ORIGINAL ARTICLE
LUNG CANCER

# High frequency of infection of lung cancer patients with the parasite *Toxoplasma gondii*

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ABSTRACT

**Background:** *Toxoplasma gondii* is an intracellular protozoan parasite that can cause a wide range of clinical conditions, including miscarriage and pneumonia. The global prevalence is 30% in humans, but varies by locality (*e.g.* in the UK it is typically 10%). The association between lung cancer and *T. gondii* infection was investigated by direct detection in lung tissue samples.

**Methods:** Lung tissue samples were taken from patients undergoing lung resection surgery (n=72) for suspected lung cancer (infection prevalence 100% (95% CI: 93.9–100%)). All 72 participants were confirmed as having lung cancer following subsequent diagnostic tests. In addition, bronchial biopsy samples were collected from non-lung cancer healthy control subjects (n=10). Samples were tested for *T. gondii* using PCR amplification of *T. gondii* specific gene markers and *T. gondii* specific immunohistochemistry.

**Results:** All 72 lung cancer patients were infected with *T. gondii* (prevalence 100% (95% CI: 93.9–100%)). Of which, 95.8% (n=69) of patients showed evidence of active parasite stages. Infection prevalence in the controls (10%) was significantly lower ($p<0.0001$).

**Conclusions:** Clinicians treating lung cancer patients should be aware of the potential presence of the parasite, the potential for induction of symptomatic complications and interference with treatment success.

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***Toxoplasma gondii* infection was found to be present in all lung tissue samples taken from 72 cancer patients (including active parasite stages in 96% of samples)** http://bit.ly/2DhPPRN

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## Introduction

*Toxoplasma gondii* is an intracellular protozoan parasite which can be found in all warm-blooded animals. This parasite can only complete its full life cycle in cats, but nevertheless around 30% of the human population, globally, is estimated to be infected [1–3]. Current estimates of human infection range from a relatively lower prevalence in countries like the UK (10%), China (10%) and the USA (10–20%) [2, 4] to areas where prevalence can exceed 40% (*e.g.* parts of continental Europe and South America) [2]. The main routes of transmission to humans are thought to be *via* ingestion of infective oocysts shed by infected cats or by ingestion of parasite cysts from undercooked meat. Congenital transmission, although reported to be infrequent, contributes to transmission and is often associated with significant neonatal pathology and possible miscarriage [5–8]. Other accidental routes of transmission have also been reported (*e.g.* blood transfusion and organ transplantation). With the exception of congenital transmission, primary infection in humans is usually asymptomatic in healthy individuals. If symptoms occur, they are usually mild influenza-like symptoms, occasionally accompanied by hepatosplenomegaly and lymphadenopathy, but are usually self-limiting [3, 9]. In immunosuppressed and immunodeficient patients *T. gondii* infection can have fatal consequences [10]. *T. gondii* can invade every type of nucleated cell in the body, but preferred target organs are the lymph nodes, brain, heart and lungs. Proliferation of tachyzoites results in the infection of neighbouring cells and necrosis [11, 12]. Common presentations include encephalitis, miscarriages, pneumonia and myocarditis [3].

Patients with cancer may have deficient cellular immunity that has allowed dysregulated proliferating cells to escape immune defences, and are potentially susceptible to opportunistic infections including *T. gondii* [5]. Not much is known about toxoplasmosis in this group of patients and few reports are available. As examples of studies of *T. gondii* infection in cancer patients [13], serological measurement of infection rates showed high prevalences in nasopharyngeal carcinoma (46.2%) and rectal cancer (63.6%), but lower rates in the other cancer groups, for example, pulmonary carcinoma (4.6%), breast cancer (9.5%), gastric carcinoma (10.0%), hepatocellular carcinoma (14.3%) and uterine cervix carcinoma (12.5%). This might suggest that there is an association between *T. gondii* infection and some types of cancer; however, these studies give little indication as to whether active infection is present, and they measure generic infection status rather than localised infection status in the cancer affected tissue. The objectives of this study were to use specific DNA based and immunohistochemical detection systems to detect the presence of the parasite, *T. gondii*, in lung biopsy samples taken from a well-characterised collection of patients with lung cancer. A secondary aim was to investigate any associations between parasite infection intensity or active/dormant infection and other recorded characteristics of these lung cancer patients (such as sex, age, presence of chronic obstructive pulmonary disease (COPD) and smoking history).

