damage, and importantly has also been linked with a more aggressive tumor phenotype as such (3). Yet, most interventions to ameliorate tumor oxygenation have failed translation into routine clinical practice (4), except for nimorazole, an oxygen-mimicking radiosensitizer that is incorporated into standard radiotherapy treatment of head and neck cancer in Denmark only (3).

More recent studies have focused on the radiosensitizing properties of metformin, a first-line treatment for diabetes mellitus, since in retrospective analyses it had been observed that diabetic patients with cancer who underwent radiotherapy had better outcomes if they were taking metformin, compared to diabetics not taking metformin (5-7). These intriguing observations were later confirmed in non-diabetic preclinical models (8,9). Metformin may affect tumor therapy response and tumor growth either directly or indirectly. The direct effect is attributed to the inhibition of the mitochondrial complex I and its downstream pathways, resulting in activation of the cellular energy sensor adenosine monophosphate-activated kinase. This in turn increases cellular catabolism and reduces anabolism (10). The indirect effect on the other hand is caused by metformin's lowering effects on blood glucose and insulin levels, two major cancer growth stimulating factors. Yet, the way in which metformin influences radiation response is still controversial and may probably be a result of different mechanisms (5-7). One of the better-established theories is that metformin can acutely reduce tumor hypoxia by inhibiting the mitochondrial respiratory chain, lowering cellular oxygen consumption, and thus reoxygenating hypoxic cells (9). In line with this, metformin has been shown to inhibit the hypoxia-driven

activation of transcription factor hypoxia-inducible factor-1, which is normally upregulated under hypoxic conditions and decreases the susceptibility of cancer cells to apoptosis by promoting progression and proliferation (11).

The validation of appropriate radiotracers as imaging biomarkers for patient selection, critically needed for further progress with clinical trials using metformin as a radiosensitizer, is still ongoing (12). The most widely spread hypoxia positron emission tomography (PET) tracers are the ^{18}F -labeled 2-nitroimidazoles. Only under hypoxic conditions those molecules undergo a series of intracellular reductions that result in intermediate metabolites with the ability to bind intracellular macromolecules. Despite the fact that 2-nitroimidazole-based tracers may detect mainly chronic rather than acute hypoxia, it has been shown in different clinical trials that tumors with a higher tracer uptake generally show a poorer radiotherapy response (13). Currently, ^{18}F -flortanidazole (^{18}F -HX4) is an established tool in this setting with correlations between tumoral uptake and reference standard immunohistochemistry markers of hypoxia (14-16), and with a more favorable kinetic profile compared to earlier 2-nitroimidazole-based hypoxia PET tracers (15). Despite its successful translation into human use in various cancer types, including NSCLC, questions remain on how ^{18}F -HX4 can be incorporated in treatment decision making of hypoxia-modulating therapies (17). Therefore, we investigated the effect of acute metformin administration on NSCLC xenograft tumor hypoxia using ^{18}F -HX4, studied the effect of a single dose of metformin prior to radiotherapy on outcome, and examined the potential of baseline ^{18}F -HX4 PET as biomarker of overall and metformin-specific treatment response.

MATERIALS AND METHODS

Animal Model

The experimental protocol was approved by the Antwerp University Ethical Committee for Animal Experiments (2015-42), and all applicable institutional and European guidelines for the care and use of animals were followed. Female CD-1 athymic nude mice (n=36; Charles Rivers Laboratories) were group-housed (up to six animals per cage) in individually ventilated cages under a 12:12 dark:light cycle, controlled temperature (20-23 °C) and humidity (50-60%) with ad libitum access to standard laboratory chow and water.

A549 NSCLC cells (ATCC) were cultured as monolayers in Dulbecco's Modified Eagle Medium enriched with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% penicillin-streptomycin (Invitrogen) at 37 °C and 5% CO₂ in a humidified incubator. Cultures were maintained in exponential growth. A549 cells were harvested by trypsinization with 0.05% trypsin-ethylenediaminetetraacetic acid, washed two times with sterile phosphate-buffered saline, counted using the Muse Cell Count and Viability Assay (Merck Millipore), and resuspended in sterile phosphate-buffered saline at a concentration of 5×10⁷ viable cells per mL. Mice (n=30) at an age of 7-9 weeks were inoculated with 100 µL of A549 cell suspension in both hind legs. Tumor growth was evaluated 3 times per week with digital caliper measurements from the moment the tumors became palpable. Tumor volume was calculated with the formula $V=0.5 \times (\text{length} \times \text{width}^2)$. Relative tumor volumes (RTV) were calculated with the formula $RTV = V_{\text{time } x} / V_{\text{baseline}}$. The tumor doubling time (TDT), i.e. the time to reach 2× the pre-irradiation tumor volume, was used as a proxy for progression-free survival and was defined as the endpoint. A minimum tumor volume of 100 mm³ was required at the start of the study. Two animals reached ethical endpoints not related to the TDT before the end of the study and were therefore sacrificed and also excluded from further analysis.

Tracer Production

^{18}F -HX4 was prepared in an automated synthesis module (Fluorsynthon I, Comecer Netherlands) by reaction of azeotropically dried ^{18}F -K(K₂₂₂)F with 17-20 mg HX4 precursor (Syncom) dissolved in a 50:50 mixture of t-butanol/acetonitrile at 110 °C for 6 minutes. After the fluorination, acetonitrile was removed under a stream of helium and vacuum and 0.1 M HCl (1 mL) was added for acidic hydrolysis at 90 °C for 5 minutes. After cooling the reaction to 75 °C, 0.7 mL of NaOAc 2 M, pH 5.5 was added and the mixture was loaded onto a high-pressure liquid chromatography loop through a preconditioned Alumina N Light cartridge (Waters). ^{18}F -HX4 was purified using a Luna C18(2) 250×10 mm, 10 μm high-pressure liquid chromatography column (Phenomenex) and 9% ethanol in saline as mobile phase at a flowrate of 3 mL/min. The fraction containing ^{18}F -HX4 was collected and transferred to a shielded laminar flow cabinet where it was diluted with saline containing 2% ascorbic acid and sterile filtered (syringe filter, 25 mm, 0.2 μm polyethersulfone membrane; VWR International).

