Original Article

# Multicentre Investigation of Prognostic Factors Incorporating p16 and Tumour Infiltrating Lymphocytes for Anal Cancer After Chemoradiotherapy

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## Abstract

*Aims:* Anal squamous cell carcinomas (ASCC) are strongly associated with human papillomaviruses. Standard of care is chemoradiotherapy at uniform doses with no treatment stratification. Immunohistochemical staining for p16INK4A (p16), a surrogate for human papillomaviruses, is prognostic for outcomes. We investigated this alongside clinical-pathological factors, including tumour infiltrating lymphocyte (TIL) scores.
*Materials and methods:* Using an independent, multicentre cohort of 257 ASCC treated with chemoradiotherapy, pretreatment biopsies were stained and scored for p16 and TIL. Kaplan—Meier curves were derived for outcomes (disease-free survival [DFS], overall survival and cancer-specific survival), by stage, p16 and TIL scores and Log-rank tests were carried out to investigate prognostic effect. A multivariate analysis was carried out using Cox regression.
*Results:* Stage, sex, p16 and TILs were independently prognostic. Hazard ratios for death (overall survival) were 2.51 (95% confidence interval 1.36—4.63) for p16 negative versus p16 positive, 2.17 (1.34—3.5) for T3/4 versus T1/2, 2.42 (1.52—3.8) for males versus females and 3.30 (1.52—7.14) for TIL1 versus TIL3 (all $P < 0.05$).
*Conclusions:* We have refined prognostic factors in ASCC. p16 adds to stratification by stage with respect to DFS in early disease and overall survival/DFS in locally advanced cancers. Our data support the role of the host immune response in mediating outcomes. These factors will be prospectively evaluated in PLATO (ISRCTN88455282).
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*Key words:* Anal cancer; anal squamous cell carcinoma; HPV; human papillomavirus; TILs; tumour infiltrating lymphocytes

## Introduction

Anal squamous cell carcinomas (ASCC) are relatively rare but are increasing in incidence [1]. They are strongly associated with high-risk subtypes of human papillomaviruses (HPV) [2] and share biological and clinical features with other HPV-associated neoplasms [3]. Phase III trials

determined chemoradiotherapy (CRT) with mitomycin and 5-fluorouracil as the standard of care (although notably with lower doses of radiotherapy than comparable head and neck or cervical cancers), replacing radical surgery and permanent colostomy as primary curative treatment.

Clinical and pathological features that are prognostic for inferior outcomes following CRT include primary tumour and lymph node stage, male gender [4], smoking, lack of concurrent chemotherapy and radiotherapy treatment intensity and compliance [5]. Within these parameters, however, there exists a spectrum of responses and a major

opportunity to improve prognostication through better characterisation of relevant biomarkers [6] and use this to develop strategies to improve outcomes.

In parallel with oropharyngeal cancer, where HPV involvement confers a significantly improved prognosis [7,8], HPV status has been investigated as a prognostic marker in ASCC. Single institution studies [9–12] show that HPV involvement or its surrogate – immunohistochemical staining for p16INK4A – are associated with improved outcomes and an individual patient data meta-analysis (eight studies/666 anal cancer patients) facilitated multivariate analysis where HPV/p16-positive status remained prognostic [13]. Specifically, negative p16 immunohistochemistry conferred a hazard ratio for relapse of 2.38 (95% confidence interval 1.64–3.33). Unlike oropharyngeal cancers, however, the vast majority of ASCC are HPV positive (81% were p16 positive in the meta-analyses), so the clinical utility of HPV/p16 is limited. Importantly, prior studies have not investigated the additional prognostic benefit of p16 immunohistochemistry to stage, i.e. in early or locally advanced disease.

The sensitivity of HPV-associated tumours to CRT may arise from their wild-type p53 status (p53 mutations are mutually exclusive with HPV involvement across the HPV-associated cancers [14]). An alternative is that host immune recognition (potentially driven by viral neoantigens) modulates the response to CRT and subsequent disease control. Consistent with this, pretreatment haematological parameters, such as neutrophil/lymphocyte ratios, have been shown to have prognostic value [15,16], and in previous single-centre studies a higher degree of tumour infiltrating lymphocytes (TIL) appeared to confer a strong beneficial effect [17,18]. Again, this mirrors the situation in oropharyngeal cancer, where TIL scores add prognostic value to p16/HPV status [19]. Specifically, Ward and co-workers [19] showed that patients with HPV-positive/TIL high tumours had excellent rates of disease control and survival, whereas HPV-positive but TIL low cancers conferred outcomes similar to HPV-negative cases.

In order to better understand the relationship between these clinical and tissue prognostic biomarkers, both to guide the analysis of clinical outcomes and frame scientific questions to determine their predictive value within randomised studies of treatment alteration, we investigated these factors (immunohistochemistry for p16 and scoring of TIL) and how they relate to clinical stage groupings and outcomes in an entirely separate cohort of patients treated with standard of care CRT from four UK centres.

## Materials and Methods

### Ethics and Data Collection

Ethical approval for this work was granted by the UK National Research Ethics Service (15/LO/0603). Data were retrieved from each institution using a standardised template, recording patient sex, T (1–4) and N (1/0) stage at presentation (patients with metastatic disease were excluded from the study), smoking status, confirmation of completion of CRT with date and status at point of censor (disease free, locoregional/metastatic relapse, death with cause and date of death). We followed the recommendations of Simon and co-workers [20] in the design and conform with the REMARK guidelines in reporting this study [21].

### p16 and Tumour Infiltrating Lymphocyte Scoring

Samples (formalin fixed and paraffin embedded) were retrieved under anonymous study numbers and reviewed by specialist pathologists (MW, KS, SB) to confirm the presence of invasive ASCC.

Slides were cut from a representative block from each tumour and stained for p16 using the automated Dako Autostainer Link 48 system. Staining for p16 was carried out using mouse antihuman monoclonal p16 antibody within the p16 CINtec Histology V.Kit (mtm laboratories AG, Heidelberg, Germany) with cervical cancer specimens as a positive control. Slides were categorised as p16 positive or negative by pathologists blinded to clinical outcomes. p16 was considered absent if <5% cells stained positive.