## Materials and methods

### *Study subjects and sample processing*

In total, 72 tissue samples were collected from patients undergoing lung resection surgery at University Hospital of South Manchester as part of their clinical care in the National Health Service (NHS). These patients were not specifically recruited for this study but were referred to the hospital with suspected lung cancer and biopsies were taken as part of the diagnostic process. This centre serves a large catchment area covering referrals for lung diseases across the north-west of England. The samples were taken for exploratory investigations as part of suspected lung cancer diagnosis and were taken prior to any anti-cancer drug therapy. All 72 patients were subsequently confirmed as having lung cancer following all subsequent diagnostic tests. Routine diagnosis for cancer does not involve serological testing for *Toxoplasma* infection, thus none of these patients were tested in this way. To act as controls, a further 10 bronchial biopsy samples were obtained from healthy subjects without any history or evidence of lung cancer who were recruited specifically as healthy controls. The potential risk of biopsy to healthy subjects made it difficult to gain a large sample size of control subjects. The control subjects were selected from the same population catchment area as the lung cancer patients and covered a comparable age range, although they have a lower average age (table 1). We recognise the limitations of the control sample, the relatively small numbers, the younger average age range and different tissue type (bronchial rather than lung biopsy), but it was not possible with this study to recruit more appropriate controls. These limitations are discussed later in this article. The overall study methodology is presented in figure 1. The studies were approved by the local South Manchester research ethics committee (03/SM/396, lung tissue collection) and the NRES Committee North West – Greater Manchester South (06/Q1403/156, control sampling). All subjects provided written informed consent. The study also received ethical approval from the University of Salford Research Governance and Ethics (CST 12/37 and ST16/124). For each sample, data was available on age, sex, lung conditions (*e.g.* COPD) if present, pack-years smoking history, and inhaled medication use including bronchodilators and inhaled corticosteroids.

TABLE 1 Summary of patient demographics

| | Males/ females | Age years | $FEV_1$ L | $FEV_1$ %pred | FVC L | $FEV_1$/FVC ratio | Smoking history pack-years | Lung cancer lesion | Recorded medications | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | SAB | LAB | ICS |
| **COPD, current smoker** | 10/6 | 70.9 (60–82) | 1.9 (0.9–3.5) | 74.6 (53–96) | 3.3 (1.7–5.4) | 59.0 (46–75) | 56.9 (9–124) | Yes | 7 | 4 | 4 |
| **COPD, ex-smoker** | 17/2 | 72.1 (60–80) | 1.7 (1.3–2.5) | 64.8 (45–118) | 3.0 (2.4–4.5) | 56.3 (42.5–69.3) | 48.7 (11–112) | Yes | 9 | 12 | 9 |
| **No airflow obstruction, current smoker** | 4/13 | 64.4 (44–78) | 2.3 (1.6–3.3) | 105.2 (70–131) | 3.1 (2.1–4.4) | 73.1 (66.9–82.5) | 44.2 (15–90) | Yes | 0 | 0 | 0 |
| **No airflow obstruction, ex-smoker** | 9/8 | 72.1 (57–84) | 2.1 (1.2–3.2) | 91.9 (47–127) | 2.9 (1.8–4.1) | 71.2 (55–85.8) | 37.3 (2.1–117) | Yes | 1 | 1 | 1 |
| **Never smoker** | 0/3 | 68.3 (65–71) | 1.9 (1.8–2.0) | 108.3 (100–113) | 2.7 (2.2–3.6) | 82.5 (77–91) | 0 (0) | Yes | 1 | 0 | 1 |
| **Average for lung cancer group** | 40/32 (total) | 69.8 (44–84) | 2.0 (0.87–3.5) | 84.8 (45–131) | 3.1 (1.69–5.4) | 65.5 (42.5–90.9) | 44.1 (0–124) | Yes | 18 | 17 | 15 |
| **Healthy, never smoker controls** | 7/3 | 52.2 (31–75) | 3.3 (2.3–4.18) | 107 (82.7–148.6) | 4.3 (2.9–5.6) | 78.6 (70.8–95.1) | 0.0 (0) | No | 0 | 0 | 0 |

Subject demographics of cancer patients (n=72) and healthy nonsmoker control subjects (n=10). Data are presented as the mean (range) or n. $FEV_1$: forced expired volume in 1 s; FVC: forced vital capacity; SAB: short-acting bronchodilators; LAB: long-acting bronchodilators; ICS: inhaled corticosteroid.

Tissue sections were obtained from the lung as far distal to the tumour as possible, as determined by an NHS pathologist. Lung tissue was washed in sterile PBS prior to use. A portion of the tissue was fixed with 10% formalin in PBS buffer and embedded in paraffin, using a Leica TP1020 automatic tissue processor (Leica Microsystems (UK) Ltd, Milton Keynes, UK). Tissues were sectioned into 5 µm slices and lifted