^{18}F -HX4 was obtained with a radiochemical purity of >95% and a radiochemical yield of 47±5% (decay corrected to end-of-bombardement; n=7). The molar activity was 137.3±12.6 GBq/μmol (decay corrected to end-of-synthesis; n=7).

Experimental Set-Up

Acute Metformin Administration and its Radiosensitizing Effects. The study design is shown in [Fig. 1](#). A baseline ^{18}F -HX4 μPET/computed tomography (CT) scan was performed to determine baseline tumor hypoxia (=Day 0). Details of the scan protocol are represented in Fig. 1A. Approximately 18.5 MBq of ^{18}F -HX4 in a final volume of 200 μL saline was administered as a bolus intravenous (IV) injection via the tail vein. During ^{18}F -HX4 μPET/CT acquisition, which started 180 min after tracer administration (18), mice were anesthetized with isoflurane (induction

5%, maintenance 1-2%; Abbott) and medical O₂ (100%) and body temperature was kept constant via a heating bed. Respiration was continuously monitored during image acquisition. Static PET acquisition (20 min), followed by an anatomical CT acquisition (10 min) was performed on an Inveon μ PET/CT scanner (Siemens Preclinical Solutions). The next day, mice were administered 100 mg/kg metformin hydrochloride (ABC Chemicals) in a final volume of 100 μ L saline IV. Thirty min later, mice were injected with approximately 18.5 MBq of ¹⁸F-HX4 IV, whereupon a second ¹⁸F-HX4 μ PET/CT scan was performed to assess changes in tumor hypoxia.

PET images were reconstructed using 4 iterations \times 16 subsets of a three-dimensional - ordered subset expectation maximization (OSEM3D) algorithm following Fourier rebinning. Dead time, normalization, scatter and attenuation correction were applied. The PET/CT images were analyzed in PMOD v3.3 software (PMOD Technologies). An elliptic volume-of-interest that enclosed the entire tumor was positioned manually and was centered on the tumor area that showed maximal uptake. Then, three-dimensional isocontours at 60% of the maximum pixel value within this elliptic volume-of-interest were generated automatically. Tumor-to-background ratios (TBR) were determined using heart as reference region, whereby the heart was manually delineated on the CT images of each individual mouse.

Two days later, mice were categorized into three groups: a control group (1; n=7), a radiotherapy group (2; n=6), and a metformin+radiotherapy group (3; n=8). Animals were administered saline (groups 1-2) or 100 mg/kg metformin hydrochloride (group 3) IV, whereupon a single dose of radiotherapy was administered 30 min after metformin treatment (groups 2-3). Control animals of group 1 received sham irradiation. In brief, during irradiation animals were anesthetized with isoflurane (5% induction, 1-2% maintenance) and positioned within the self-contained X-ray system X-RAD 320 (Precision X-Ray). The whole body of the animals was shielded using lead protection, except for the tumors. Irradiation was delivered at a rate of 100 cGy/minute with 320 kV X-rays. Tumors received a single dose of 10 Gy. Control animals that received sham irradiation were anesthetized and positioned in the X-ray system for 10 min but

were not irradiated. After irradiation, monitoring of the tumor growth was continued until tumors reached a volume of 1500 mm³ (the ethical endpoint of the study), whereupon animals were sacrificed and tumor tissue was resected, formalin-fixed and paraffin-embedded. Tissue sections of 3 µm thick were mounted on SuperFrost microscope slides (Menzel-Glaser) for hematoxylin and eosin staining and Ki67 immunohistochemistry. Immunostaining and scorings were performed as previously described (19).

¹⁸F-HX4 Biodistribution Analysis. In order to rule out the possibility that metformin altered ¹⁸F-HX4 uptake by disturbing its biodistribution, a ¹⁸F-HX4 ex vivo study was performed in six mice (Fig. 1B). In brief, nude mice were injected IV with either 100 mg/kg metformin (n=3) or saline (n=3), and 30 min later with approximately 18.5 MBq of ¹⁸F-HX4. 170 min later, mice were anesthetized with isoflurane and 10 min later, blood was collected by cardiac puncture, immediately followed by sacrifice of the animals by cervical dislocation. All main organs and tissues were rapidly removed, rinsed in phosphate-buffered saline, blotted dry, weighed and counted for radioactivity in an automated Wizard² 2480 gamma counter (PerkinElmer). Activity was expressed as a percentage of injected dose per gram of sample (%ID/g).

Statistics

Prism v6 software (GraphPad Software) was used for analyzing changes in ¹⁸F-HX4 uptake values with a Wilcoxon matched pairs signed rank test, and for analyzing differences in parameters between groups using log-rank tests or using a Kruskal-Wallis test with Mann-Whitney U post-hoc analyses. Using SAS System v9 software (SAS Institute Inc.), a general linear mixed model including time, treatment group and their interaction with step-down Bonferroni correction for post-hoc multiple comparison was performed to determine whether there was a significant effect of the treatment on the RTVs. Cox proportional hazards regression was used to assess the

effect of treatment and ^{18}F -HX4 uptake on TDT (Stata 14.2; StataCorp LLC). Metformin-specific effects were assessed by estimating the interaction term of treatment and ^{18}F -HX4 uptake. Model checks for goodness-of-fit and proportional hazards assumption were performed as appropriate. P-values <0.05 were considered statistically significant. All data are expressed as mean \pm SEM.