Slides stained with haematoxylin and eosin (H&E) were used to assess infiltration of lymphocytes into the tumours [19], scored by pathologists trained on slides from the previous study [17] and blinded to patient demographics or outcomes. Using a low-power magnification (× 2.5 objective), TILs were scored as high (3 – diffuse, present in >80% tumour/stroma), moderate (2 – patchy, present in 20–80% tumour/stroma) or low (1 – weak/absent, present in <20% tumour/stroma).

### Statistical Analysis

The cohort was characterised by clinical-pathological factors, specifically sex, smoking history, stage (TNM 2018) and early versus locally advanced (T1/2N0 representing early disease and T3/4 or node-positive locally advanced; groupings used to determine current radiotherapy doses and reflecting the design of the PLATO trial), immunohistochemical staining for p16 and TIL score. Correlation between factors was investigated using Pearson chi-squared tests.

A univariate Log-rank analysis to explore prognostic ability of these clinical and pathological factors was carried out with respect to the recent international consensus core outcome set for clinical trials of CRT interventions for anal cancer (CORMAC) [22]. Specifically, these were disease-free survival (DFS; defined by time to ASCC recurrence or death), overall survival and cancer-specific survival (CSS; defined by time to ASCC death). Outcomes at 2 and 5 years were described and hazard ratios reported. Survival curves were generated for DFS, CSS and overall survival by stage, p16 and TIL status. A multivariate analysis of all clinical pathological characteristics was carried out using Cox regression. A 5% significance level was used to declare statistical significance. No adjustments were made for multiple testing due to the hypothesis-generating nature of the research. All analyses were carried out in STATA.

# Results

## Demographics

The cohort of 257 ASCC patients was broadly representative of previous reports, with a median age of 58 (range 23–96) and a female preponderance (64% cases). Demographics for the whole cohort are shown in Table 1.

In total, 29/255 evaluable cases were p16 negative, i.e. 226 or 88.6% patients were p16 positive; 52/251 evaluable cases showed high levels of TIL, 123/251 scoring moderate and 76/251 low. p16 staining and TIL scores were independent of stage, with similar rates of p16-positive cases in both early (T1/2N0) and locally advanced (T3/4 or N+) cases. TIL scores were equally distributed between early and locally advanced tumours (Table 1). TIL groupings were similarly distributed to previous reports.

Significant correlations were seen between higher T stage (3/4 versus 1/2) and node positivity. Female patients were more likely to have higher T-stage tumours (chi-squared coefficient = 4.6, $P = 0.033$) and male patients more likely to have tumours that were p16 negative (chi-squared = 11.9, $P = 0.001$).

## Outcomes (Table 2, Figure 1)

DFS and overall survival rates were commensurate with results from the phase III ACT2 trial [23]. With a median follow-up of 42.8 months (interquartile range 28.4–62.4), 80/257 patients experienced relapse; the 2-year DFS was 75.0% (95% confidence interval 69.1–79.9) falling to 68.7% (95% confidence interval 62.3–74.2) by 5 years. Thirty-six patients relapsed at the primary site alone and a further 19 patients relapsed locoregionally (within the pelvis), of whom five had evidence of synchronous metastatic disease. Twenty-five patients presented with metastatic disease as their first site of recurrence. This mirrors recently published data characterising ASCC relapses following intensity-modulated radiotherapy [24]. The CSS for ASCC was 87.6% (95%

**Table 1**
Demographics and clinical pathological details

| Total | | | | n | % | | |
|---|---|---|---|---|---|---|---|
| | | | | 257 | | | |
| Age | | | Median 58 (range 23–86) | | | | |
| Sex | | | Male | 94 | 36.6 | | |
| | | | Female | 163 | 63.4 | | |
| Smoker | | | Smoker | 109 | 42.4 | | |
| | | | Non-smoker | 51 | 19.8 | | |
| | | | Unknown | 97 | 37.7 | | |
| Tumour stage | | | T1 | 23 | 8.9 | | |
| | | | T2 | 98 | 38.1 | | |
| | | | T3 | 88 | 34.2 | | |
| | | | T4 | 42 | 16.3 | | |
| | | | Unknown | 6 | 2.3 | | |
| Nodal stage | | | N0 | 147 | 57.2 | | |
| | | | N1 | 37 | 14.4 | | |
| | | | N2 | 45 | 17.5 | | |
| | | | N3 | 21 | 8.2 | | |
| | | | Unknown | 7 | 2.7 | | |
| Stage groupings | | | T1/2 N0 | 91 | 35.4 | | |
| | | | T3/4 or N+ | 162 | 63.0 | | |
| | | | Unknown | 4 | 1.6 | | |
| p16 immunohistochemistry | | | Positive | 226 | 87.9 | | |
| | | | Negative | 29 | 11.3 | | |
| | | | Unknown | 2 | 0.78 | | |
| TIL score | | | 1 | 76 | 29.6 | | |
| | | | 2 | 123 | 47.9 | | |
| | | | 3 | 52 | 20.2 | | |
| | | | Unknown | 6 | 2.3 | | |
| T1/2 N0 (total n = 91) | p16-positive | 80 | 87.9% | TIL 1 | 27 | 30.0% | |
| | p16-negative | 11 | 12.1% | TIL 2 | 44 | 48.9% | |
| | | | | TIL 3 | | 21.1% | |
| T3/4 or N+(total n = 162) | p16-positive | 142 | 88.8% | TIL 1 | 47 | 29.9% | |
| | p16-negative | 18 | 11.2% | TIL 2 | 78 | 49.7% | |
| | | | | TIL 3 | | 20.4% | |