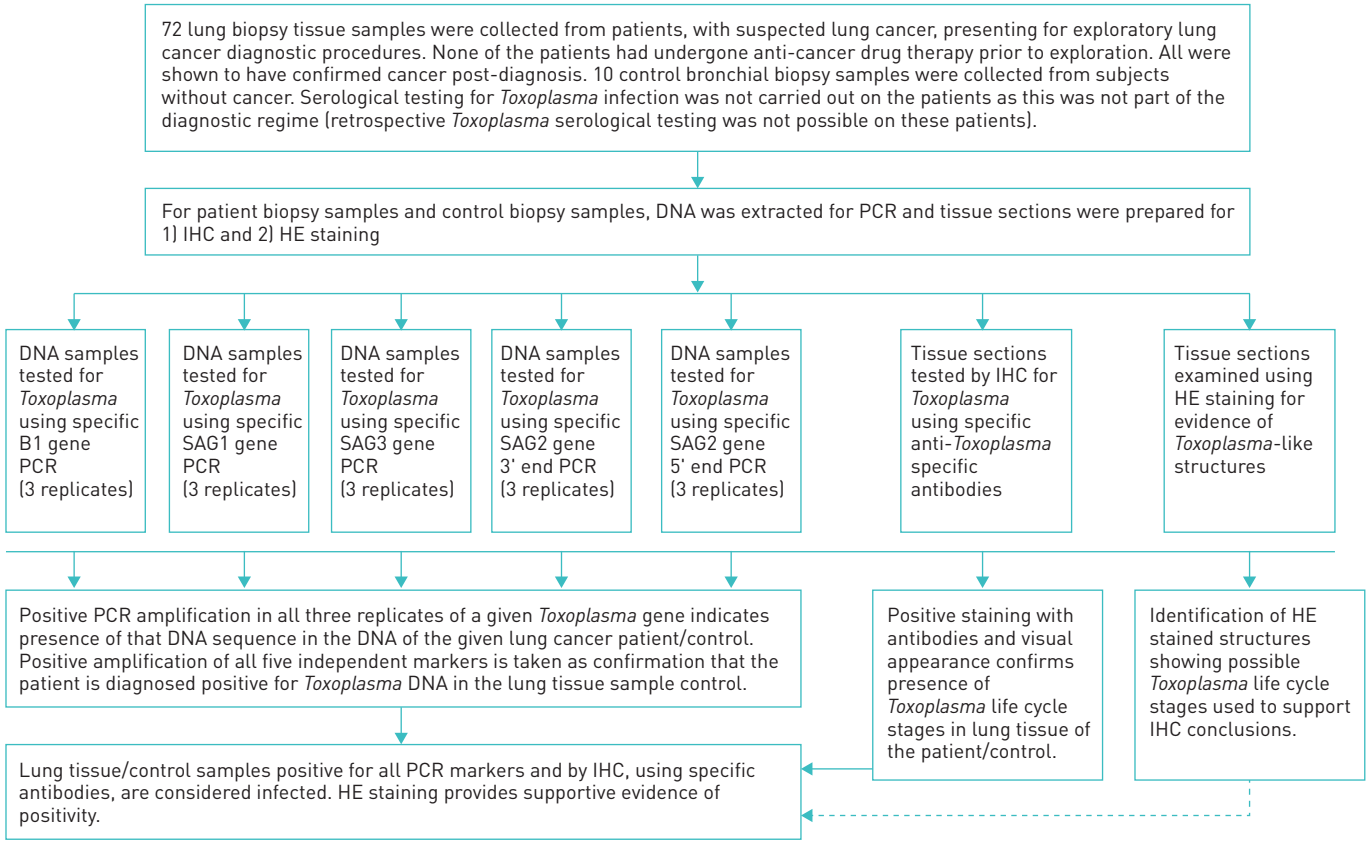


FIGURE 1 Flowchart showing the study methodology. IHC: immunohistochemistry; HE: haematoxylin and eosin.

onto poly-L-lysine glass slides. Portions of tissue were snap frozen in liquid nitrogen, stored at −80°C and used later for DNA extraction. Control bronchial biopsies were collected from subjects and immediately fixed using 10% neutral buffered formalin (CellPath, Newtown, UK), processed and paraffin embedded. 4 µm sections were cut and lifted onto poly-L-lysine coated glass slides (Surgipath, Peterborough, UK).

### *PCR detection of* T. gondii *in human lung samples*

DNA from 72 lung cancer patients and 10 control subjects was extracted from small blocks of snap frozen tissue or directly from sections on poly-L-lysine microscope slides, using proteinase K lysis followed by phenol/chloroform extraction as previously described [14]. Extracted DNA was tested using a mammalian α-tubulin PCR to ensure the viability of the DNA for PCR amplification [15]. Protocols and processes were applied to prevent cross contamination of PCR reactions as previously described [16–19]. The presence of the parasite was tested with five markers at four genetic loci: SAG1, SAG2 (the 3′ and 5′ ends were tested separately), SAG3 and B1 [20–22] as previously described [19]. All of these markers are commonly used specific PCR diagnostic markers for *T. gondii*. Pure parasite DNA from the *T. gondii* RH strain and from a type II strain, isolated from a goat in Slovakia [19] were used as positive controls. Negative controls (water) were interspersed throughout the PCR reactions to detect any possible false amplification and DNA extraction controls from sham blocks were also included as negative controls. Any experiment in which the negative controls showed amplification was discarded and repeated. PCR amplifications were conducted in replicates; each sample was tested three times. PCR products were visualised by agarose gel electrophoresis using standard methods and were sequenced to confirm that the correct amplicons were amplified. The DNA samples were considered to be positive for *T. gondii* if they successfully amplified in all three reactions with all five *Toxoplasma* specific markers.