RESULTS

Acute Metformin Administration and its Radiosensitizing Effects

The mean tumor volume at baseline was 342 ± 33 mm³. Thirty min post-metformin treatment, ^{18}F -HX4 TBR could demonstrate a significant change in A549 tumor hypoxia with a mean intratumoral reduction in ^{18}F -HX4 TBR from 3.21 ± 0.83 to 2.87 ± 0.83 ($p=0.0001$), as depicted in Fig. 2A. Importantly, the background tracer uptake was not affected by metformin (0.06 ± 0.01 to 0.07 ± 0.01 ; $p=0.09$). Representative baseline and follow-up ^{18}F -HX4 images of five mice are shown in Fig. 2B.

Two days after their follow-up scan, animals were divided into three treatment groups with comparable baseline parameters that are summarized in Table 1. The tumor growth curves of the A549 xenografts are shown in Fig. 3A. Overall, RTV over time differed across treatment groups ($p<0.0001$), with the metformin+radiotherapy group having significantly lower RTV than controls from day 7 post-therapy onwards (0.68 ± 0.05 vs 1.43 ± 0.08 resp.; $p=0.006$), and metformin+radiotherapy-treated tumors from day 12 post-therapy onwards compared to radiotherapy-treated tumors (0.77 ± 0.06 vs 1.27 ± 0.08 , resp.; $p=0.03$). From this time, RTVs of radiotherapy-treated tumors were also significantly lower than control tumor RTVs (1.65 ± 0.13 ; $p=0.01$). These results were confirmed by log-rank tests as the median doubling-free survival of radiotherapy-treated animals compared to controls was significantly increased (34 vs 19 days, respectively; 95% CI 3.0-18.8; log-rank $p=0.0002$); whereas addition of metformin to the treatment

regimen further increased the median doubling-free survival to 52 days (95% CI 1.7-11.3; log-rank $p=0.005$) as compared to radiotherapy alone, as clearly shown in Fig. 3B. The tumor proliferation at sacrifice assessed with Ki67 immunohistochemistry was numerically higher in controls ($55\pm 2\%$) as compared to radiotherapy-treated tumors ($51\pm 2\%$) and metformin+radiotherapy-treated tumors ($47\pm 3\%$; $p=0.2$; Fig. 4A). Accordingly, tumor necrosis at sacrifice was numerically lower in control tumors ($34\pm 7\%$) as compared to radiotherapy-treated tumors ($42\pm 9\%$) and metformin+radiotherapy-treated tumors ($42\pm 7\%$; $p=0.6$; Fig. 4B). No correlations were found between the immunohistochemistry parameters and the volumetric outcome parameters (i.e. RTV and TDT; data not shown). Fig. 4C and 4D show representative immunohistochemistry images.

^{18}F -HX4 as a Biomarker of Tumor Doubling Time

Overall baseline ^{18}F -HX4 TBR was a prognostic biomarker for TDT, independent of treatment and adjusting for baseline tumor volume (HR 2.0 for every unit increase in TBR; 95% CI 1.2-3.2; $p=0.0004$). In addition, a reduction in TBR $\geq 5\%$ ($\Delta^{18}\text{F}$ -HX4) after metformin administration was also prognostic for TDT across treatment groups (HR 0.39; 95% CI 0.16-0.95; $p=0.04$). Out of these two, baseline ^{18}F -HX4 performed slightly better than $\Delta^{18}\text{F}$ -HX4 TBR in predicting TDT (Harrell's C 0.86; 95% CI 0.81-0.91 vs. C 0.81; 95% CI 0.76-0.85, respectively; $p=0.02$).

As clearly shown in Fig. 5, focusing specifically on the treatment effect of metformin, baseline ^{18}F -HX4 TBR could predict the synergistic effect of metformin+radiotherapy over radiotherapy alone, revealing that treatment modulation was dependent on baseline tumor hypoxia. This means that across the spectrum of observed baseline hypoxia values, in tumors with lower ^{18}F -HX4 TBR (≤ 2.5) adding metformin to radiotherapy resulted in a relative reduction in the risk of tumor doubling of 72% over radiotherapy alone (HR 0.28; 95% CI 0.09-0.92; $p=0.04$),

whereas this modulatory effect was attenuated in tumors at the higher end of baseline TBR (>2.5) values (HR 0.55; 95% CI 0.11-2.82; p=0.5).

¹⁸F-HX4 Biodistribution Analysis

In both the metformin-treated group and the control group, the quantitative ¹⁸F-HX4 biodistribution study 180 min post-injection showed a high accumulation of radioactivity in urine (0.23±0.14 vs 0.92±0.31 %ID, respectively) and the large intestine (2.36±0.26 vs 2.66±0.24 %ID/g, respectively), indicating a combined renal and intestinal clearance. Blood pool activity was very low (0.05±0.00 vs 0.07±0.02 %ID/g, respectively). Fig. 6 shows that in general, no significant differences in the ¹⁸F-HX4 biodistribution profile could be observed between the metformin-treated mice and the control group.