**Table 2**
Outcomes (disease-free survival, cancer-specific survival and overall survival) in 257 anal squamous cell carcinomas treated with chemoradiotherapy by sex, stage, p16 status and tumour infiltrating lymphocyte scores

| | Disease-free survival | | | | | | Cancer-specific survival | | | | | | Overall survival | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2-year (95% CI) | 5-year (95% CI) | Univariate | | Multivariate | | 2-year (95% CI) | 5-year (95% CI) | Univariate | | Multivariate | | 2-year (95% CI) | 5-year (95%CI) | Univariate | | Multivariate | |
| | | | HR | P | HR | P | | | HR | P | HR | P | | | HR | P | HR | P |
| Overall | 75.0% (69.1−79.9) | 68.7% (62.3−74.2) | n/a | | | | 87.6% (82.7−91.1) | 78.8% (72.6−83.7) | n/a | | | | 81.6% (76.2−85.9) | 66.3% (59.5−72.2) | n/a | | | |
| Female | 80.7% (73.7−86.0) | 72.8% (64.9−79.2) | 1.62 (1.04−2.52) | 0.034 | 2.12 (1.30−3.45) | 0.002 | 93.0% (87.7−96.1) | 82.6% (74.9−88.1) | 1.90 (1.08−3.33) | 0.025 | 2.53 (1.36−4.71) | 0.003 | 91.2% (85.6−94.7) | 73.9% (65.4−80.6) | 2.16 (1.41−3.32) | <0.001 | 2.42 (1.52−3.86) | <0.001 |
| Male | 64.4% (53.3−73.5) | 61.6% (50.2−71.0) | | | | | 77.4% (66.9−85.0) | 71.9% (60.6−80.4) | | | | | 65.0% (54.2−73.8) | 52.9% (41.5−63.1) | | | | |
| T1/2 | 84.9% (77.1−90.2) | 82.1% (73.9−88.0) | 2.69 (1.65−4.39) | <0.001 | 2.27 (1.32−2.90) | 0.003 | 95.7% (90.0−98.2) | 90.0% (82.5−94.3) | 3.24 (1.68−6.27) | <0.001 | 2.90 (1.41−5.98) | 0.004 | 92.4% (85.8−95.9) | 77.4% (67.6−84.6) | 2.12 (1.34−3.37) | 0.001 | 2.17 (1.34−3.52) | 0.002 |
| T3/4 | 64.1% (55.9−72.8) | 57.2% (47.5−65.7) | | | | | 79.6% (71.1−85.9) | 69.7% (59.7−77.6) | | | | | 72.1% (63.4−79.1) | 57.6% (47.8−66.2) | | | | |
| N0 | 81.8% (74.5−87.3) | 79.0% (71.0−85.0) | 2.55 (1.61−4.06) | <0.001 | 2.27 (1.37−3.74) | 0.001 | 94.3% (89.0−97.1) | 86.3% (78.6−91.3) | 2.45 (1.35−4.43) | <0.001 | 2.14 (1.14−4.03) | 0.018 | 89.6% (83.4−93.6) | 71.7% (62.6−78.9) | 1.54 (0.98−2.42) | 0.060 | NS | |
| N+ | 65.2% (54.8−73.8) | 57.2% (46.5−66.5) | | | | | 78.4% (68.5−85.5) | 71.1% (60.3−79.4) | | | | | 72.6% (62.6−80.3) | 63.3% (52.7−72.2) | | | | |
| T1/2 N0 | 88.7% (80.0−93.8) | 87.5% (78.5−92.9) | 3.48 (1.92−6.33) | <0.001 | n/a† | | 97.8% (91.4−99.4) | 92.5% (83.9−96.6) | 3.70 (1.65−8.26) | <0.001 | n/a† | | 93.3% (85.6−96.9) | 75.9% (64.1−84.4) | 1.67 (1.03−2.71) | 0.038 | n/a† | |
| T3/4 or N+ | 67.1% (59.1−73.9) | 59.4% (51.0−67.0) | | | | | 81.8% (74.6−87.2) | 72.8% (64.4−79.5) | | | | | 75.8% (68.2−81.7) | 62.8% (54.2−70.2) | | | | |
| p16-positive | 78.2% (72.1−83.1) | 71.1% (64.3−76.8) | 2.30 (1.26−4.18) | 0.007 | 2.61 (1.37−4.94) | 0.003 | 89.7% (84.7−93.1) | 80.3% (73.7−85.3) | 2.09 (0.98−4.47) | 0.057 | 2.67 (1.15−6.15) | 0.021 | 84.5% (79.0−88.7) | 67.9% (60.6−74.2) | 2.15 (1.21−3.82) | 0.009 | 2.51 (1.36−4.63) | 0.003 |
| p16-negative | 49.0% (28.7−66.5) | 49% (28.7−66.5) | | | | | 72.1% (50.0−85.6)% | 67.6% (45.3−82.4) | | | | | 61.7% (41.5−76.7) | 53.7% (33.7−70.1) | | | | |
| TIL3 | 84.3% (71.0−91.8) | 84.3% (71.0−91.8) | | | | 0.0284* | 92.0% (79.9−96.9) | 92.0% (79.9−96.9) | | | | 0.0013* | 86.2% (73.2−93.2) | 81.6% (67.5−90) | | | | 0.0039* |
| TIL2 | 75.6% (66.8−82.4) | 67.6% (58.0−75.5) | 1.93 (0.94−3.99) | 0.075 | 2.43 (1.11−5.30) | | 89.6% (82.4−94.0) | 80.3% (71.2−86.8) | 2.49 (0.86−7.21) | 0.092 | 3.68 (1.08−12.54) | | 86.5% (78.9−91.5) | 70.7% (60.7−78.6) | 1.56 (0.77−3.14) | 0.216 | 2.04 (0.95−4.38) | |
| TIL1 | 69.5% (57.4−78.8) | 61.5% (48.9−71.9) | 2.53 (1.20−5.36) | 0.015 | 2.60 (1.16−5.82) | | 84.5% (73.7−91.1) | 69.3% (55.4−79.6) | 3.62 (1.23−10.63) | 0.02 | 4.91 (1.41−17.20) | | 74.7% (63.2−83) | 51.6% (38.6−63.2) | 2.66 (1.31−5.39) | 0.007 | 3.30 (1.53−7.14) | |