### *Immunohistochemical detection of* T. gondii *in human lung sections*

Using established approaches [23], immunohistochemistry (IHC) was performed on paraffin embedded tissue using commercial anti-*T. gondii* polyclonal antibodies produced in rabbits (Thermo Fisher Scientific, Catalogue number PA1-38789, Rockford, IL, USA). This antibody was generated from a whole *Toxoplasma gondii* lysate, has been validated for IHC (Thermo Fisher) and used in previous studies (*e.g.* [24]). The 5 µm tissue sections were cut and mounted on positively charged glass slides then dewaxed in Histoclear (2×5 min), rehydrated in alcohols (ethanol), 100% (5 min), 90% (3 min), 75% (2 min) and 50% (1 min). They were finally rinsed in tap water to remove the ethanol for 5 min. Antigen retrieval was performed in 1% trypsin/calcium chloride (pH 7.8) at 37°C for 30 min in a humidified chamber [25]. After incubation, the sections were left to cool at room temperature for 10 min, then washed in PBS Tween 20 twice for 2 min. Endogenous peroxidase activity was blocked by incubating slides in 0.3% hydrogen peroxide for 30 min at room temperature followed by washing in TBS. Nonspecific antibody binding was blocked using normal goat serum (Vectastain ABC Systems, Vector Laboratories, Peterborough, UK) for 30 min at room temperature and followed by incubation in diluted (1/100) polyclonal rabbit anti-*T. gondii* antibodies for 1 h at room temperature. Following this incubation, the slides were washed in TBS Tween for 3×3 min and incubated in biotinylated goat anti-rabbit secondary antibody (Vectastain ABC Systems) for 30 min at room temperature followed by another wash in TBS Tween (3×3 min). Slides were incubated in ABC-Px mix (Vectastain ABC Systems) for 30 min, and re-washed ×3 in TBS. The resulting complex was visualised using 3-3′-diaminobenzidine (DAB) for a maximum of 10 min. The intensity of the tissue staining was monitored using light microscopy and the DAB reaction quenched in distilled water when optimal staining was reached. Sections were then washed with running water for 5 min, counterstained with haematoxylin for 45 s, washed with water for another 5 min, dehydrated with alcohols, 50% (1 min), 75% (2 min), 95% (4 min), and 100% (5 min), cleared in Histoclear (2×5 min) and mounted, using cover slips, in DPX. Three negative controls were used for each staining. These were lung sections from *T. gondii* negative wood mouse (*Apodemus sylvaticus*), human lung sections with primary antibodies omitted and cells derived from a C2C12 culture (mouse myoblast cell line, free of *T. gondii*) with both primary antibodies present and absent. Specific *T. gondii* staining was not observed in any negative controls. Cell culture derived *T. gondii* RH strain tachyzoites and lung tissue from a *T. gondii* infected wood mouse were used as positive controls [19]. Specific *T. gondii* staining was observed in all positive controls. Immunostained slides were also assessed using quantitative criteria. The program ImageJ (https://imagej.nih.gov/ij/) was used to calculate a percentage score which described the degree of coverage of infected tissue on each slide. For each patient, three microscope fields of view (×400 magnification) were randomly selected, photographed and quantified using ImageJ software (as a percentage of stained pixels with respect to total pixels). The mean percentage of pixels in the stained areas was calculated for each slide. According to the calculated mean (a measure of parasite intensity) the patients were divided into three grades of staining. Grade 1 had a staining of <10% of the area covered, grade 2 between 10 and 20% and grade 3 intensity of >20%. In addition to overall percentage cover, slides were analysed in more detail and percentage coverage for different parasite life cycle stages were recorded

(*T. gondii* cysts, intracellular infection of macrophages (or other cell types) and free tachyzoites). Finally, haematoxylin and eosin (HE) staining was used to confirm that structures compatible with *T. gondii* stages could be observed within sections [26].

### *Statistical analyses*

To compare infection status of lung cancer patients (n=72) and non-lung cancer control patients (n=10), 2×2 contingency tables were used. Fisher's exact test was used to calculate p-values and values of <0.05 were considered statistically significant. In order to investigate any relationships between *T. gondii* infection and demographic data collected from within the lung cancer patient cohort (n=72), logistic regression (generalised linear model with a binomial distribution) was used to avoid the statistical pitfalls of conducting multiple univariate analyses. Model selection was based on backwards selection with only those factors remaining significant at a level of p<0.05 being included in the final model. This approach was used to avoid the pitfalls of confounding variables. As all lung cancer patients were infected, this analysis used the following variables for parasite infection status: *Toxoplasma* infection intensity (*i.e.* grades 1–3) and active/inactive parasite stages. Intensity grades were assessed by IHC staining using Image J analysis of pixel coverage, as described above, and also included distinction between active (presence of tachyzoites and infected cells) and inactive stages (presences of cysts alone). Dependent variables used in the model for parasite infection were inactive *versus* active infection. Data on patient status was also recorded as follows: sex, age, presence of COPD and smoking history. Lung cancer patients were placed into two categories (dependent variables), "No COPD" subjects (no airflow obstruction as determined by normal spirometry) or "COPD" subjects (Global Initiative for Chronic Obstructive Lung Disease criteria), and considered against factors that predicted individuals in each category. Factors considered included age, sex of the individual and a number of measures of smoking and *Toxoplasma* infection intensity and active/inactive stage status. Patient-associated dependent variables were as follows: smoker, "pack-year history" (1 pack-year is defined as 20 cigarettes per day for 1 year) and non-smoker; current smoker *versus* non-smoker; prior medication *versus* non-medication. Only one patient- or *Toxoplasma*-associated variable was considered at a time and all combinations were considered. Model selection was based on backwards selection with only those factors remaining significant at a level of p<0.05 being included in the final model. All analyses were undertaken using R 3.01 (RCore Team, 2013) [27].