DISCUSSION

In an A549 NSCLC xenograft model, we first looked at changes in intratumoral hypoxia after acute metformin administration and found a mean reduction in ¹⁸F-HX4 uptake of more than 10%, implying that a single dose of metformin can immediately improve tumor oxygenation. Our result is in line with previous observations in a colorectal cancer xenograft model, in which the uptake of the hypoxia tracer ¹⁸F-fluoroazomycin arabinoside was compared between tumors treated with metformin or saline IV (9).

Second, we observed that administration of metformin 30 min prior to radiotherapy significantly improved long-term treatment outcome. Others made similar observations in the colorectal cancer xenograft model (9). Accordingly, in an A549 xenograft model it had already been observed that long-term daily administration of 300 mg/kg metformin via the animal's drinking

water sensitized tumors to the effects of a single radiotherapy dose of 10 Gy (8), but to date the effects of a single dose of metformin on radiotherapy outcome were not explored in A549 tumors.

It has been hypothesized that metformin can accumulate up to 500-fold in the mitochondria, resulting in mitochondrial concentrations of the drug in the mM-range (20,21) which should be adequate for inhibiting the respiratory chain complex I (11,20,22) and consequently improving tumor oxygenation.

Despite our own definite observations that are in line with this theory, the direct inhibition of tumor cell respiration by biguanides has lately been questioned. A recent study showed that tumor retention of an intraperitoneally administered mixture of unlabeled metformin and trace amounts of ^{11}C -labeled metformin (^{11}C -metformin; total dose equaling 250 mg/kg) was low and did not affect tumor hypoxia. Moreover, the same study showed that an IV-administered bolus of ^{11}C -metformin cleared rapidly from the circulation (23). These authors extrapolated their observations to a metformin dose of 100 mg/kg (which we used in our set-up) and concluded that the resulting plasma levels of 50 μM 30 min post-administration are insufficient to evoke direct respiratory responses (23). However, they compared in vitro and in vivo observations which we believe can be misleading, as ample evidence shows the existence of different dynamics of response to metformin in cell cultures and in vivo (21).

We demonstrated that a higher tumoral baseline ^{18}F -HX4 uptake significantly correlated with a poorer survival independently from the therapy that was administered, supporting ^{18}F -HX4 as prognostic biomarker in the A549 xenograft model. This observation confirms previous clinical findings made with other 2-nitroimidazole PET imaging in different types of cancer, including NSCLC (1,13,24). However, to the best of our knowledge the potential of the pharmacokinetically superior hypoxia PET tracer ^{18}F -HX4 as a prognostic biomarker has not been assessed to date (25,26).

Interestingly, we also demonstrated ^{18}F -HX4 PET to be a predictive biomarker for metformin-specific therapeutic effects. In other words, the therapeutic benefit of

metformin+radiotherapy over radiotherapy alone in our set-up was found to dependent on the baseline degree of tumor hypoxia and most pronounced in tumors with lower values of baseline ^{18}F -HX4 TBR (≤ 2.5). There are some potential explanations for this apparent differential therapeutic effect of metformin. First, the administered dose and/or the applied therapy regimen may have been inadequate, particularly for tumors with a higher degree of baseline hypoxia in which the increase in tumor oxygenation resulting from metformin may still be too limited for effective radiosensitizing effects. Second, it is well established that mitochondrial inhibition by metformin not only results in more oxygen in cancer cells, it also activates the adenosine monophosphate-activated kinase pathway, which eventually results in inhibition of mammalian target of rapamycin, a central cellular regulator of cell growth and survival. In this way, metformin may suppress tumor proliferation independently from hypoxia (5,7). However, under highly hypoxic conditions, metformin may be unable to activate adenosine monophosphate-activated kinase or to inhibit mammalian target of rapamycin (27). Taken together, ^{18}F -HX4 shows great promise as a tool to investigate and predict metformin-specific therapeutic effects and tailor patient treatment selection.

However, it should be noted that on top of the complex drug dynamics and kinetics which are not fully understood yet, our study design may have been limited by the high baseline degree of tumor hypoxia. The baseline ^{18}F -HX4 TBR was ≥ 1.4 for all tumors and TBR thresholds as low as 1.2 have been reported to represent hypoxia, limiting our ability to fully assess treatment-specific effects across the entire range of tumor oxygenation (28-30). Indeed, Graves et al. have shown that ^{18}F -fluoroazomycin arabinoside uptake could only be detected in subcutaneous, but not in orthotopic A549 NSCLC xenograft models (31). The accessibility of oxygen via the alveoli in orthotopic, but not subcutaneous lung tumors, may explain this phenomenon (32).

CONCLUSIONS

Using ^{18}F -HX4 PET in an NSCLC xenograft model, we demonstrated that tumor hypoxia significantly decreased immediately after IV administration of a single dose of metformin. Administering metformin prior to irradiation significantly increased TDT. Additionally, we showed that baseline ^{18}F -HX4 PET shows great promise as an imaging biomarker; both prognostic for survival and predictive for metformin-specific therapeutic effects.

DISCLOSURE

We have no conflict of interest to declare.

