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Original Research Article

# Nitroglycerin as a radiosensitizer in non-small cell lung cancer: Results of a prospective imaging-based phase II trial

Bart J.T. Reymen [a,1], Marike W. van Gisbergen [g,1], Aniek J.G. Even [a], Catharina M.L. Zegers [a,f], Marco Das [c], Erik Vegt [d], Joachim E. Wildberger [c], Felix M. Mottaghy [c,e], Ala Yaromina [g], Ludwig J. Dubois [g], Wouter van Elmpt [a], Dirk De Ruysscher [a], Philippe Lambin [b,c,*]

[a] Department of Radiation Oncology (MAASTRO), GROW - School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands
[b] The D-Lab & The M-Lab, Department of Precision Medicine, GROW – School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands
[c] Department of Radiology and Nuclear Medicine, Maastricht University Medical Center, Maastricht, The Netherlands
[d] Department of Radiology and Nuclear Medicine, Erasmus University Medical Center, Rotterdam, The Netherlands
[e] Department of Nuclear Medicine, University Hospital, RWTH Aachen University, Aachen, Germany
[f] Institute of Data Science, Maastricht University, The Netherlands
[g] The M-Lab, Department of Precision Medicine, GROW – School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands

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ABSTRACT

*Background:* Nitroglycerin is proposed as an agent to reduce tumour hypoxia by improving tumour perfusion. We investigated the potential of nitroglycerin as a radio-sensitizer in non-small cell lung cancer (NSCLC) and the potential of functional imaging for patient selection.

*Material and methods:* Trial NCT01210378 is a single arm phase II trial, designed to detect 15% improvement in 2-year overall survival (primary endpoint) in stage IB-IV NSCLC patients treated with radical (chemo-) radiotherapy and a Transiderm-Nitro 5 patch during radiotherapy. Patients underwent dynamic contrast-enhanced CTs (DCE-CT) and HX4 (hypoxia) PET/CTs before and after nitroglycerin. Secondary endpoints were progression-free survival, toxicity and the prognostic value of tumour perfusion/hypoxia at baseline and after nitroglycerin.

*Results:* The trial stopped after a futility analysis after 42 patients. At median follow-up of 41 months, two-year and median OS were 58% (95% CI: 44–78%) and 38 months (95% CI: 22–54 months), respectively. Nitroglycerin could not reduce tumour hypoxia. DCE-CT parameters did not correlate with OS, whereas hypoxic tumours had a worse OS ($p = 0.029$). Changes in high-uptake fraction of HX4 and tumour blood flow were negatively correlated ($r = -0.650$, $p = 0.022$). The heterogeneity in treatment modalities and patient characteristics combined with a small sample size made further subgroup analysis of survival results impossible. Toxicity related to nitroglyerin was limited to headache (17%) and hypotension (2.4%).

*Conclusion:* Nitroglycerin did not improve OS of NSCLC patients treated with (chemo-)radiotherapy. A general ability of nitroglycerin to reduce hypoxia was not shown.



*Abbreviations:* BF, blood flow; BV, blood volume; CI, confidence interval; CoR, coefficient of repeatability; DCE-CT, dynamic contrast-enhanced CT; FHV, fraction of hypoxic volume hypoxic fraction of the GTV; GTV, gross tumour volume; GTVln, gross tumour volume of the lymph nodes; GTVp, gross tumour volume of the primary tumour; HX4, 2-nitroimidazole [$^{18}$F]-HX4 (flortanidazole, 3-[18F]fluoro-2-(4-((2-nitro-1Himidazol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)-propan-1-ol); HX4-HF, HX4 hypoxic fraction; HX4-HV, HX4 hypoxic volume; INDAR, individualized accelerated radiotherapy; IQR, interquartile range; LRPFS, loco-regional progression free survival; MFS, metastasis-free survival; NO, nitric oxide; NSCLC, non-small cell lung cancer; OS, overall survival; PET, positron emission tomography; $SUV_{max}$, maximum standardised uptake value; $SUV_{mean}$, mean standardised uptake value; TTD, total tumour dose; TBR, tumour-to-blood ratio.

* Corresponding author at: The D-Lab & The M-Lab, Department of Precision Medicine, Maastricht University, Universiteitssingel 40/29, room 4.549, 6229 ER, Maastricht, The Netherlands.
*E-mail address:* philippe.lambin@maastrichtuniversity.nl (P. Lambin).
[1] Authors contributed equally.