## Results

DNA and tissue samples, for IHC, were collected from resected lung tissues from lung cancer patients (n=72) and non-lung cancer healthy control subjects (n=10). Patient demographics are shown in table 1 and figure 1 illustrates the overall methodology used to examine the samples. Initially, DNA was extracted successfully from tissue from all subjects. All samples were tested for the absence of PCR inhibition using amplification of the mammalian α-tubulin gene. All samples showed successful PCR amplification of the α-tubulin gene and were used for *T. gondii* DNA PCR detection using three replicated experiments of five genetic markers at four independent loci: SAG1, SAG2 (3′ and 5′ ends), SAG3 and B1. Multiple independent markers were used to rule out the possibility that individual markers could nonspecifically amplify as can happen with *Toxoplasma* in single marker PCR or qPCR. All tested lung cancer patient samples (32 females, 40 males) were found to be *T. gondii* positive, giving a prevalence of 100% with all five genes. Of the 10 healthy control subjects, one sample showed positive amplification for all *T. gondii* markers and the remaining nine did not amplify with any of the *T. gondii* specific primers. To confirm the presence of the parasite, using an independent detection system, the tissue sections were examined by IHC using specific anti-*T. gondii* antibodies. This was followed by secondary confirmation using HE staining. IHC was performed on all tissue sections enabling both detection of the parasite and identification of the life cycle stage. Infected tissue could be identified as containing *T. gondii* cysts, intracellular infection of macrophages (or other cell types) and free tachyzoites (figure 2). All 72 lung cancer patient tissue samples showed positive staining with the anti-*T. gondii* antibody and thus confirmed the PCR detection results of 100% (95% CI: 95.19–100%) prevalence in this cohort. Only one sample in the control group showed specific staining with the anti-*T. gondii* antibody and this was the same sample that showed positive amplification for the five *T. gondii* specific PCR amplifications. There was an extremely significant difference in prevalence between the lung cancer group (n=72) and the non-cancer control group (n=10) (p<0.0001, Fisher's exact test). A surprisingly high proportion of lung cancer patients (95.8%; n=69) showed evidence of an active form of infection, as defined by the presence of tachyzoites or infected alveolar macrophages (or other cell types). Only three lung cancer subjects (4.2%) had the dormant cyst stage as the only stage present. This is indicative of a latent infection in these three patients. Image J was used to measure the proportion of infected cells and infected areas of the lung tissue. A quantitative score was calculated for each type of life cycle stage individually and an overall score determined (see materials and methods) for each lung tissue sample (table 2). Both *T. gondii* cysts and infected cells were observed in the single infected non-cancer control sample. All samples were also stained with HE and observed

