Lesson of the month: novel method to quantify neutrophil uptake in early lung cancer using SPECT-CT

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Disclosures

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Keywords: lung cancer, neutrophil, squamous cell carcinoma, adenocarcinoma, SPECT-CT
Abstract

Neutrophils play an important role in the lung tumour microenvironment. We hypothesised that radiolabelled neutrophils coupled to single-photon emission computed tomography (SPECT) may non-invasively quantify neutrophil uptake in tumours from patients with non-small cell lung cancer. We demonstrated increased uptake of radiolabelled neutrophils from the blood into tumours compared to non-specific uptake using radiolabelled transferrin. Moreover, In-111-neutrophil activity in the tumour biopsies also correlated with MPO-positive neutrophils. Our data support the utility of imaging with In-111-labelled neutrophils and SPECT-CT to quantify neutrophil uptake in lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Inflammation has emerged as a hallmark of tumourigenesis, contributing to the development and progression of a range of cancers. Neutrophils are known to be important players in the tumour microenvironment and these tumour-associated neutrophils (TANs) are broadly classified into an N1 tumour-suppressing phenotype or an N2 tumour-promoting phenotype. While neutrophils may exert both pro- and anti-tumoural functions, the prevailing view is that they serve to exclude T cell access and hence promote tumour progression. In murine studies, Ly-6G-specific depletion of neutrophils reduced the metastatic burden in the lungs,\(^1\) while in \(k\text{ras}\)-driven mouse models of lung cancer, Gr1\(^+\) neutrophils were found to favour tumour growth, reduce T cell homing, and antagonise anti-PD1 immunotherapy.\(^2\) Furthermore, neutrophil
derived mediators have been shown to be pro-tumourigenic; neutrophil elastase contributes to tumour proliferation,[3] MMP-9 increases VEGF release (from the ECM) to promote angiogenesis,[4] and neutrophil extracellular traps (NETs) can sequester circulating tumour cells in the pulmonary microvasculature, promoting the development of metastasis.[5]

Although evidence exists that infiltrating neutrophils accumulate in NSCLC,[6] direct non-invasive imaging and quantification of neutrophils within patient tumours has never been described. Such quantification could aid in predicting the therapeutic response and clinical outcome of patients. Here we describe quantitative SPECT-CT using indium-111-radiolabelled autologous neutrophils to quantify neutrophil uptake in tumours of patients with early stage lung cancer, using indium-labelled transferrin as a marker for non-specific uptake of non-cell-bound activity. We also correlated tissue concentrations of radiolabelled neutrophils with histological evidence of MPO+ neutrophils. These findings have important implications for the use of quantitative SPECT-CT to monitor the extent of neutrophil uptake by tumours.

**Clinical Cases**

Six patients were selected for entry into the study met the following criteria: (i) Positron Emission Tomography or biopsy confirmed pre-operative stage I-II lung cancer in the mid-upper zones of the lung, (ii) no prior chemo- or radio-therapy, and (iii) no other active malignancy. Biopsies were performed ≥ 1 month prior to acquisition of SPECT images. Tumours in the mid-upper zones were chosen to avoid potential overlap of the signal with physiological uptake of radiolabelled cells in the liver. Patient characteristics can be found in Supplementary Table 1 and the study protocol is summarised in Supplementary Figure 1.
In four volunteers, neutrophils were isolated from peripheral venous blood using plasma-Percoll gradients and radiolabelled with indium-111-tropolonate, as described in Supplementary Methods. Given that increased vascularity of tumours could contribute to enhanced uptake of In-111-neutrophils,[7] we also assessed the uptake of In-111-transferrin into tumours. Transferrin can be taken up by tumours non-specifically through highly permeable blood vessels. Hence, in two further patients, In-111-transferrin was prepared as a control for non-specific uptake (Supplementary Methods), so that specific focal accumulation of In-111-neutrophils in the tumour area can be compared.