HR, hazard ratio (95% confidence interval).
*P-value combined across TIL1−3.
† Not applicable as T and N stage used as independent variables.
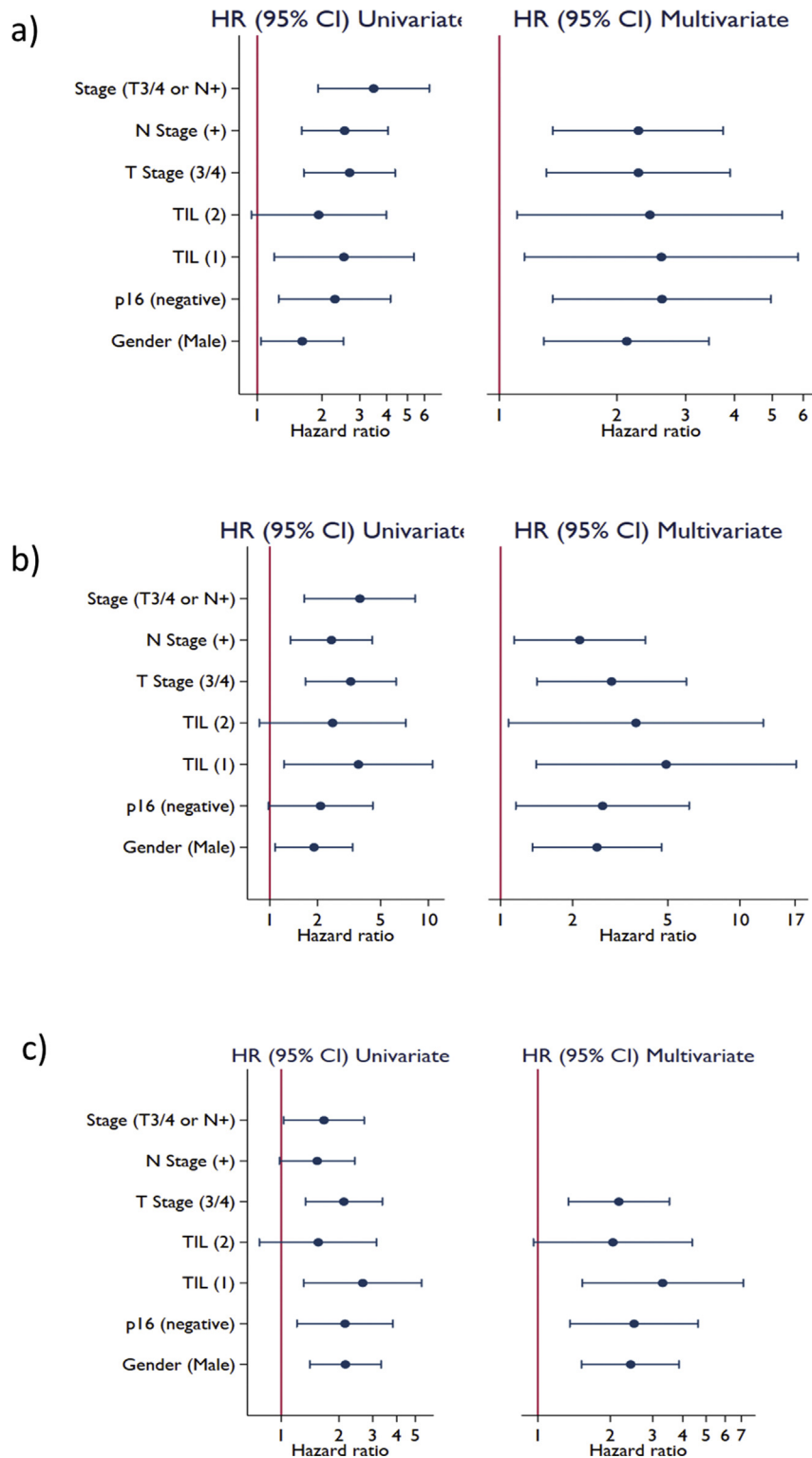
**Fig 1.** Forest plots of hazard ratios (and 95% Confidence Intervals) for univariate and multivariate analysis of prognostic factors in ASCC. a) Disease Free Survival, b) Cancer Specific Survival, c) Overall Survival.

confidence interval 82.7−91.1) at 2 years and 78.8% (95% confidence interval 72.6−83.7) at 5 years (overall 49/257 patients died of anal cancer). For the whole cohort, the overall survival was 81.6% (95% confidence interval

76.2−85.9) at 2 years and 66.3% (95% confidence interval 59.5−72.2) at 5 years; non-ASCC deaths included eight from second malignancies, nine from cardiovascular disease and 18 from a range of other causes.

*Outcomes and Clinical-Pathological Parameters (Table 2, Figure 2)*

Stage, sex, p16 status and TIL score all showed prognostic value with respect to DFS and overall survival (Log-rank $P <$ 0.05 on univariate analyses) as shown in Table 2 and Figure 2. Smoking status (recorded in 62% of cases) was not independently prognostic for outcomes ($P = 0.611$).

Patients with early stage (T1/2N0) cancers showed a higher relapse-free rate compared with those with locally advanced disease (88.7% versus 67.1% at 2 years; hazard ratio 2.69; 95% confidence interval 1.65−4.39). CSS was 97.8% at 2 years in early stage disease and 81.8% in locally advanced cases (92.5% versus 72.8% at 5 years; hazard ratio 3.24; 95% confidence interval 1.68−6.27). Two-year overall survival in patients with locally advanced disease was 75.8% as compared with 93.3% in T1/2N0 cases; overall survival at 5 years was 62.8% versus 75.9%. It is notable that of 23 deaths in the T1/2 N0 group, only seven were as a result of anal cancer.

Male patients had worse DFS than female patients (hazard ratio 1.62; 95% confidence interval 1.04−2.52). Male patients also had an increased risk of death (hazard ratio 2.16; 95% confidence interval 1.41−3.32).