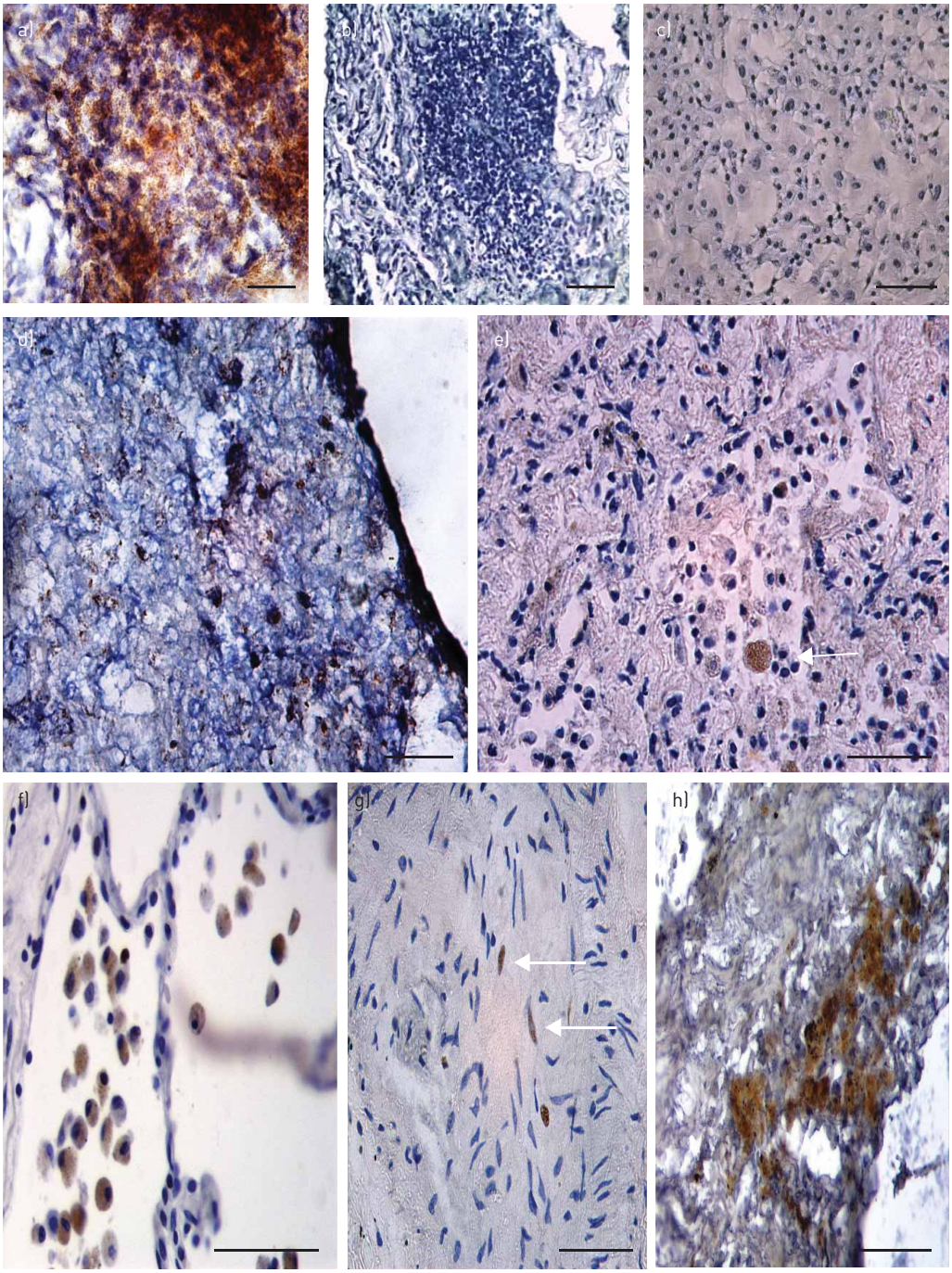


FIGURE 2 Anti-*Toxoplasma gondii* antigen immunostaining of human lung and control tissues. a) Cell culture derived *T. gondii* RH strain tachyzoites stained with polyclonal anti-*T. gondii* antibodies. Brown staining indicates detection of *T. gondii*. Positive control (×400 magnification, scale bar = 100 µm). b) Human lung section stained with polyclonal anti-*T. gondii* antibodies with primary antibodies omitted. Negative control (×400 magnification, scale bar = 100 µm). c) Cells derived from a C2C12 culture (mouse myoblast cell line) which is *T. gondii* free and stained with polyclonal anti-*T. gondii* antibodies. Negative control (×400 magnification, scale bar = 100 µm). d) Lung tissue from a *T. gondii* infected wood mouse (*Apodemus sylvaticus*) stained with polyclonal anti-*T. gondii* antibodies. Brown staining indicates detection of *T. gondii*. Positive control (×400 magnification, scale bar = 100 µm). e) Human lung section, from subject 1045, stained with polyclonal anti-*T. gondii* antibodies. *T. gondii* cysts can be seen (examples indicated with white arrows) (×400 magnification, scale bar = 100 µm). f) Human lung section, from subject 1040, stained with polyclonal anti-*T. gondii* antibodies. Alveolar macrophages infected with *T. gondii* can be seen (×400 magnification, scale bar = 100 µm). g) Human lung section, from subject 1028, stained with polyclonal anti-*T. gondii* antibodies. By observation of cell morphology, fibroblasts infected with *T. gondii* can be seen (examples indicated with white arrows) (×400 magnification, scale bar = 100 µm). h) Human lung section, from subject 975, stained with polyclonal anti-*T. gondii* antibodies. Ruptured *T. gondii* cysts and free *T. gondii* tachyzoites can be seen (×400 magnification, scale bar = 100 µm).

TABLE 2 Life cycle stages of *Toxoplasma gondii* and type of infection in lung cancer patients (n=72) as identified by immunohistochemistry

| Infection type | Subjects n | Range of percentage score[#] | Infection status |
|---|---|---|---|
| **Cysts** | 3 | 0.2–2.9% | Inactive |
| **Tachyzoites** | 3 | 0.8–8.7% | Active |
| **Macrophages or other cells** | 18 | 0.6–34.2% | Active |
| **Mixture** | 48 | 1.1–44.0% | Active |
| **Total infections** | 72 | | |

[#]: the score was determined as described in the materials and methods.

under the light microscope. Although less specific as a diagnostic technique than the IHC and PCR, the presence of structures consistent with infection by the parasite was confirmed in 67 out of 72 tissue sections from the lung cancer patients and in one out of 10 of the non-cancer control group. In the latter case, this corresponded to the sample that was positive for the PCR amplifications and IHC. The remaining lung cancer samples could not be reliably confirmed as potentially infected by this method, but could have possessed less visible structures such as tachyzoites. Using the HE staining technique, infected cells, macrophages and some tissue cysts were observed (figure 3) but no free tachyzoites could be detected.