Over 40 min of dynamic gamma camera imaging, labelled neutrophils displayed physiological kinetics in liver, lungs and spleen, in line with previous results from healthy volunteers (Supplementary Fig. 2A).[8] The recoveries of radiolabelled neutrophils from the peripheral blood 45-min after injection (Supplementary Fig. 2B) were comparable to published data, indicating that the cells were not activated by isolation and radiolabelling. As expected, In-111-transferrin behaved as a labelled plasma protein (Supplementary Figs. 2A and B).

SPECT-CT showed very clear and evident uptake of neutrophils into the tumour of a patient with squamous cell carcinoma (Case 1; Figs. 1A-1D). As shown in Fig 1E, tumour uptake increased in patients with injected In-111-neutrophils with a median of 0.0038 mL/min/mL (IQR; 0.0231-0.0531) compared with a median of 0.0009 mL/min/mL for In-111-transferrin (IQR; 0.0004-0.0013). SPECT-CT with attenuation correction (Fig 1F) showed an overall increase in In-111-neutrophil clearance into tumour with a median of 0.0103 mL/min/mL (IQR; 0.0021-0.0387) compared with a
median of 0.0037 mL/min/mL for In-111-transferrin clearance (IQR; 0.0008-0.0066). Of note, SPECT-CT without attenuation correction increased In-111-neutrophil clearance compared to In-111-transferrin clearance in all four patients, but only in two patients following attenuation correction. Attenuation correction of SPECT images is known to improve quantitative accuracy of malignant lesions in the body by reducing soft tissue artefacts.[9] Therefore, we propose that the attenuation corrected values provide a more representative quantification of neutrophil clearance.

The median peripheral neutrophil count was higher in the In-111 neutrophil patient group compared to the In-111 transferrin group (Supplementary Table 1); however the correlation coefficient between the neutrophil count and the non-attenuation corrected Patlak-Rutland slope (r=0.6, p=0.24) or the attenuation corrected Patlak-Rutland slope (r=0.20, p=0.71) was not significant for the 6 cases.

On cell counting of tissue *ex vivo*, Case 1 displayed substantial accumulation of neutrophils in the centre (median 21 MPO$^+$ neutrophils per hpf (IQR; 14-35) and periphery (median 19 MPO$^+$ neutrophils per hpf (IQR; 15-29) of the tumour compared to the background parenchyma (median 5 MPO$^+$ neutrophils per hpf (IQR; 4-11) (Figs. 1G and 1H). Similar findings were obtained in one further patient with a squamous cell carcinoma (Supplementary Fig. 3). There was no discernible neutrophil infiltration at the tumour centre or periphery in the patient with adenocarcinoma (Supplementary Fig. 3). When all four cases were combined, the radiolabelled neutrophil gamma counts positively correlated with the number of MPO$^+$ neutrophils (Fig. 1I; r=0.50, 95% CI: 0.291-0.666; p<0.0001). Of interest, neutrophils were
detected by IHC when the gamma radiation counts were low, suggesting the presence of tissue resident neutrophils.

Discussion

In the present study, we demonstrated quantitative in vivo SPECT-CT imaging of radiolabelled neutrophil uptake in tumours of patients with lung cancer. Our study also established that MPO$^+$ neutrophils in lung resections correlated with radiolabelled neutrophil counts. Such a non-invasive imaging strategy could have important implications for monitoring neutrophil infiltration into tumours, and assessing the impact of neutrophil-targeted therapies in NSCLC.

Two previous studies have reported enhanced uptake of In-111 mixed leukocytes and In-111 granulocytes into a number of localised malignant neoplasms such as pulmonary sarcoma, albeit without quantitative measurements and a subjective scoring protocol.[10,11] There is current interest in quantifying tissue concentration of single-photon emitting tracers using attenuation-corrected SPECT-CT, with the aim of bringing SPECT-CT in line with PET-CT; we believe ours is the first attempt to do so in cell trafficking studies. The importance of our approach lies in the ability to fully exploit techniques such as Patlak-Rutland analysis, which, as in PET-CT, rely on absolute tissue quantification. In our previous studies of neutrophil trafficking in lung tissue, we circumvented this by normalising the Patlak-Rutland slope to the intercept of the plot, which reflects distribution volume of tracer in the tissue (the marginated pulmonary neutrophil pool). However, in solid tissue such as tumour, the quantitative nature of the intercept is uncertain. Quantitative SPECT-CT has general value in studies based on labelled neutrophils because quantification of neutrophil
accumulation in enclosed spaces, like abscesses (as opposed to open tracts), uses crude indices such as abscess/background ratios at arbitrarily selected time points post-injection.