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## 1. Introduction

Nitroglycerin is a commonly used vasodilator used in angina pectoris or heart failure, which has been proposed as a potentially valuable adjuvant drug in non-small cell lung cancer (NSCLC) treatment. Yasuda et al. showed a significant survival benefit of adding nitroglycerin to chemotherapy in stage IV non-small cell lung cancer in a randomized phase II study [1]. The authors hypothesized this was due to increased tumour perfusion, also based on decreased VEGF-levels found in surgically treated NSCLC tumours in patients pre-treated with nitroglycerin. This result stimulated others to initiate phase II trials investigating the addition of a nitric oxide donor to different treatment regimens for NSCLC, all based on the rationale of improved tumour perfusion resulting in decreased tumour hypoxia [2–5].

We initiated a trial to test the effect of nitroglycerin on the OS of NSCLC patients treated with radiotherapy. Because the proposed beneficial effects of nitroglycerin on tumour perfusion and hypoxia were never formally established in humans, patients were asked to undergo a hypoxia PET scan and dynamic contrast enhanced CT (DCE-CT) scan, both before and after treatment with nitroglycerin [6]. We analysed the prognostic value of hypoxia HX4-PET and DCE-CT imaging at baseline. Additionally, we assessed nitroglycerin effects on hypoxia and tumour perfusion by comparing baseline and nitroglycerin scans. The changes in imaging parameters between the two time points were explored as a potential predictive marker [7].

## 2. Materials and methods

### 2.1. Clinical experiments

#### 2.1.1. Study design

Patients with NSCLC stage Ib-IV referred to Maastro Clinic for radical radiotherapy were eligible for inclusion in the prospective trial NCT01210378 (see supplementary Table S1 for inclusion criteria). The regional staging protocol includes $^{18}$FDG-PET-CT for all patients and a brain MRI for patients with stage III-IV NSCLC. In this trial, a nitroglycerin patch (Transiderm Nitro 5 mg, Novartis) was applied on each day of irradiation, starting on day 1. These patches contained 25 mg/10 cm$^2$ and released nitroglycerin at 0.2 mg/h, identical to the patches used in the positive Yasuda trial. Patients applied a patch at least 2 h prior to the first radiation session of the day and removed the patch only after the last session of the day in case of bi-daily treatments. Patients were asked to undergo facultative scans to measure effects of nitroglycerin on hypoxia and perfusion of the primary tumour. Hypoxia was evaluated by the 2-nitroimidazole flortanidazole (3-[$^{18}$F]fluoro-2-(4-((2-nitro-1Himidazol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)-propan-1-ol), referred to as HX4-PET scans. Tumour perfusion was investigated by dynamic contrast enhanced CT scanning (DCE-CT). DCE-CT and HX4-PET scans were made at two time-points before start of radiotherapy: once to measure baseline tumour perfusion and hypoxia and a second time at least 48 h later, at a minimum of 1 h after application of a nitroglycerin patch (Fig. S1) [6]. The minimum inter-scan interval of 48 h was chosen to allow sufficient time for kidney recuperation and wash-out of iodine contrast and HX4 (HX4 biological T1/2 = 4.3 h, T1/2 $^{18}$F = 110 min) to allow accurate retesting [29].

#### 2.1.2. Follow-up and analysis

All patients were followed up according to standard follow-up protocol including a CT-scan at 3 months post-treatment, repeated yearly and whenever clinically indicated. Overall survival (OS), loco-regional progression-free survival (LRPFS) and metastasis-free survival (MFS) were determined for all patients. OS was defined as time between pathological diagnosis and death. LRPFS and MFS were defined from the time of pathology until first progression on imaging, which was respectively a recurrence in primary tumour or regional lymph nodes, or distant metastases.

Kaplan Meier curves were constructed to analyse OS, LRPFS and MFS for the HX4-PET and DCE-CT scans at baseline and after nitroglycerin application. Survival of patients with hypoxic tumours and non-hypoxic tumours was analysed separately. For the DCE-CT scans, the median BF and BV were used for patient stratification. In addition, baseline median GTV and median FDG $SUV_{max}$ and $SUV_{mean}$ were tested for prognostic value.

The response to nitroglycerin was assessed by comparing baseline and nitroglycerin HX4-PET and DCE-CT scans. Changes in hypoxia and perfusion were marked as significant if they exceeded the previously determined coefficient of repeatability (CoR). The absolute CoR for HX4 was 0.30 for the TBR (tumour-to-blood ratio) and 14.9% for the fraction of hypoxic volume: hypoxic fraction of the GTV (FHV) with a threshold of TBR > 1.2. For perfusion the CoR of Larici et al were adopted (BF 16.4%, BV 9.3%) [8,9].

