

# Is It Unnecessary to Assess Tumor Stroma-Infiltrating Lymphocytes in Localized Lung Adenocarcinomas?

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**Background:** During the last decade, more attention was paid to the tumor cell microenvironment, especially to tumor stroma-infiltrating lymphocytes (TILs). This study aimed to assess the prognostic impact of different TILs subpopulations and PD-L1 positive tumor cells in localized lung adenocarcinomas.

**Materials and Methods:** We conducted a retrospective descriptive study, which included localized adenocarcinomas diagnosed in the department of pathology and resected in the Thoracic Surgery Department of the same hospital between 2015 and 2020. TILs were analyzed using the immunohistochemical method for Fox-P3, CD4, CD8, CD20, and CD3. Besides, the PD-L1 antibody was used to assess tumor cell expression. Intra-tumoral and stromal labeled lymphocytes were quantified by manual counting at high magnification (X400). Fox-P3+/CD8+, Fox-P3+/CD4+, FoxP3+/PD-L1+, and CD8+/CD4+ ratios were subsequently calculated. The prognostic value of TILs was assessed with respect to overall survival (OS) and recurrence free survival (ReFS).

**Results:** A total of 44 localized adenocarcinomas were included. In the univariate analysis, the prognostic factors influencing OS included gender, adenocarcinoma subtype, Tumor-Infiltrating Lymphocyte (TIL) score, TIL grade, PD-L1 expression, PD-L1 grade, tumor immunity in the microenvironment, and the expressions of various immune markers: CD3, CD4, CD8, CD20, and FoxP3. The analysis also considered the FoxP3 ratio, FIL score, and different ratios involving immune markers, such as CD8/CD4 ratio, FoxP3/CD8 ratio, FoxP3/CD4 ratio, and FoxP3/PD-L1 ratio. The prognostic factors influencing the ReFS consisted of gender, the adenocarcinoma subtype, TIL score, TIL grade, PD-L1, PD-L1 grade, TIM, CD8 expression, CD20 expression, FIL score, Fox-P3/CD8 ratio, Fox-P3/CD4 ratio, and Fox-P3/PD-L1 ratio. Multivariate analysis revealed no independent predictive factors of OS or ReFS.

**Conclusion:** Despite the limitations of this study, the results highlighted that TILs may not represent an independent prognostic factor in localized adenocarcinomas and don't play a major role in comparison to the tumor stage.

**Keywords:** Tumor stroma-infiltrating lymphocytes; Adenocarcinomas; Lung cancer; Prognosis; Immunohistochemistry

## INTRODUCTION

Tumor stroma-infiltrating lymphocytes (TILs) are constant elements of the tumor microenvironment. They play a key role in the anti-tumor immune response. Their

prognostic impact has been reported in different cancers including lung cancer and induced the notion of immune score. Different methods of quantification have been reported in the literature without a real consensus. The

TILs interaction with tumor cells represents the target of immunotherapy which induced promising clinical results in limited groups of patients.

TILs consist of B lymphocytes and T lymphocytes. T lymphocytes are represented by cytotoxic lymphocytes, memory lymphocytes, or auxiliary lymphocytes. Fox-P3 T lymphocytes belong to the transcription factors family, "forkhead/winged-helix". They represent a T CD4+ regulatory lymphocytes sub-population accounting for 5 to 10% of the T lymphocytes. They play a key role in the inhibition of the majority of the immune cells including the T effectors lymphocytes. They interact directly with the tumor cells. They produce inhibiting molecules such as IL-10, TGF- $\beta$ , and adenosine. Regulatory T lymphocytes are essential in the immune tolerance phenomenon. The absence of regulatory T lymphocytes induced by the loss of FOXP3 is implicated in many auto-immune diseases (1-4).

Geng et al. reported that a high density of Tregs FOXP3+ in the tumor stroma was correlated to an unfavorable prognosis in lung cancer (5). Tao et al. reported that the accumulation of regulatory T lymphocytes in the tumor stroma of non-small cell lung cancers was significantly correlated to a decrease in overall survival and recurrence-free survival (6). The density of FOXP3 + TILs has been reported to be an independent prognostic factor of overall survival in multiple studies. CD8 receptor is expressed by natural killer, dendritic cells, and cytotoxic T lymphocytes. The latter plays a central role in the cellular immune response through cell mediation. They are implicated in the decrease of growth of many tumors and the decrease of the metastatic potential through a modification in the tumor stroma and the direct action on tumor cells.

In non-small cell lung carcinomas, the stromal density of CD8+ TILs have been reported as a prognostic marker. Numerous studies have reported that high levels of CD8 TILs was significantly associated to a better overall survival, progression-free survival, and recurrence-free survival. CD4+ T-lymphocytes activate CD8+ T-

lymphocytes and stimulate their proliferation. They play an important role in the anti-tumoral response by enabling CD8+ T-lymphocytes to penetrate the tumor and inhibit tumor angiogenesis. A significant correlation between the number of CD4+ T-lymphocytes and CD8+ T-lymphocytes has been reported in the different tumor compartments. High levels of intra-epithelial CD4 TILs have been correlated to better overall survival in patients with lung cancer. Zeng et al. reported that high levels of CD4 TILs were correlated to better overall survival in non-small cell lung carcinomas without a significant difference in recurrence-free survival (7).