Of interest, we observed inter-tumour heterogeneity with respect to neutrophil uptake into tumours by both imaging and immunohistochemistry approaches, consistent with previous immunohistochemistry studies. A larger scale study will be required to understand the prognostic implication of such variable neutrophil infiltration. Some authors have suggested that, in addition to neutrophil abundance, the specific location of neutrophils within the tumour has prognostic relevance. Sody et al., using a murine model of neutrophil trafficking, found that neutrophils exhibit distinct migratory patterns depending on their localisation to intra-tumoural or peri-tumoural regions.[12]

Given the broadly pro-tumourigenic role of neutrophils, modulation of neutrophil migration into tumours is an attractive therapeutic approach in NSCLC. Indeed, targeting neutrophil recruitment may be beneficial as adjuvant therapy. The CXCR2 pathway has a major role in the control of neutrophil recruitment into tumours, and antagonists of the CXCR2 receptor have reduced tumour growth in murine studies. Phase II clinical trials using CXCR2 inhibitors in NSCLC patients are on-going. [13] Our findings thus offer the prospect of imaging neutrophils in clinical trials to better understand the therapeutic response of targeting neutrophils.

Our study has several limitations. First, our results are drawn from a small sample size, and follow-up will be required to confirm the utility of neutrophil imaging in
NSCLC. Second, as tumour-associated neutrophils exhibit significant heterogeneity, our study could not detect the maturity or activation status of the trafficked cells.

Third, there are reports of increased transferrin receptor expression on lung cancer cells,[14] which could limit the use of radiolabelled transferrin as a negative control. However, as both radiolabelled transferrin and radiolabelled neutrophils are injected in plasma, this will likely increase the baseline uptake in both cases.

In conclusion, we demonstrate how radiolabeled neutrophils and SPECT-CT can be combined to allow non-invasive quantification of neutrophil accumulation in lung cancer. Further studies of in vivo neutrophil trafficking in NSCLC are warranted.

Patient Consent
All subjects gave written informed consent. The study was approved by the Cambridgeshire Research Ethics Committee (13/EE/0130) and the Administration of Radioactive Substances Advisory Committee of the United Kingdom (83/400/33426) and conducted in accordance with the Declaration of Helsinki.

Contributors
NF, NT, LSCL, DMR, MS, SP and CL performed experiments and analysed data. NF drafted the manuscript, with contributions from the other authors as appropriate. ERC, AMP and CS designed the study. JB, UH, SH, RCR and DG contributed to study design, analysis and interpretation. RCR recruited patients for the study. ERC and CS were responsible for the conduct of the study. All authors discussed the results and commented on the manuscript.

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References


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Figure 1. Quantification of radiolabelled neutrophils and radiolabelled transferrin in lung tumours of patients with early stage lung cancer.

SPECT-CT images showing A) transaxial, B) coronal with 3D reconstruction of tumour, C) sagittal and D) coronal plane from a patient with squamous cell carcinoma (Case 1). Images show accumulation in the tumour (outlined in green) and physiological uptake in the liver (L), spleen (S) and vertebra (V) 20 hours after reinjection of In-111-labelled neutrophils. E) and F) Quantification of tumour uptake of In-111-labelled neutrophils (Cases 1-4) and In-111-labelled transferrin (Cases A and B) using non-attenuation corrected (E) and attenuation corrected values (F). G) Tissue gamma counts and MPO$^+$ neutrophil counts from the background parenchyma, peri-tumoural parenchyma, tumour periphery, and tumour centre of a subject with squamous cell carcinoma (Case 1). H) Background parenchyma and tumour centre cores from Case 1 (upper panels) alongside representative MPO immunohistochemical staining (lower panels). Magnification x400. I) Correlation between gamma radiation counts and MPO$^+$ neutrophil counts in four subjects. MPO$^+$ neutrophils were counted in four high powered fields. Correlation coefficients were calculated using Spearman correlation analysis.
Figure 1