### 2.2. Statistics

Primary endpoint was a 15% improvement in 2-year OS assuming a 50% 2-year overall survival OS based on historical controls treated at Maastro Clinic in 2010, with a one-sided alpha-value of 0.10 and a power of 0.80. Reference survival assumed a distribution of 30% stage I, 10% stage II, 60% stage III and <5% stage IV patients included in the trial population. This required inclusion of 53 evaluable patients of whom 32 should be alive at 2 years after diagnosis. Survival data were analysed using R (v3.3.2, Vienna, Austria; survival package v2.38). For the imaging parameters, the median and interquartile range (IQR) of the group of patients are provided. Correlation coefficients were calculated using Spearman's correlation coefficient. Survival statistics are presented as the median with the 95% confidence interval (CI). Survival differences between groups were tested using a log-rank test. For all analyses, the significance level was set at a two-tailed p-value $\leq$0.05.

## 3. Results

We enrolled 47 of the initially planned 53 patients between December 2011 and June 2016, 42 of which were evaluable: 3 withdrew consent, 2 were excluded because of wrongful inclusion (re-irradiation of a recurrence after prior radical radiotherapy). In July 2016 we performed an interim analysis after the NVALT-12 publication indicating a potentially negative effect of nitroglycerin on survival [2]. At that time, median follow-up was 30 months and 18/42 patients had died. To reach 65% 2-year survival, all patients still alive had to reach 2 years survival and at least 9 of 11 patients still to be included would have to survive for 2 years. Institutional Review Board and the Medical Ethical Trial Committee decided that a benefit was highly unlikely and the trial was halted in July 2016.

### 3.1. Patient characteristics

Patient and treatment details are given in Table 1 and are described in supplementary material and methods. Median age was 60 years (range 36–82), 98% of patients had a WHO-PS $\leq$1. Adenocarcinoma was the predominant histology (40%), while 26% of patients had squamous cell carcinoma (see Table 1). Most patients (64%) had stage III disease and 26% stage IV. Eight of 11 patients with stage IV disease were staged M1a (cervical nodes

**Table 1**
Patient and treatment characteristics.

| | | |
|---|---|---|
| Gender | Male | 24 (57%) |
| | Female | 18 (43%) |
| Age (mean, range; years) | | 60 (36–82) |
| GTV (median, range; $cm^3$) | Tumor | 22 (0–477) |
| | Nodes | 15 (0–251) |
| | Total | 64 (6–497) |
| WHO-PS | 0 | 9 (21%) |
| | 1 | 32 (76%) |
| | 2 | 1 (2%) |
| Charlson Comorbidity Index | 0 | 22 (52%) |
| | 1 | 16 (38%) |
| | 2 | 3 (8%) |
| | 3 | 1 (2%) |
| Smoking | Active | 8 (19%) |
| | Never | 1 (2%) |
| | Quit | 31 (74%) |
| | Unknown | 2 (5%) |
| Treatment (thoracic) | Radiotherapy | 4 (10%) |
| | Stereotactic radiotherapy | 2 (5%) |
| | Sequential chemoradiation | 4 (10%) |
| | Concurrent chemoradiation | 32 (76%) |
| Radiotherapy schedules | 60 Gy/2 Gy/QD | 1 (2%) |
| | 60 Gy/7.5 Gy/3 fractions per week | 2 (5%) |
| | INDAR:1.8 Gy/BID | 8 (19%) |
| | INDAR: 1.5 Gy/BID + 2 Gy/QD | 31 (74%) |
| TNM (T) | 1 | 1 (2%) |
| | 2 | 12 (29%) |
| | 3 | 10 (24%) |
| | 4 | 16 (57%) |
| | X | 3 (7%) |
| TNM (N) | 0 | 6 (14%) |
| | 1 | 1 (2%) |
| | 2 | 18 (43%) |
| | 3 | 17 (41%) |
| TNM (M) | 0 | 31 (74%) |
| | 1 | 11 (26%) |
| Site of metastases | M1a | |
| | Cervical nodes | 4 (10%) |
| | Contralateral lung | 4 (10%) |
| | M1b | |
| | Adrenal | 2 (5%) |
| | Brain | 1 (2%) |
| Stage | I | 2 (5%) |
| | II | 2 (5%) |
| | III | 27 (64%) |
| | IV | 11 (26%) |
| Pathology | Adenocarcinoma | 17 (40%) |
| | Squamous cell carcinoma | 11 (26%) |
| | Large-cell carcinoma | 10 (24%) |
| | NSCLC NOS | 4 (10%) |

or contralateral lung metastases). One patient died prior to radiotherapy due to neutropenic sepsis, but was included in the intent-to-treat survival analyses. All other patients completed radiotherapy. Four patients (10%) stopped nitroglycerin prior to conclusion of radiotherapy, mostly after side-effects during concurrent chemoradiation (nausea in 2, pancytopenia in 1) and pulmonary embolism in 1 patient.