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REFERENCES

1. Grootjans W, de Geus-Oei L-F, Troost EGC, Visser EP, Oyen WJG, Bussink J. PET in the management of locally advanced and metastatic NSCLC. *Nat Rev Clin Oncol*. 2015;12:395-407.
2. Bayer C, Shi K, Astner ST, Maftai C-A, Vaupel P. Acute versus chronic hypoxia: why a simplified classification is simply not enough. *Radiat Oncol Biol*. 2011;80:965-968.
3. Siemann DW, Horsman MR. Modulation of the tumor vasculature and oxygenation to improve therapy. *Pharmacol Ther*. 2015;153:107-124.
4. Overgaard J. Hypoxic radiosensitization: adored and ignored. *J Clin Oncol*. 2007;25:4066-4074.
5. Koritzinsky M. Metformin: a novel biological modifier of tumor response to radiation therapy. *Radiat Oncol Biol*. 2015;93:454-464.
6. Samsuri NAB, Leech M, Marignol L. Metformin and improved treatment outcomes in radiation therapy – a review. *Cancer Treat Rev*. 2017;55:150-162.
7. Lin A, Maity A. Molecular pathways: A novel approach to targeting hypoxia and improving radiotherapy efficacy via reduction in oxygen demand. *Clin Cancer Res*. 2015;21:1995-2000.
8. Storozhuk Y, Hopmans SN, Sanli T, et al. Metformin inhibits growth and enhances radiation response of non-small cell lung cancer (NSCLC) through ATM and AMPK. *Br J Cancer*. 2013;108:2021-2032.

9. Zannella VE, Dal Pra A, Muaddi H, et al. Reprogramming metabolism with metformin improves tumor oxygenation and radiotherapy response. *Clin Cancer Res*. 2013;19:6741-6750.
10. Makowski L, Hayes DN. Role of LKB1 in lung cancer development. *Br J Cancer*. 2008;99:683-688.
11. Wheaton WW, Weinberg SE, Hamanaka RB, et al. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *eLife*. 2014;3:e02242.
12. Troncone M, Cargnelli SM, Villani LA, et al. Targeting metabolism and AMP-activated kinase with metformin to sensitize non-small cell lung cancer (NSCLC) to cytotoxic therapy: translational biology and rationale for current clinical trials. *Oncotarget*. 2017;8:57733-57754.
13. Horsman MR, Mortensen LS, Petersen JB, Busk M, Overgaard J. Imaging hypoxia to improve radiotherapy outcome. *Nat Rev Clin Oncol*. 2012;9:674-687.
14. Dubois LJ, Lieuwes NG, Janssen MHM, et al. Preclinical evaluation and validation of [¹⁸F]HX4, a promising hypoxia marker for PET imaging. *Proc Natl Acad Sci U S A*. 2011;108:14620-14625.
15. Carlin S, Zhang H, Reese M, Ramos NN, Chen Q, Ricketts SA. A comparison of the imaging characteristics and microregional distribution of 4 hypoxia PET tracers. *J Nucl Med*. 2014;55:515-521.
16. Wack LJ, Mönnich D, van Elmpt W, et al. Comparison of [¹⁸F]-FMISO, [¹⁸F]-FAZA and [¹⁸F]-HX4 for PET imaging of hypoxia – a simulation study. *Acta Oncol*. 2015;54:1370-1377.

17. Challapalli A, Carroll L, Aboagye EO. Molecular mechanisms of hypoxia in cancer. *Clin Transl Imaging*. 2017;5:225-253.
18. Peeters SGJA, Zegers CML, Lieuwes NG, et al. A comparative study of the hypoxia PET tracers [¹⁸F]HX4, [¹⁸F]FAZA, and [¹⁸F]FMISO in a preclinical tumor model. *Radiat Oncol Biol*. 2015;91:351-359.
19. De Bruycker S, Vangestel C, Van den Wyngaert T, et al. Baseline [¹⁸F]FMISO μ PET as a predictive biomarker for response to HIF-1 α inhibition combined with 5-FU chemotherapy in a human colorectal cancer xenograft model. *Mol Imaging Biol*. 2016;18:606-616.
20. Chandel NS, Avizonis D, Reczek CR, et al. Are metformin doses used in murine cancer models clinically relevant? *Cell Metabolism*. 2016;23:569-570.
21. Dowling RJO, Lam S, Bassi C, et al. Metformin pharmacokinetics in mouse tumors: Implications for human therapy. *Cell Metabolism*. 2016;23:567-568.
22. He L, Wondisford FE. Metformin action: concentrations matter. *Cell Metabolism*. 2015;21:159-162.
23. Iversen AB, Horsman MR, Jakobsen S, et al. Results from ¹¹C-metformin-PET scans, tissue analysis and cellular drug-sensitivity assays questions the view that biguanides affects tumor respiration directly. *Sci Rep*. 2017;7:9436.
24. Bollineni VR, Wiegman EM, Pruijm J, Groen HJM, Langendijk JA. Hypoxia imaging using positron emission tomography in non-small cell lung cancer: implications for radiotherapy. *Cancer Treat Rev*. 2012;38:1027-1032.
25. Yip C, Blower PJ, Goh V, Landau DB, Cook GJR. Molecular imaging of hypoxia in non-small-cell lung cancer. *Eur J Nucl Med Mol Imaging*. 2015;42:956-976.

26. Szyszko TA, Yip C, Szlosarek P, Goh V, Cook GJR. The role of new PET tracers for lung cancer. *Lung Cancer*. 2016;94:7-14.
27. Garofalo C, Capristo M, Manara MC, et al. Metformin as an adjuvant drug against pediatric sarcomas: hypoxia limits therapeutic effects of the drug. *PLoS ONE*. 2013;8:e83832.
28. Busk M, Horsman MR, Overgaard J. Resolution in PET hypoxia imaging: voxel size matters. *Acta Oncol*. 2008;47:1201-1210.
29. Michalski MH, Chen X. Molecular imaging in cancer treatment. *Eur J Nucl Med Mol Imaging*. 2010;38:358-377.
30. Even AJG, Reymen B, La Fontaine MD, et al. Predicting tumor hypoxia in non-small cell lung cancer by combining CT, FDG PET and dynamic contrast-enhanced CT. *Acta Oncol*. 2018;56:1595-1600.
31. Graves EE, Vilalta M, Cecic IK, et al. Hypoxia in models of lung cancer: implications for targeted therapeutics. *Clin Cancer Res*. 2010;16:4843-4852.
32. Maity A, Koumenis C. Location, location, location - makes all the difference for hypoxia in lung tumors. *Clin Cancer Res*. 2010;16:4685-4687.