By quantifying the *Toxoplasma* infection intensity among the 72 lung cancer patients, we were able to evaluate any relationship between parasitic load and presence of COPD or other demographic factors. Our cohort did not show any significant association between *Toxoplasma* infection load with patient smoking history (both total exposure ($p>0.05$) or current exposure ($p>0.05$)) or airflow obstruction in COPD ($p>0.05$). All other analyses conducted had non-significant p-values ($p>0.05$) except for a parasitologically unrelated association between COPD and sex (males>females; $p<0.05$).

## Discussion

In this study, we investigated the prevalence of *T. gondii* in clinical samples from patients with lung cancer using PCR and *T. gondii* specific IHC. Surprisingly, of the 72 subjects admitted to hospital for examination for lung cancer, all (100%) showed evidence of infection of lung tissue by the parasite *T. gondii*. A significant difference was observed between the prevalence in these lung cancer patients and in a non-cancer control group. Of the lung cancer patients, 95.8% showed evidence of active infection as opposed to being in the dormant (cyst) stage. Four stages of infection were observed by IHC: cysts, infected macrophages, other cell types infected and free-living tachyzoites. Given that the background levels of infection in the UK are considered to be low at 10% and the global infection rate is 30% [2], this represents an extremely high infection rate in these cancer patients. While detailed prevalence figures have not recently been determined for the catchment area sampled, it is unlikely that it significantly deviates

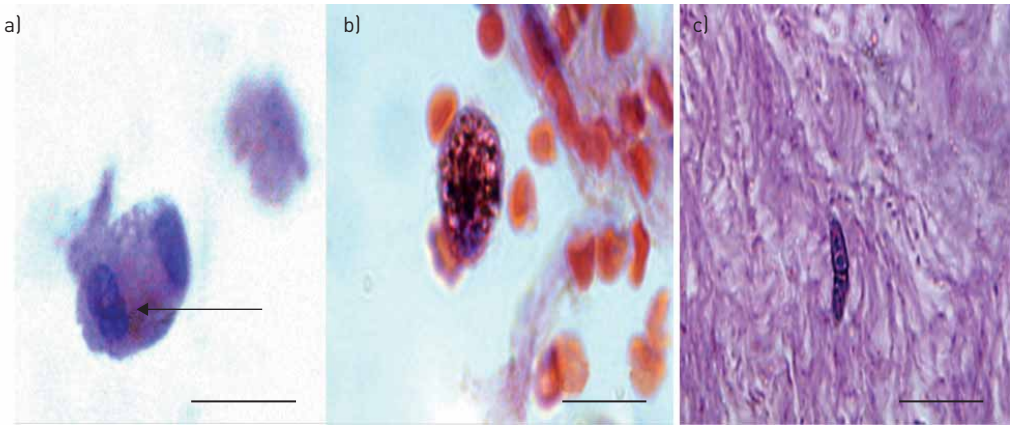


FIGURE 3 Haematoxylin and eosin staining of human lung sections. a) Infected alveolar macrophage with four visible tachyzoites (red arrow) in subject 1040 (×400 magnification, scale bar = 10 µm). b) Tissue cyst from subject 1070 (×400 magnification, scale bar = 10 µm) and c) young tissue cyst in subject 1028 (×400 magnification, scale bar = 10 µm).

from the national average. The most recent studies in the UK show infection in a range of 7–34% [2, 28–30]. In our study, we were able to evaluate *Toxoplasma* infection in 10 samples from healthy subjects without cancer who were specifically recruited as controls and were similarly age range matched with the cancer patients (although with a lower average age). We acknowledge some limitations in the control group; however, it was difficult to achieve a suitably sized and matched control group due to the potential risks of lung biopsy techniques. For example, one limitation was the comparison of lung tissue *versus* bronchial tissue. However, as *T. gondii* can infect any nucleated cell types and that both tissue types will have the same exposure to parasite infection, we consider minimal impact of these limitations on the conclusions. We also acknowledge potential bias due to the relatively small control sample size and younger average age of the control population, which is again related to the difficulty and risks associated with sampling healthy subjects. However, taken alongside the many studies of prevalence in the UK [2, 28–30], this study has demonstrated an unexpectedly high prevalence of *Toxoplasma* infection in lung cancer patients compared with control subjects and with the expected UK prevalence in people without cancer.