### 3.2. Survival results

At a median follow-up of 41 months (range: 11–65 months), 21/42 patients had died. Two-year OS was 58% (95% CI: 44–78%), median OS was 38 months (95% CI: 22–54 months) and PFS was 25 months (95% CI: 8–42 months). For stage III patients, the 2-year OS was 62% and the median OS 36 months (95% CI: 18.6–53.3 months). Twenty-four patients (57%) developed progressive disease; mainly in the form of distant metastases. The disease progressed inside the PTV in 8 patients (19%). Main causes of death (Table 2) were disease progression (14 patients, 33%) and pulmonary infection (4 patients, 10%).

**Table 2**
Survival and progression data.

| | | |
|---|---|---|
| Nr of patients | | 42 |
| Death | Yes | 21 (50%) |
| | No | 21 (50%) |
| Progressive disease | Yes | 24 (57%) |
| | No | 18 (43%) |
| Site of first progression | Loco-regional | 7 (16%) |
| | Distant | 13 (31%) |
| | Loco-regional + distant | 4 (10%) |
| Progression in PTV | Yes | 8 (19%) |
| | - Tumour | 2 (5%) |
| | - Nodes | 3 (7%) |
| | - Tumour + nodes | 3 (7%) |
| | No | 34 (81%) |
| Treatment at progression | Radical intent | 9 (21%) |
| | - Surgery + chemo | 1 (2%) |
| | - Radiotherapy | 7 (16%) |
| | - Chemoradiation | 1 (2%) |
| | Palliative intent | 9 (21%) |
| | - Nivolumab | 4 (10%) |
| | - Gefitinib | 1 (2%) |
| | - Gemcitabin-Cisplatin | 1 (2%) |
| | - Pemetrexed | 2 (5%) |
| | - Radiotherapy | 1 (2%) |
| | Best supportive care | 6 (14%) |
| Cause of death | Progressive disease | 14 (33%) |
| | Infection | 5 (12%) |
| | Pneumonitis | 1 (2%) |
| | Terminal dementia | 1 (2%) |

### 3.3. Toxicity

All recorded adverse events are presented in Table S2. Hematologic toxicity was most frequent: 34% of patients developed grade $\geq$3 neutropenia, while grade $\geq$3 thrombopenia were noted in 16% and anemia in 19% of patients respectively. Grade 3 esophagitis was seen in 4 patients (10%), all stage IV patients receiving concurrent chemoradiation.

Nitroglycerin related toxicity occurred in 7 patients (17%) mentioning headache and 1 patient with symptomatic hypotension (2%).

### 3.4. Imaging results

Acquisition and analysis of images are described in the supplementary material and methods. Baseline and post-nitroglycerin HX4-PET/CT scans were made in 32 and 25 patients respectively, while baseline and post-nitroglycerin DCE-CTs were acquired in 22 and 13 patients respectively. The median interval between baseline and second HX4 and DCE-CT scans was 4 and 5 days respectively (range 2–7 days). No patients received chemo- or radiotherapy during this interval. In Fig. 1, HX4-PET/CT scans and BF and BV DCE-CT maps at baseline and after nitroglycerin in an example patient are shown. Hypoxia was present in 25/31 primary tumours (80%) while 16/25 nodal volumes (64%) were hypoxic. Baseline HX4-TBR and HF showed a negative correlation with blood flow ($r = -0.451$, $p = 0.046$ and $r = -0.573$, $p = 0.008$) (Fig. S2).

For patients with baseline and post-nitroglycerin HX4-PET imaging and a primary tumour (n = 24), the median TBR remained unchanged after nitroglycerin: 1.4 (IQR: 1.2–1.8) vs 1.4 (IQR: 1.3–1.8). Likewise, other hypoxia features did not show any significant changes (Fig. 2). Numerically, more tumours and nodes were hypoxic after nitroglycerin: 19/24 tumours (79%) vs 21/24 tumours (87.5%) and 9/18 nodal volumes (50%) vs 10/18 nodal volumes (55%). A reduction in hypoxia exceeding the CoR was found in only 1 GTVp based on the TBR threshold and in 2 GTVp based on the HX4-HF threshold. The HX4-TBR increased by more than CoR in