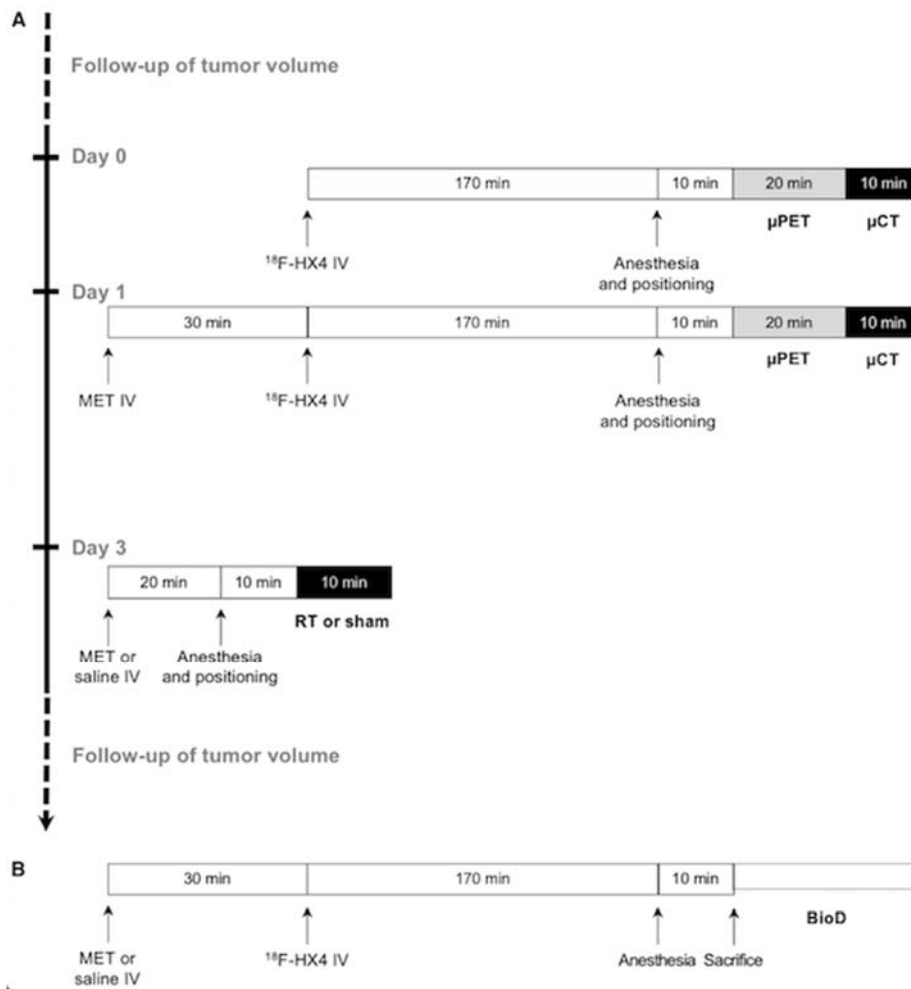


FIGURE 1. Experimental set-up. (A) Acute metformin administration in A549 xenografts. (B) Biodistribution study. Hypoxia was quantified using $^{18}\text{F}\text{-HX4}$. $^{18}\text{F}\text{-HX4}$ = ^{18}F -flortanidazole; BioD=biodistribution study; CT=computed tomography; IV=intravenous; MET=metformin; PET=positron emission tomography; RT=radiotherapy.

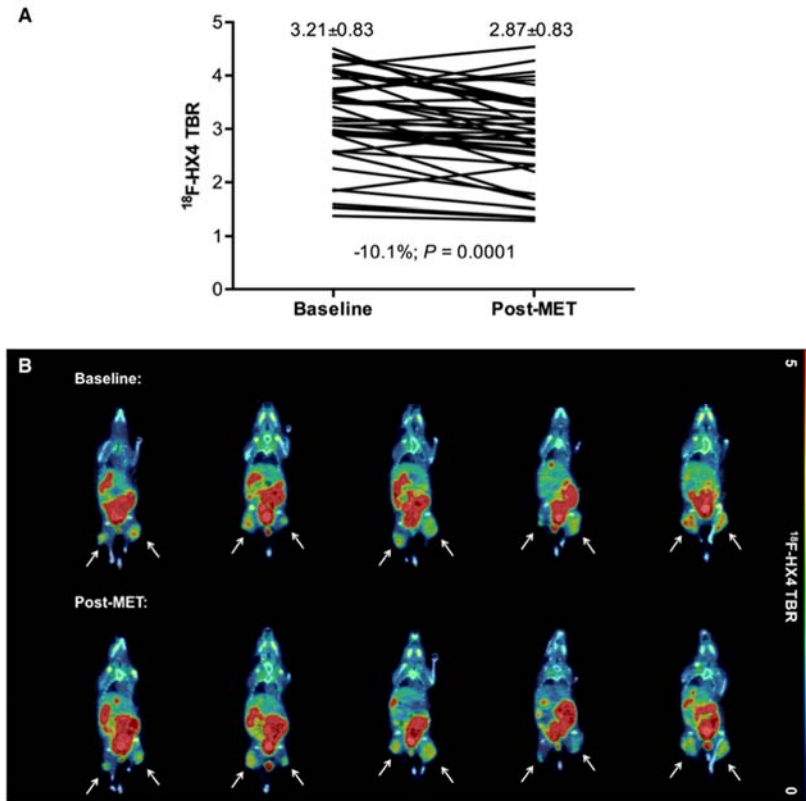


FIGURE 2. Metformin improves tumor oxygenation. (A) A significant decrease in $^{18}\text{F-HX4 TBR}$ could be observed 30 min after IV administration of 100 mg/kg metformin ($p=0.0001$). (B) Representative $^{18}\text{F-HX4 PET/CT TBR}$ -corrected images (coronal view) of five mice before (upper row) and after (lower row) IV administration of 100 mg/kg metformin. Arrows indicate tumors. $^{18}\text{F-HX4} = ^{18}\text{F-flortanidazole}$; MET=metformin; TBR=tumor-to-background ratio.