Patients with p16-positive tumours had a 2-year relapse-free rate of 78.2%, as opposed to 49.0% in p16-negative cases (hazard ratio 2.30; 95% confidence interval 1.26−4.18). The effect of p16 was of borderline significance with respect to CSS, but the overall trend was conserved (2-year CSS 89.7% versus 72.1% in p16 positive versus p16 negative).

Significant differences in overall survival were also conferred by p16 status (2-year overall survival of 61.7% in p16-negative cases versus 84.5% when p16 positive).

The 2-year relapse-free rate for differing TIL scores was 84.3%, 75.6% and 69.5% for high, moderate and low TILs, respectively (hazard ratio 2.53, 95% confidence interval 1.2−5.36 for TIL1 versus TIL3; hazard ratio 1.93, 95% confidence interval 1.94−3.99 for TIL2 versus 3). Again, the effect on CSS did not reach statistical significance (CSS 92.0%/89.6%/84.5% in TIL high, moderate and low cases), although a significant effect on overall survival was seen (2-year overall survival ranging from 74.7% in TIL low tumours to 86.2% in those scoring TIL high).

*Multivariate Analyses of Clinical-Pathological Parameters*

In multivariate analysis for DFS using Cox regression, stage (T and N status), p16 status and TIL score all retained independent prognostic ability. Advanced stage disease conferred an increased risk for recurrence with a hazard ratio of 2.69 (95% confidence interval 1.65−4.39; $P = 0.003$) for T3/4 versus T1/2 and 2.55 (95% confidence interval 1.61−4.06; $P = 0.001$) for node positive versus negative cases. Male sex was also associated with increased risk of recurrence (hazard ratio 2.12, 95% confidence interval 1.30−3.44; $P = 0.002$). Negative staining for p16 showed an increased risk of recurrence (hazard ratio 2.61; 95% confidence interval 1.37−4.96; $P = 0.003$), as did lower levels of TILs (hazard ratio for disease event in TIL1 versus TIL3 2.60,
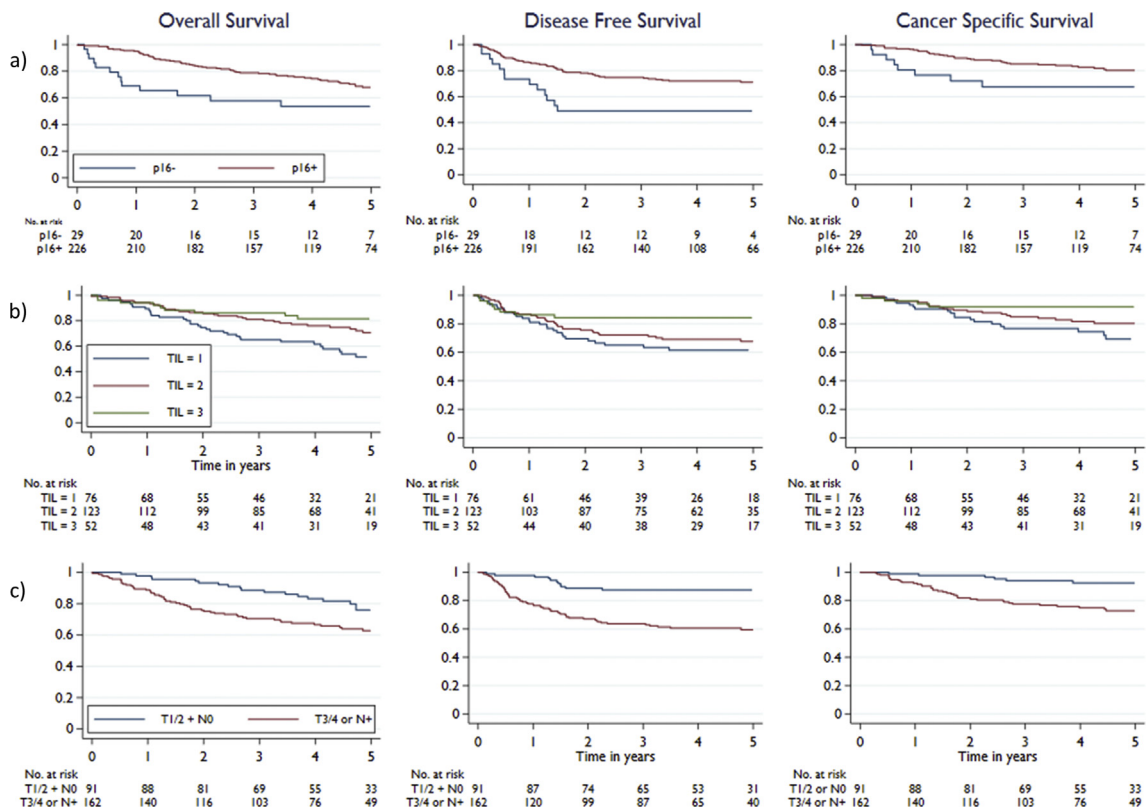


**Fig 2.** Outcomes (overall, disease free and cancer specific survival) in 257 ASCC treated with chemoradiotherapy presented by a) immunohistochemistry for p16, b) TIL = tumour infiltrating lymphocytes (1-3) and c) stage (T1/2 N0 vs T3/4 or N+).

95% confidence interval 1.15−5.82; hazard ratio for TIL2 versus TIL3 2.43, 95% confidence interval 1.11−5.30; $P = 0.028$ for TIL score overall).

In multivariate analysis, death from anal cancer was higher among individuals with more advanced disease stage (hazard ratio 2.90, 95% confidence interval 1.41−5.97 for stage T3/4 versus T1/2 and hazard ratio 2.14, 95% confidence interval 1.14−4.03 for node positive versus negative cases) and among men compared with women (hazard ratio 2.53, 95% confidence interval 1.36−4.71). Death from anal cancer was also associated with p16 status (p16 negative as compared with p16 positive, hazard ratio 2.67, 95% confidence interval 1.16−6.15; $P = 0.021$) and for TIL1/2 versus TIL3 (hazard ratio 2.49, 95% confidence interval 0.86−7.21 for TIL2 and 3.62, 95% confidence interval 1.23−10.63 for TIL1; overall $P = 0.001$.