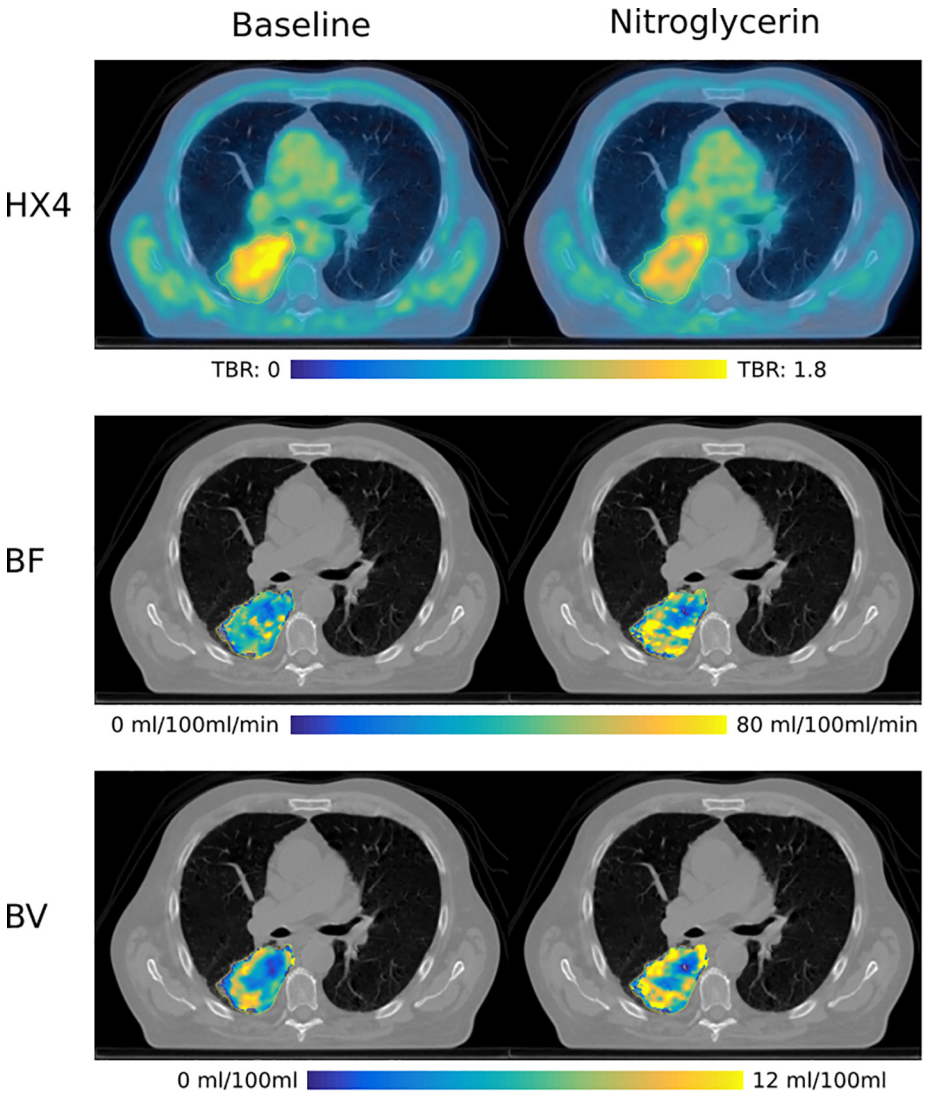


**Fig. 1.** HX4-PET/CT scan, blood flow (BF) and blood volume (BV) DCE-CT maps of a representative patient, at baseline and after applying a nitroglycerin patch. In this patient, the hypoxic volume decreased from 70 $cm^3$ to 64 $cm^3$, while the average BF increased from 37 ml/100 ml/min to 54 ml/100 ml/min, and the average BV increased from 5.8 ml/100 ml to 8.6 ml/100 ml.

3/24 (12.5%) and an increase CoR of the HF was seen in 1 GTVp (4%). The effect of nitroglycerin on HX4-TBR in GTVp correlated with the effect in GTVn in the same patients ($r = 0.701$, $p = 0.002$).

For the 13 patients with baseline and post-nitroglycerin DCE-CT scans, there was no difference between the median BF before or after nitroglycerin: 63.6 (IQR: 52.0–81.2) vs 53.8 (IQR: 44.8–78.4) ml/100 ml/min ($p = 0.087$) or the median BV: 7.5 (IQR: 5.8–9.4) at baseline vs 7.2 (IQR: 6.9–8.6) ml/100 ml after nitroglycerin treatment ($p = 0.972$). A significant increase in BF was present in 1 tumour and a decrease was seen in 4. Blood volume increased significantly in 4 tumours and decreased in another 4 [8]. In the 12 patients who received the full set of scans we found a negative correlation between the change in HF with the change in BF ($r = -0.650$, $p = 0.022$), but not between other parameters.