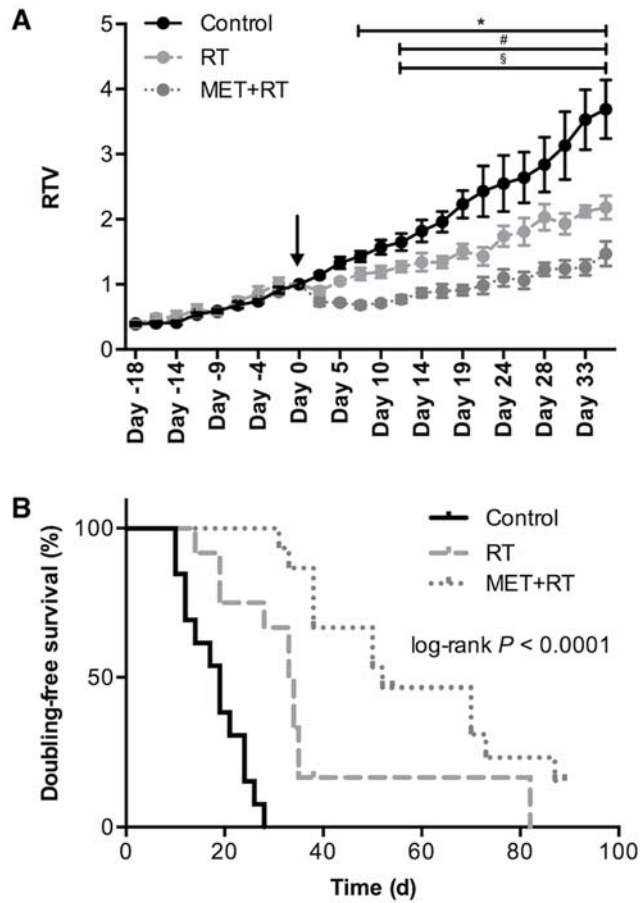


FIGURE 3. Metformin improves radiotherapy response in A549 tumors. (A) Tumor growth was followed and RTVs were calculated. The arrow indicates the moment of therapy administration. Significant differences between RTVs at the different points in time are indicated by * (metformin+radiotherapy vs control), # (metformin+radiotherapy vs radiotherapy) and § (radiotherapy vs control), respectively. (B) Kaplan-Meier representation of the doubling-free survival time (overall log-rank $p < 0.0001$). MET=metformin; RT=radiotherapy; TBR=tumor-to-background ratio.