Corresponding hazard ratios from multivariate analyses for death from any cause (overall survival) were 2.51 (95% confidence interval 1.36−4.63) for p16 negative versus positive cases, 2.17 (95% confidence interval 1.34−3.5) for T3/4 versus T1/2, 2.42 (95% confidence interval 1.52−3.8) for males versus females and 2.04 (95% confidence interval 0.95−4.38)/3.30 (95% confidence interval 1.52−7.14) for TIL2/1 versus TIL3 (all $P < 0.001$).

## Pathological Discrimination in Early or Locally Advanced Disease

In 91 patients with early stage disease (T1/2 N0), p16 status differentiated cases in terms of DFS (hazard ratio 7.58; 95% confidence interval 2.30−24.95; Figure 3a, Log-rank $P \leq 0.001$). This cohort of patients had a disproportionate number of non-anal cancer deaths and relatively few deaths attributable from anal cancer and as such p16 status was non-discriminatory for overall survival or CSS. With a small number of events, there was no additional
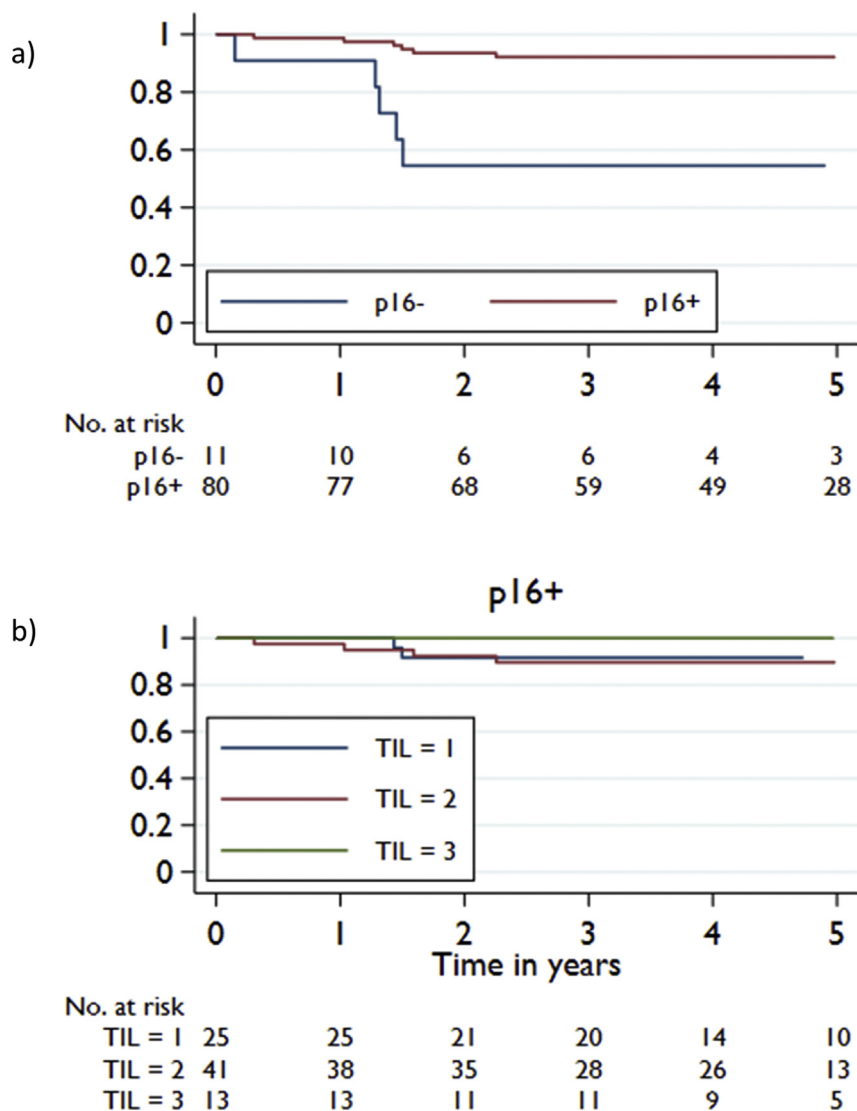


**Fig 3.** Disease free survival (DFS) in 91 patients with T1/2, N0 ASCC treated with chemoRT stratified by a) p16 (log rank p=0.0001) and then b) DFS stratified by TIL in those p16+ cases (log rank p=0.5719).

prognostic benefit in applying TIL scores to the p16-positive cases in early disease (Figure 3b, Log-rank p = 0.5719).

In locally advanced disease (160 evaluable cases) where deaths are predominately due to ASCC, p16 status was prognostic for overall survival (hazard ratio 2.58, 95% confidence interval 1.30−5.13; Figure 4a, Log-rank $P = 0.005$). Again, although the trend to improved outcomes in TIL3 patients persisted, with few events, TIL scores did not add further prognostic information to p16 status (Figure 4b, Log-rank p = 0.1448).

The independent prognostic ability of TIL scores does apply to cohorts defined by p16 status. Although by nature few in number, those p16-negative tumours with high levels of TIL (10/29 p16-negative tumours in our data) had 100% CSS (Figure 5a−c). Among the more numerous p16-positive tumours, TIL scores also conferred a statistically significant prognostic effect (Log-rank $P = 0.026$).