The Kaplan Meier curves assessing the prognostic value of baseline imaging are presented in Fig. 3 for hypoxia PET imaging and in Fig. S3 for DCE-CT imaging. A significant difference in OS ($p = 0.029$) was observed between patients with hypoxic tumours (2-year OS 47%; 95% CI: 31–72%) and non-hypoxic tumours (2-year OS 100%; 95% CI: 100–100%). Patients with hypoxic tumours also exhibited a worse MFS ($p = 0.045$) while LRPFS was not significantly different between baseline hypoxic and non-hypoxic tumours ($p = 0.23$) (Fig. 3). None of the other factors examined (total GTV, FDG-$SUV_{max}$ or FDG-) were prognostic in this patient cohort.

Kaplan Meier curves based on the scans with nitroglycerin patch are shown in Fig. S4 for hypoxia PET imaging and in Fig. S5 for the DCE-CT imaging features. For the 24 patients with a nitroglycerin scan and primary tumour, no significant differences were found between patients with post-nitroglycerin hypoxic and non-hypoxic tumours for OS ($p = 0.14$), MFS ($p = 0.19$) or LRFS ($p = 0.99$). In 13 patients with a nitroglycerin DCE-CT scan, no survival differences were found for different levels of BF or BV. Since too few patients had a hypoxia response, a separate survival analysis was irrelevant.

## 4. Discussion

In this trial we could not demonstrate a significant survival benefit from the addition of nitroglycerin to radiotherapy for NSCLC patients. After the negative results of several other simultaneous trials, ours also ended prematurely because of the inability to reach the primary endpoint. Overall, the survival, loco-regional relapse and distant metastases rates are in the range of those, previously

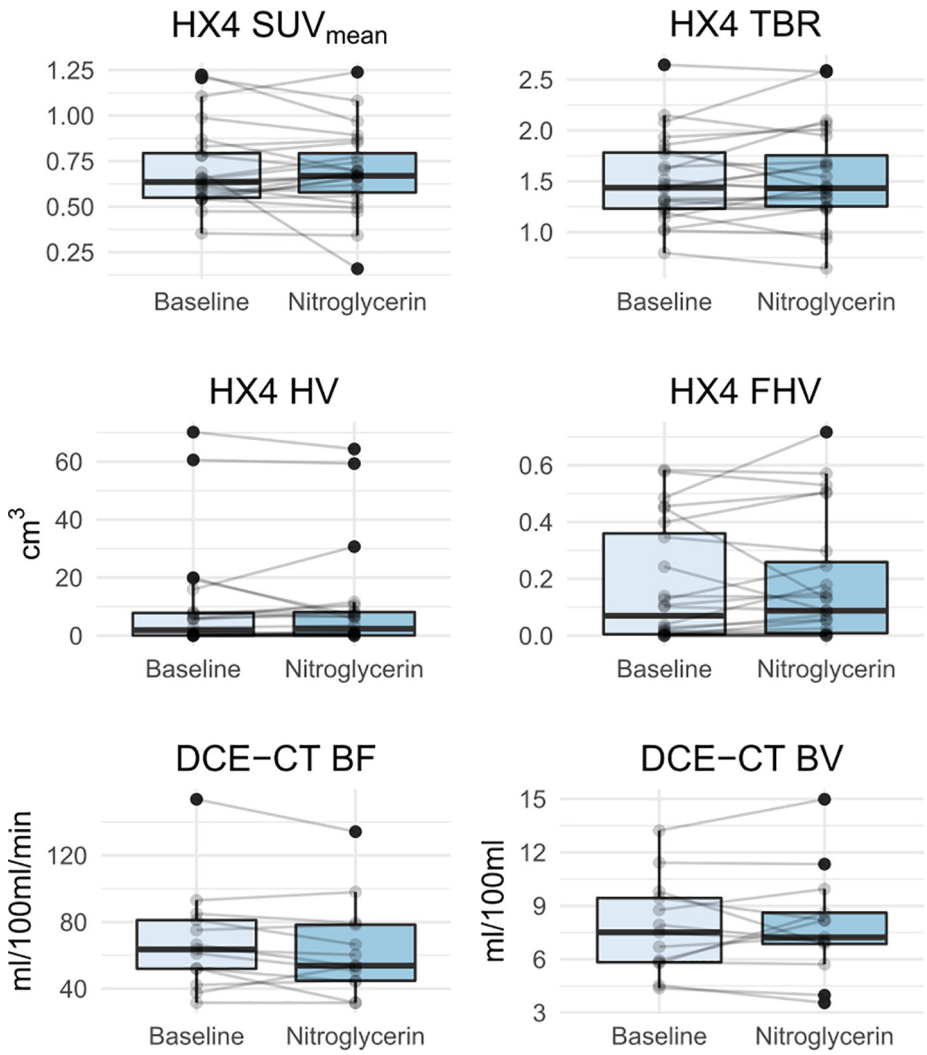


**Fig. 2.** Boxplots of HX4-PET and DCE-CT imaging characteristics for the primary tumour for patients with both a baseline and nitroglycerin scan. For the HX4-PET, the mean uptake ($SUV_{mean}$), tumour-to-background ratio (TBR), hypoxic volume (HV), and fraction of HV (FHV) are given. For the DCE-CT images, the average blood flow (BF) and blood volume (BV) are shown. The observations of patients with two scans are connected with a line. The HX4-PET imaging features are shown for 24 patients; the DCE-CT features were available for 13 patients.