E  Without attenuation correction
F  With attenuation correction

G  Gamma counts
  MPO$^+$ neutrophils

H  Background parenchyma
  Centre of tumour

I  MPO$^+$ neutrophils (per h)
1 h SPECT/CT

4 h SPECT/Planar

20 h SPECT/Planar

Multiple blood samples (1 ml)

48 h Lung resection and punch biopsies

Draw blood

Re-inject In-111-labelled neutrophils or plasma

Dynamic imaging

Neutrophil or plasma isolation & In-111 radiolabelling

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Suppl. Figure 2

A  In-111-neutrophils

- Right lung
- Left lung
- Spleen

Counts/pixel/MBq injected vs. Time post injection (min)

B  In-111-transferrin

- Right ventricle
- Liver
- Spleen

Counts/pixel/MBq injected vs. Time post injection (min)

B  Labelled activity remaining in blood at 45 min

- In-111-Neut
- In-111-Trans
**Suppl. Figure 3**

**Case 2 (Squamous cell carcinoma)**

A 

![Graph showing Gamma counts and MPO^+ neutrophils distribution across different lung regions.]

- Background parenchyma
- Peritumoral parenchyma
- Tumour periphery
- Center of tumour

B 

![Graph showing Gamma counts and MPO^+ neutrophils distribution across different lung regions.]

- Background parenchyma
- Peritumoral parenchyma
- Tumour periphery
- Center of tumour

C 

![Graph showing Gamma counts and MPO^+ neutrophils distribution across different lung regions.]

- Background parenchyma
- Peritumoral parenchyma
- Tumour periphery
- Center of tumour
Supplementary Methods, Tables and Figures

Neutrophil and transferrin radiolabelling

In four volunteers, neutrophils were isolated from peripheral venous blood using plasma-Percoll gradients (GE Healthcare, Buckinghamshire, United Kingdom) and radiolabelled with indium-111-tropolone, as described.[1] Briefly, 80 mL of blood was collected in 5% acid citrate dextrose before erythrocyte sedimentation using 5% hydroxyethylstarch (Grifols). After sedimentation, the upper layer of leukocytes was resuspended in 2 mL cell-free plasma before over layering onto Percoll iso-osmotic gradients (50%, 60%, and 65%) diluted in cell-free plasma. After centrifugation for 5 min at 150g the neutrophil layer was removed from the lower gradient interface before resuspension in cell free plasma and incubation with In-111 (Covidien Healthcare, Fareham, UK) and tropolone (0.054% w/v; Ipswich Pharmacy Manufacturing Unit, Ipswich UK) for 10 min. Prior to radiolabelling, a sample of the neutrophil layer was cytocentrifuged to assess the purity of the isolated fraction (≥ 95% neutrophil purity, with eosinophils and monocytes being the main contaminating cell types). The recovery of In-111-neutrophils from the peripheral blood was measured 45 min after injection.[2]

In two further patients, In-111-transferrin was prepared as a control for non-specific uptake from 40 mL of autologous venous blood, as described.[3]

Planar dynamic and SPECT-CT imaging

Participants were positioned in a double-headed SPECT-CT camera (GE Discovery 670, GE Healthcare), fitted with medium-energy, parallel-hole collimators. After bolus injection of indium-111-labelled neutrophils (median 12.9; interquartile range 10.4-14.8 MBq) or indium-
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**Patlak-Rutland analysis**

Patlak-Rutland analysis allows the quantification of the clearance of a tracer from the blood compartment into the tissue. It plots the ratio of tissue-to-blood concentration at known time points after tracer injection against normalised time, which is a derivative of blood concentration. The slope of the plot represents tracer clearance.