## Discussion

We assembled an independent multicentre cohort of UK patients with non-metastatic ASCC treated with radical CRT and investigated clinical-pathological factors with respect to pertinent outcomes. Key findings are the consistent prognostic effects of tumour stage (early stage tumours conferring improved outcomes), p16 status (p16-positive tumours responding better) and TIL score (high levels of TILs being associated with improved survival) with respect to outcomes. The size and multicentre nature of the cohort allowed us to combine these prognostic factors and identify
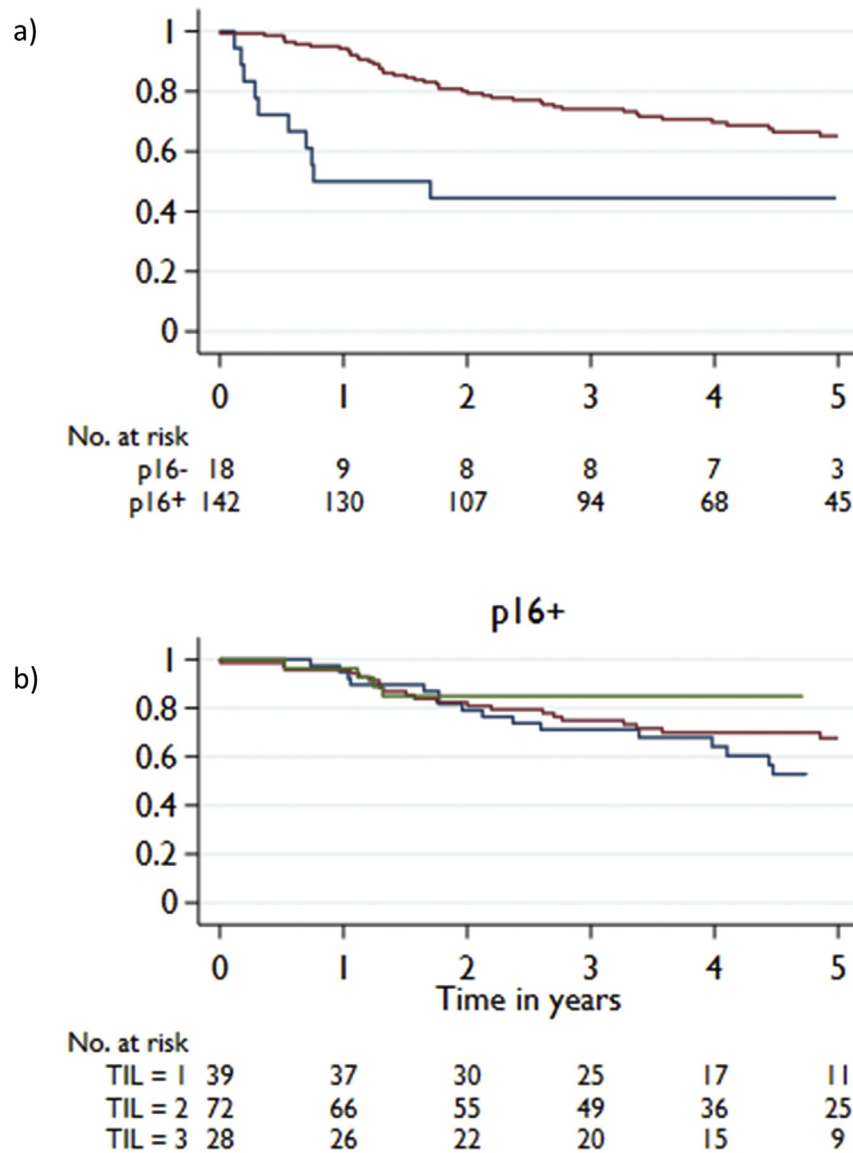


**Fig 4.** Overall survival in 162 T3/4 or N+ ASCC patients treated with chemoRT stratified by a) p16 (log rank p=0.0049) and then b) TIL in p16+ cases (p=0.1448).

a)

b)