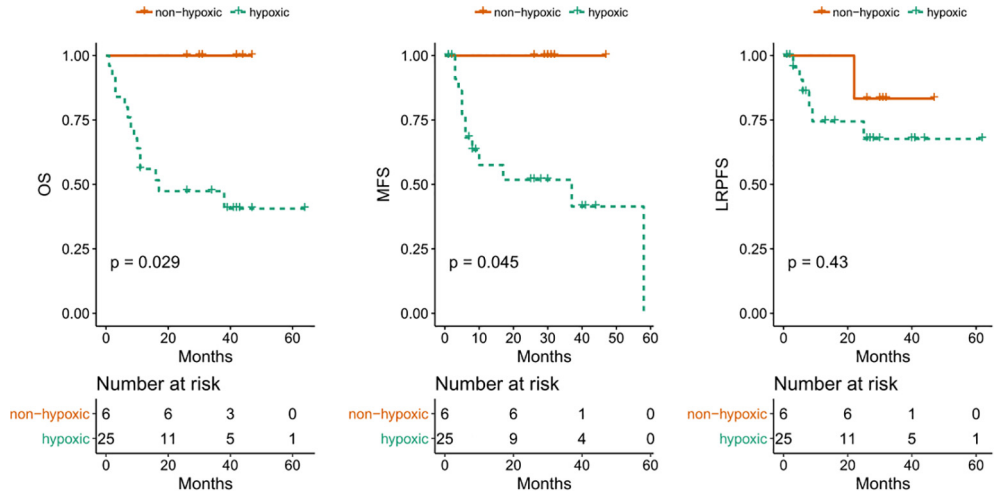


**Fig. 3.** Prognostic value of baseline HX4-PET imaging of the primary tumour, with from left to right the overall survival (OS), metastasis-free survival (MFS) and loco-regional progression free survival (LRPFS). In total 32 patients received a baseline HX4-PET/CT. The 31 patients with a primary tumour are displayed in the graph.

published by our group in patients with stage III disease treated with concurrent individualized accelerated radiotherapy (INDAR) and the ESPATU-trial on which the INDAR schedule was based [10,11]. Our trial was designed in 2010, and a 2-year overall survival of 65% was thought to represent a clinically relevant improvement relative to standard treatment to follow through with a phase III trial. In more recent trials where treatment at relapse also incorporates targeted therapy, 2-year survival rates of 60% and higher for stage III patients are regularly reported [11,12]. This aggressive approach of relapsing patients can also be seen in our trial, where 2/3 of patients with progressive disease received second line treatment, half of which had radical intent. Therefore, at interim analysis the trial staff decided that even a 65% 2-year survival would not represent an improvement over standard care to justify phase III testing.

Combining nitroglycerin with standard treatment, toxicity was mild. The most frequent toxicity was neutropenia. Our rate of 39% in patients receiving chemotherapy is in the range of the 30–60% rates reported in other trials using cisplatin-doublet chemotherapy with nitroglycerin. Headache was only reported by 17% of patients and did not exceed grade 2. This is in line with the results of other trials using a 25 mg patch. In the NVALT-12 trial the rate of $\geq$grade 3 headache was 12.1% and 20% of patients stopped nitroglycerin because of headache. In that trial however, a patch containing 75 mg of nitroglycerin was used [1,2,5].

FDG-PET, DCE-CT imaging parameters and total GTV did not correlate with survival in this cohort, but patients with baseline hypoxic primary tumours had a worse prognosis than patients with normoxic tumours. Other nitro-imidazole based PET tracers, FMISO, FAZA and FETNIM [13–16], already showed to be prognostic in NSCLC, but this is the first prospective trial to find this correlation for HX4. Whether this is independent of other factors (such as GTV or pathology) should be investigated in larger cohorts, since the limited number of patients with baseline hypoxia precludes extensive multivariate analysis. The difference in survival rate between patients with hypoxic and non-hypoxic primary tumours is related to a difference in metastasis free survival, rather than loco-regional control rate. All metastases developed in patients with a hypoxic primary tumour, which correlates with the observation that hypoxia selects cells with a more aggressive and metastasis-prone phenotype and enables metastatic spread [17,18].