Toxoplasmosis has been reported to increase the fatality rate in a variety of cancers such as Hodgkin's disease, leukaemia, melanoma and brain cancer [31–35]. However, *Toxoplasma* infection causing complications in lung cancer has been reported only rarely. A case report demonstrated that a *T. gondii* infection was detected in a patient with lung cancer [36]. The diagnosis was based on tachyzoites present in bronchoalveolar lavage and detection of specific IgM antibodies. Most of the studies that investigate the link between cancer and *T. gondii* infection are based on serological prevalence detection of the parasite in cohorts of cancer patients rather than by direct investigation of tissue samples. Overall 8.38% of examined patients with malignant neoplasms in China were seropositive for antibodies against *T. gondii*. However, when nested PCR detection was used on the same samples, only 3.55% of these patients were positive [37]. In another study from China [38], much higher prevalence was detected, with 35.56% of the cancer patients overall being positive for anti-*T. gondii* IgG. The highest prevalence of infection, in this study [38], was observed in lung cancer patients (60.94%) followed by cervical cancer patients (50%). Among 356 cancer patients, 21 (5.9%) cases were found to be IgG-positive and 8 (2.3%) were IgM-positive, and five of them were found to have both IgG and IgM antibodies [39]. The total seroprevalence of *Toxoplasma* infection in this study was 6.8% [39]. A study in Iran concluded that 45.2% of cancer patients were seropositive for *T. gondii* [40]. High seropositivity rates were detected in women with breast cancer (86.4%) [41]. In a study comparing national figures from 37 countries [42], it was found that brain cancers are 1.8 times more common in countries where *T. gondii* infections are more prevalent than in those where it is virtually absent. Overall, the studies that investigate cancer and *T. gondii* infection generally show no particular link, although these studies are rarely specifically addressing the link or are controlled against a healthy cohort. As far as we can determine, our study is the first that specifically investigates the link between cancer affected lung tissue and *T. gondii*. Unfortunately, while we recognise the value of it, we were not able to investigate the seropositivity of our cohort of cancer patients since there is no formal process of *Toxoplasma* testing as part of lung cancer diagnostic protocols. Subsequent follow-up is not possible due to some subjects having passed away since diagnosis and the length of time since diagnosis could complicate the serological outcomes. Our study suggests that routine serological testing for *Toxoplasma* may be of value in lung cancer diagnostic protocols.

Using additional data associated with our sample set, the relationship between patient health and *T. gondii* infection was investigated to see if there were any further factors associating with patient health. We investigated which factors were associated with predicting whether an individual had normal lung function (no COPD) or "patients" (COPD) using logistic regression. This multiple regression analysis takes into account that the data are not normally distributed and follows a binomial distribution. We investigated the effects of age and sex, as well as a range of different smoking parameters (smoker, non-smoker and pack-year smoking history) and *Toxoplasma* infection measures (*Toxoplasma* intensity of infection, presence of free tachyzoites, acute or active infection). We only looked at one smoking and one infection related measure at a time, but considered all possible combinations (*e.g.* smoker and parasite intensity, then smoker and free tachyzoites, then smoker and acute infection) and each factor on its own (*e.g.* smoker). The final model we selected included only sex as a factor and we showed that males were more likely to have an obstruction than females in our cohort. There was no significant effect of smoking or stage/extent of *Toxoplasma* infection on the likelihood of being a lung "patient" (*i.e.* having COPD) in this sample set. While the sex relationship is clearly of interest in relation to lung disease, it does not have relevance to the *T. gondii* infection reported here. COPD has been linked to a higher risk in the male population, until more recently when it is now predicted that incidence in females will overtake that of males as a possible result of an increased proportion of female smokers in Western societies [43]. We recognise that there are limitations in our regression analyses and there were many potentially confounding parameters where no data were available. For example, most studies on *T. gondii* infection

include risk factors for infection. As no previous studies on lung cancer patients have revealed such striking prevalence levels as this study, there has been little reason to investigate parasitological parameters. In the future, detailed studies are required which involve more specific questions pertinent to the results presented here.

The high frequency of *T. gondii* infection in these lung cancer patients raises questions about whether the two conditions are linked. It is unlikely that there is a direct cause and effect linkage as there are no reported causative effects of *T. gondii* infection on producing cancers, as far as we are aware. However, many types of cancer can cause immunomodulatory effects on affected tissues and individuals [44] and *Toxoplasma* infection may also provoke a state of immunosuppression by affecting thymic related T-cell activity as systemic *Toxoplasma* infection triggers a long-term defect in the generation and function of naïve T-lymphocytes [5, 45–48]. Furthermore, pulmonary toxoplasmosis is generally considered to be rare in immunocompetent hosts [49], further supporting the idea that these patients are immunocompromised (at least locally within the lung tissue). Based on the observations reported in this article, a high proportion of lung cancer patients potentially could be at risk of acute infection or reactivation of chronic infection from *T. gondii*. This could lead to complications such as pulmonary toxoplasmosis, a serious condition causing a high mortality rate, which could seriously affect general wellbeing and interfere with treatment. Further research is required to establish the wider significance of these findings but in the meantime, we suggest that all lung cancer patients (and possibly patients with other cancers) should be considered at risk of *T. gondii* infection and, if necessary, monitored to prevent further complications during their treatments.

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