c)
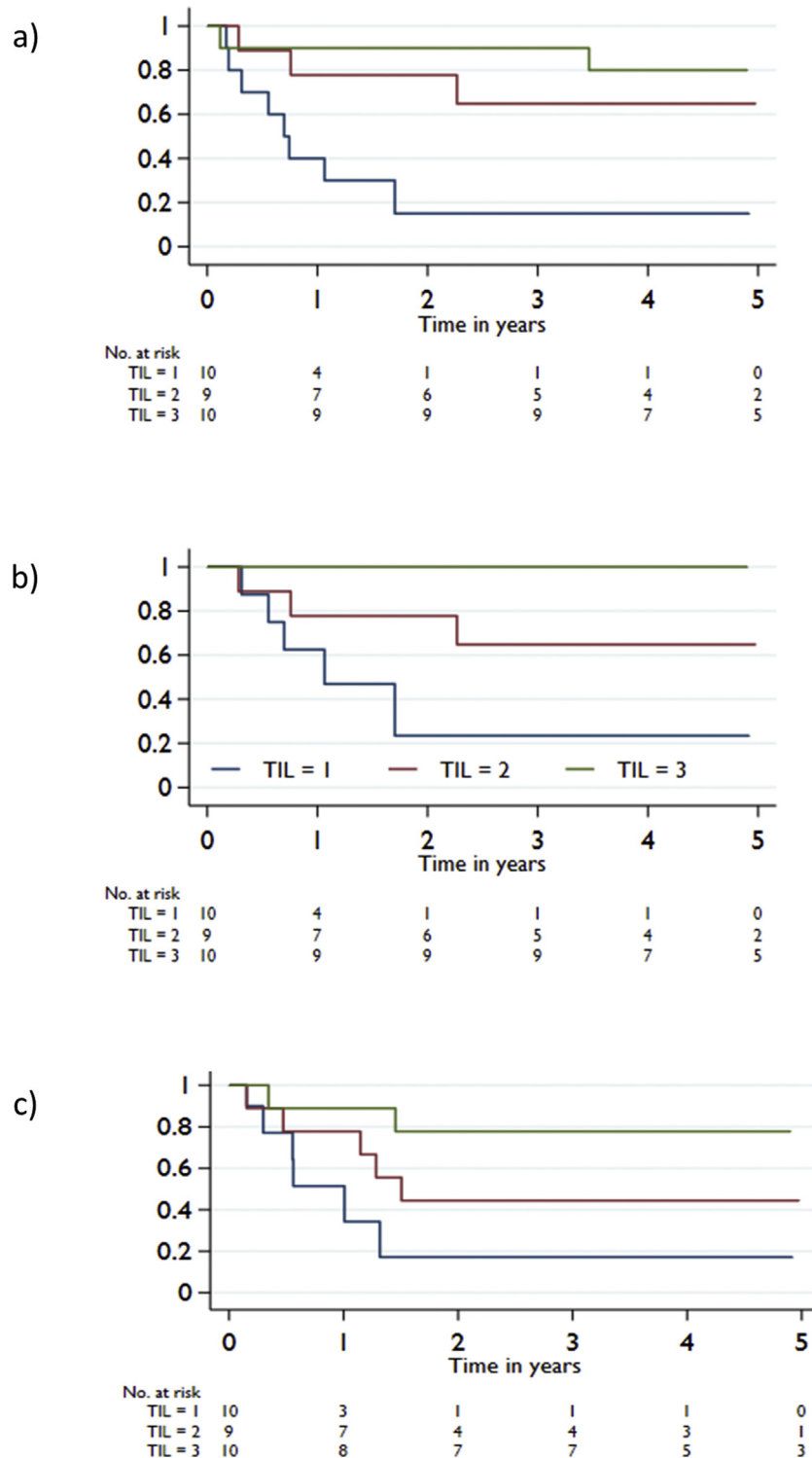


**Fig 5.** Kaplan–Meier curves for p16-negative cohort of anal squamous cell carcinoma (ASCC) patients ($n = 29$) stratified by tumour infiltrating lymphocyte (TIL) scores: (a) overall survival for p16-negative cohort of ASCC patients ($n = 29$) treated with chemoradiotherapy (Log-rank $P = 0.004$); (b) cancer-specific survival for p16-negative cohort of ASCC patients ($n = 29$) treated with chemoradiotherapy (Log-rank $P = 0.007$); (c) disease-free survival for p16-negative cohort of ASCC patients ($n = 29$) treated with chemoradiotherapy (Log-rank $P = 0.038$).

clinically relevant subgroups. Specifically, we have defined the role for p16 in terms of DFS (although not CSS or overall survival) in early stage disease, and in the stratification of all cancer outcomes in locally advanced disease. This finding is supported by recent data from the US National Cancer Database [25], where using a propensity score matched analysis, HPV status only conferred a significant benefit for overall survival in locally advanced cancers.

The independent prognostic role for TIL scores shown here is supported by data from a large Belgian study [26] and supports host lymphocyte recognition in meditating outcomes after CRT. Studies comparing the circulating components of the immune system also support the role of lymphocytes (as opposed to an inflammatory phenotype) in mediating outcomes. Leukocytosis (raised white cell count) is associated with increased risk of relapse [4,15,16]. The neutrophil:lymphocyte ratio is a strong negative prognostic factor, where higher neutrophils are associated with a worse outcome (and higher proportions of lymphocytes a greater likelihood of cure). The relationship between circulating immune cells and those within tumours was demonstrated [27] in a study of 79 patients with ASCC treated with CRT, where the peripheral blood differential counts mirrored the intra-tumoural findings. Pretreatment leukocytosis (total white cell count greater than the median) was associated with higher stage disease and lower infiltration of the tumour with CD8+ lymphocytes, which in itself was associated with an increased risk of relapse. CD8+ TIL levels were inversely correlated with the amount of peri-tumoural tumour-associated neutrophils.

To date, although clinical and pathological prognostic factors have been identified, they are not used to alter treatment. The highest level evidence for CRT as the standard of care utilises a defined curative dose of radiotherapy combined with chemotherapy for all patients. Building on the prognostic groupings identified here, the next step is to develop a multivariate prognostic nomogram that can be prospectively validated across the current generation of randomised trials in ASCC and assess the predictive role in novel treatment approaches.

The introduction of intensity-modulated radiotherapy [28,29] reduces acute toxicity and allows dose escalation without altering overall treatment time. The PLATO platform (ISRCTN88455282) includes two randomised trials that test dose alteration and provide the opportunity to validate prognostic biomarkers and study their predictive value [30]. ACT4 is a randomised phase II trial comparing standard versus reduced radiotherapy dose CRT in node-negative tumours <4 cm. ACT5 is a randomised phase III trial testing two radiotherapy dose escalation schedules versus standard dose CRT in locally advanced tumours. Integrated translational work is prospectively analysing tumours for HPV/p16 and TILs enabling, for the first time, an understanding of the predictive value of these biomarkers in the context of dose modulation.

HPV-associated cancers seem to have relatively high response rates to immune checkpoint inhibition [31]; PD1 inhibitors have efficacy in metastatic ASCC [32,33], PD1/PDL1 is associated with resistance to CRT in head and neck cancers [34] and phase I/II studies combining immune checkpoint inhibition with CRT are underway in ASCC, although further studies of disease biology are important to inform the optimal design. In particular, a detailed understanding of the interplay between immune checkpoint inhibition and the pre-existing tumour microenvironment will be key to patient selection, either around better determining outcomes following CRT or ideally predictive biomarkers of response to immune checkpoint inhibition.

The data share the limitations of retrospectively collected series of a relatively rare cancer. Non-cancer related deaths are frequent, in particular in the cohort of patients with early stage disease, representing comorbidities and societal factors that are disproportionately associated with ASCC. We used p16 as a surrogate for HPV involvement; it may be that alternative methods yield subtle differences in prognostic effect. Small numbers make subgroup analyses challenging, reinforcing the need for broad collaboration in this area of study and underlining the importance of prospectively collected PLATO data that will facilitate validation of p16 and TILs with respect to the range of pertinent outcomes.

In summary, we have shown the consistent prognostic effect of p16 immunohistochemistry and TIL scores in a novel cohort of patients with ASCC treated with CRT. Rates of p16+ and lymphocyte infiltration appear to be independent of tumour stage. Although patients with early tumours appear to do well with CRT, p16-negative cases do have higher rates of relapse. In locally advanced cases, the prognostic effect is more clearly seen and impacts on overall survival. Prospective randomised studies should determine whether dose escalation or additional therapies improve outcomes in this high-risk group and investigate a predictive role for these biomarkers in treatment intensification.

## Conflicts of Interest

The authors declare no conflicts of interest.

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