The main rationale underlying all trials investigating nitroglycerin as an adjunct to standard treatment modalities for NSCLC is enhanced tumour perfusion attributed to the vasodilating properties of nitroglycerin [1,2,4,5]. This was also proposed by Yasuda to explain the lower VEGF levels observed in NSCLC tumours in operated patients pre-treated with nitroglycerin and in the blood of patients treated with nitroglycerin prior to chemotherapy [19]. Although enhanced tumour perfusion was previously shown, our results do not support a general tumour perfusion increase in human NSCLC: most tumours showed no significant changes. Moreover, one in six volumes exhibited a significant increase in HX4 uptake after treatment with nitroglycerin and a significant decrease in BF and BV was found in almost a third of tumours. This observation correlates with warnings that NO should be considered a 'double-edged sword' in cancer treatment [20]. As NO is not a targeted agent focusing its actions solely on the tumour vasculature, its systemic effect on peripheral vessels could cause a steal phenomenon in adequately perfused tumours, shifting blood away from the tumour to the systemic vasculature [21,22]. Blood pressure measurements at the time of scanning could have offered more information, but were not obtained. According to several investigators an alternative mechanism of action of nitroglycerin could be NO-mediated inhibition of mitochondrial oxygen consumption [23–26]. In a separate in vitro experiment (see supplements) we also didn't observe a reduced oxygen consumption rate upon exposure to human plasma levels of nitroglycerin or even 100,000 fold higher (Fig. S6), which is in contrast with earlier reports [27]. This can be explained by our use of stabilisation agent free nitroglycerin. Often saccharides are used to stabilize nitroglycerin [28], which can upregulate the glucose metabolism and surpass mitochondrial respiration.

Our observations present an argument against the general application of nitroglycerin in unselected patients and highlight the need patient stratification and selection. We hypothesized that nitroglycerin-induced differences in hypoxia levels, as measured on HX4-PET/CT scans, could aid selection of patients for nitroglycerin treatment, but due to the limited number of patients and the heterogeneous patient group, we could not confirm this hypothesis.

There are caveats to this study. We used a TBR of 1.2 to distinguish between hypoxic and non-hypoxic tumours. However, a formally established threshold is missing and several different thresholds have been proposed for nitro-imidazole based PET tracers [29]. The threshold used to divide the patients in nitroglycerin responders and non-responders based on their hypoxia status is arbitrary and may have been too strict for this study, limiting detection of responders. Also, the HX4-PET scans used by Zegers et al. to calculate the coefficients of repeatability were acquired shortly after injection of the PET tracer [9]. These scans will have less optimal contrast to noise ratios compared to the scans used in this study [29]. The expected lower noise levels in this study will arguably yield a higher reproducibility, thus smaller changes in hypoxia levels could be ascribed to nitroglycerin administration. The averaging of all imaging features over the whole tumour is also a limiting factor: tumour vasculature is highly irregular and differences in perfusion and hypoxia levels can be local and heterogeneous [30,31]. By averaging the imaging features over a large region, regional nitroglycerin effects might be left unappreciated. Possibly not the average blood flow, but the distribution and redistribution of the blood flow might be more relevant for reducing hypoxia and improving chemotherapy accessibility [31]. Sub-regional tumour analysis could yield valuable information on local differences, however, our small patient group limits this more advanced analysis [32,33].

The studied patient group is small and more heterogeneous than expected beforehand: NSCLC patients (stage IB-IV) were included, receiving a wide range of (combined) treatments, making interpretation of survival for subgroups difficult. While multiple factors thus could have influenced survival, further subgroup analysis or multivariate analysis is restricted due to the limited number of patients. Randomisation with a placebo study group could have limited these influences, but we chose the single arm format to encourage patient participation in view of several simultaneously running, but slowly recruiting randomized phase III trials (eg NVALT-11 and PET-BOOST).

In conclusion, this study adds to the list of trials that could not demonstrate a benefit of adding nitroglycerin as a sensitizing agent to classical treatment of NSCLC patients. We did show for the first time the negative prognostic significance of increased uptake of the hypoxia tracer HX4. In an exploratory analysis we demonstrated that nitroglycerin can exert both increases and decreases in hypoxia, varying between individuals and correlated with both negative or positive variations induced in tumour BF. Selection of patients who could benefit from treatment with nitroglycerin based on imaging parameters wasn't possible, but in any case these results don't support the hypothesis that nitroglycerin can serve as a general hypoxia sensitizing agent in unselected patients.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctro.2019.12.002.

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