Peripheral blood samples were taken at 2, 4, 6, 8, 10, 15, 30, 45, 90, 120, 240, 360, and 1200 min after injection. Regions of interest were drawn on SPECT-CT images. As previously described for whole lung neutrophil clearance, Patlak-Rutland was used to measure In-111-neutrophil and In-111-transferrin clearances from the blood into tumours.[4] For the first time in relation to labelled blood cell trafficking, attenuation correction was performed using Volumetrix (Version MI, GE Healthcare) and SPECT-CT co-registration using 3D Slicer (Version 4.10.1, NIH). Co-registration of the SPECT images before reconstruction used iterative reconstruction (OSEM 2 iterations 10 subsets). Clearances were then expressed without and with attenuation correction, the latter giving clearance in real units of mL/min/mL.

**Tissue quantification of radiolabelled neutrophils**

Punch biopsy cores (4 mm) were taken from formalin-fixed resected tumours ≤24 h after surgery. Cores were taken from the background parenchyma, peri-tumoural parenchyma,
tumour periphery and tumour centre. The number of cores collected from each case is shown in Supplementary Table 2; core number was dependent on lobe size. Furthermore, care was taken to ensure enough samples were available for tumour, node and staging classification.

After collection the cores were measured in a gamma counter (Wallac 1480, Perkin Elmer, MA, USA). Radioactivity counts were adjusted for weight, decay-corrected, and expressed as counts/g tissue/MBq.

**Immunohistochemistry**

Four micrometer tissue sections were incubated with polyclonal rabbit anti-human myeloperoxidase antibody (Dako Cytomation, Ely, UK), labelled using dextran-coupled peroxidase and 3-3’diaminobenzidine (Dako Cytomation, Ely, UK), and counterstained with haematoxylin. MPO+ neutrophils were counted in a blinded manner.

**Statistical analysis**

Statistical analyses were undertaken using GraphPad Prism (6.0d, San Diego, CA, USA).
Table S1. Patients and tumour characteristics.

Data are presented as median with the interquartile range in brackets. Tumour, node and metastasis staging was classified using The Eighth Edition Lung Cancer Stage Classification.[5]

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Table S2. Number and location of lung tissue cores collected per clinical case.

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References


Supplementary Figure legends

Supplementary Figure 1. Summary of study protocol.
Schematic diagram of the study protocol. Four patients were imaged using In-111 radio-labelled neutrophils and two patients with In-111 radio-labelled transferrin. Lobectomy was performed ≥24 hours following the final SPECT/planar scan. SPECT, single-photon emission computed tomography.

Supplementary Figure 2. Dynamic planar imaging in patients with resectable lung cancer.
A) Distribution of radioactivity over 20 h for the right lung (green), left lung (orange), liver (pink), spleen (red) and right ventricle (blue) after reinjection of In-111-labelled neutrophils (left) or In-111-labelled transferrin (right). Data show median and interquartile range. B) Proportion of In-111-labelled neutrophils (n=4) and In-111-labelled transferrin (n=2) remaining in the blood 45 minutes after reinjection.

Supplementary Figure 3. In-111-labelled neutrophil radioactivity and MPO+ neutrophil counts in resected lungs.
Tissue gamma counts and MPO+ neutrophil counts from the background parenchyma, peritumoural parenchyma, tumour periphery, and tumour centre from Patients 2, 3, and 4.
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### References


Supplementary Figure legends

Supplementary Figure 1. Summary of study protocol.
Schematic diagram of the study protocol. Four patients were imaged using In-111 radio-labelled neutrophils and two patients with In-111 radio-labelled transferrin. Lobectomy was performed ≥24 hours following the final SPECT/planar scan. SPECT, single-photon emission computed tomography.

Supplementary Figure 2. Dynamic planar imaging in patients with resectable lung cancer.
A) Distribution of radioactivity over 20 h for the right lung (green), left lung (orange), liver (pink), spleen (red) and right ventricle (blue) after reinjection of In-111-labelled neutrophils (left) or In-111-labelled transferrin (right). Data show median and interquartile range. B) Proportion of In-111-labelled neutrophils (n=4) and In-111-labelled transferrin (n=2) remaining in the blood 45 minutes after reinjection.

Supplementary Figure 3. In-111-labelled neutrophil radioactivity and MPO⁺ neutrophil counts in resected lungs.
Tissue gamma counts and MPO⁺ neutrophil counts from the background parenchyma, peritumoural parenchyma, tumour periphery, and tumour centre from Patients 2, 3, and 4.