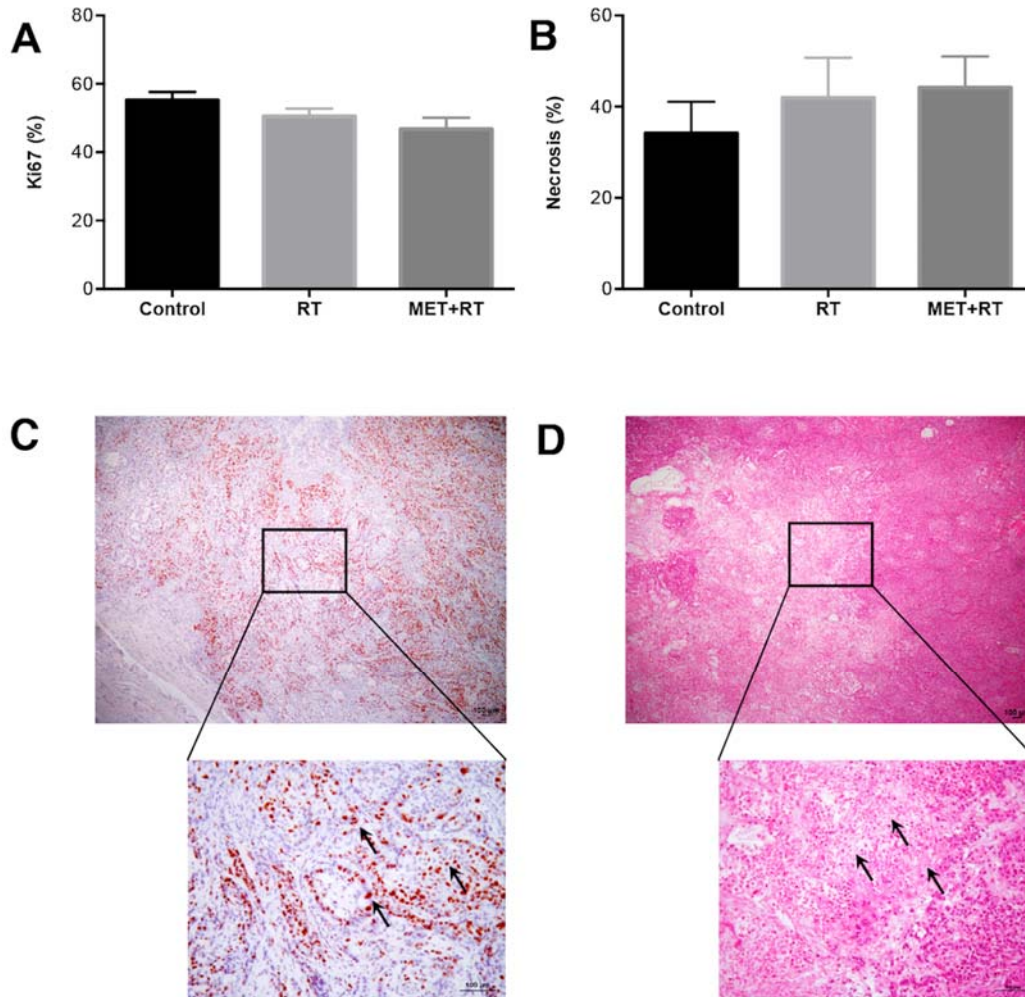


FIGURE 4. Tumor proliferation as assessed with Ki67 immunohistochemistry and tumor necrosis at sacrifice. (A) No major differences in tumor proliferation could be observed between the different treatments groups. (B) Same conclusions could be drawn from necrosis scoring. (C) Representative example of a Ki67 staining. Arrows indicate positively stained nuclei. (D) Representative example of a hematoxylin and eosin staining. Arrows indicate areas of necrosis. MET=metformin; RT=radiotherapy.

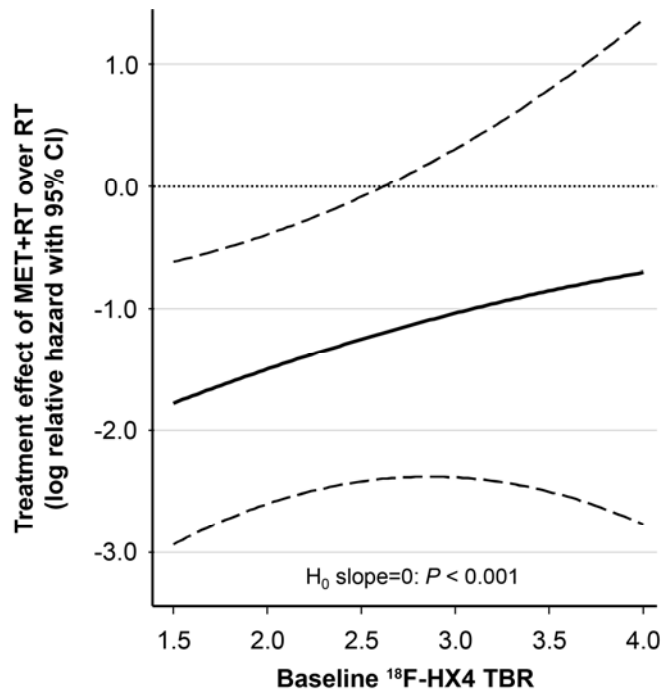


FIGURE 5. The therapeutic benefit of metformin was dependent on the baseline degree of tumor hypoxia and could be predicted with baseline ¹⁸F-HX4 PET. ¹⁸F-HX4=¹⁸F-flortanidazole; MET=metformin; RT=radiotherapy; TBR=tumor-to-background ratio.

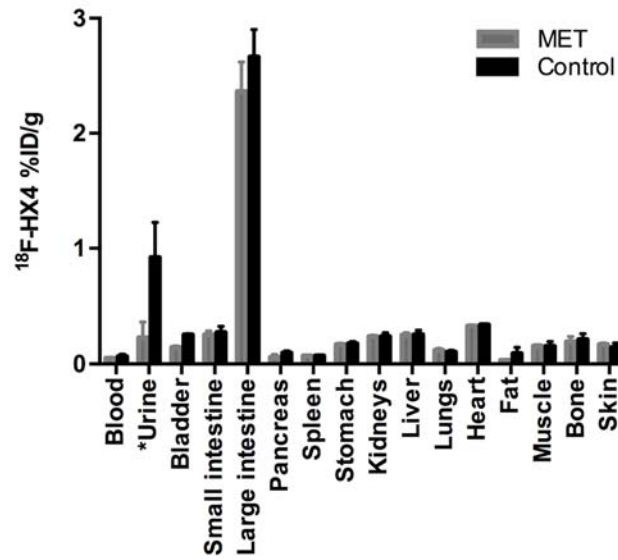


FIGURE 6. Results of the ^{18}F -HX4 biodistribution study after acute metformin administration. No major differences could be observed in the ^{18}F -HX4 biodistribution profile between the metformin-treated mice and the control group. ^{18}F -HX4= ^{18}F -flortanidazole; MET=metformin; %ID/g=percentage injected dose per gram of sample. *Values shown in %ID due to variability in urine production.

TABLE 1. Overview of the baseline parameters of the different treatment groups of the A549 xenografts.

	Radiotherapy	Metformin + radiotherapy	Control	P-value
Tumor volume (mm³)	395±57	297±51	329±65	0.5
Animal weight (g)	29.6±0.8	29.7±0.9	29.4±1.1	1.0
Baseline ¹⁸F-HX4 TBR	3.60±0.17	2.74±0.26	3.47±0.20	0.3
Δ¹⁸F-HX4 TBR (%)	-13±4	-4±4	-14±3	0.2

Data are expressed as mean±SEM. ¹⁸F-HX4=¹⁸F-flortanidazole; TBR=tumor-to-background ratio; Δ¹⁸F-HX4

TBR=change in ¹⁸F-HX4 TBR post-metformin.



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