PD-L1 lymphocytes have been reported to be associated with high-grade tumors with a low CD8 density without a correlation to overall survival or progression-free survival. Non-small cell lung carcinomas (NSCLC) with low PD-L1+/CD8 ratio have been reported to be associated with a high tumor grade and advanced tumor stages.

Many researchers reported a significant prognostic value of the ratios. Teng et al. reported better recurrence-free survival in patients with low FoxP3 /CD8 ratio (8). Jackute et al. reported that CD8+/FoxP3 and CD4+/CD8+ ratios were positively correlated to the overall survival with an impact on the density of CD4 + or CD8+ T lymphocytes alone (9). The major advantage of using ratios is their utility to highlight the collaboration between immune cells. Different methods have been described in the literature to quantify TILs including morphologic, immunohistochemical, or flow-cytometry-based methods. Morphological criteria have been initially used in invasive breast carcinomas (10). Immunohistochemical techniques have been reported using quantitative assessment (11,12) or semi-quantitative assessment (13). Manual assessment and digital one have also been reported (14,13).

The research question of this study was the assessment of the collaboration between Fox-P3, CD3, CD20, CD4, and CD20 TILs in localized lung adenocarcinomas. Our main objective was to assess the prognostic impact of different TILs subpopulations and PD-L1 positive tumor cells in

localized lung adenocarcinomas. Our secondary objectives consisted of quantifying Fox-P3, CD4, CD8, and CD20 TILs into the tumor stroma and evaluating the expression of PD-L1 in the adenocarcinomas.

## MATERIALS AND METHODS

### Patients and samples

This study included patients presenting localized adenocarcinomas (stage I or IIa) diagnosed in a pathology lab and resected in the thoracic surgery department of the same hospital during the period between 2015 and 2020. From the medical records, the authors retrieved patients' clinical data including gender, age, adenocarcinoma subtype, and follow-up information. Exclusion criteria consisted of incomplete data or cases with insufficient tumor material. Expert pathologists (FM, MM) re-reviewed hematoxylin-eosin-stained slides from all cases and corresponding formalin-fixed, paraffin-embedded specimens and counted the TILs after the immunohistochemical staining.

### Immunohistochemistry

All immunohistochemical staining were done on 5-um whole standard tissue sections. The different details concerning the CD4, CD8, CD3, CD20, PD-L1, and Fox-P3 antibodies used are represented in Table 1. All staining were performed on a Ventana Benchmark Ultra autostainer using the Ultra View diaminobenzidine kit (Ventana).

### TILs quantification

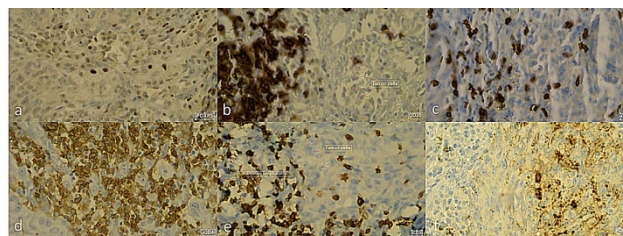
The authors performed a manual quantification of TILs. It was independently realized by 2 researchers (FM, MM) without taking into account the clinical records.

- TIL score: TIL score was assessed according to Giatromanolaki et al. method (15). For every slide, lymphocytes into the stroma were counted into the most representing 5 high power fields (x400). The reported density was the mean of the total number of lymphocytes. The TIL score was grade as: grade 1 (mean: 1-10 lymphocytes), grade 2 (mean: 10-70 lymphocytes), grade 3 (mean: 70-150 lymphocytes), and grade 4 (mean>150 lymphocytes).

**Table 1.** The different antibodies used in the study.

	Antibodies	Clone	Firm	Recommended dilutor/used	Internal marker	Staining
CD4	Anti-CD4	SP35	Ventana	1:50/ 1:50	-	cytoplasmic
CD8	Anti-CD8	SP57	Ventana	1:25/ 1:25	-	cytoplasmic
Fox-P3	Anti-Fox-P3	SP97	Spring	1:100/ 1:100	-	nuclear
CD20	Anti-CD20	SP34	Ventana	1:50/1:50	-	Cytoplasmic
PD-L1	Anti-PD-L1	-	Roche	1:50/1:50	Macrophages, tumor cells	Cytoplasmic

- PD-L1 expression assessment: It was measured quantitatively using an established immunohistochemistry assay (Ventana SP 263) which had been published. The stained sections were scores in five randomly selected areas containing tumor cells, which showed membranous and cytoplasmic staining (Figure 1). The percentage of positive tumor cells was graded on a scale of 0-2: 0 (<1%), 1 (1-5%), and 2 (>5%). The intensity of staining was scored as: 0 (no staining), 1 (weak staining), and 2 (moderate or strong staining).



**Figure 1.** The assessment of TILs in a case (ref 161): a/ Expression of Fox-P3 by TILs (x400), b/ expression of CD20 by TILs, the tumor cells are highlighted by arrows (x400), c/ expression of CD8 by TILs (x400), d/ expression of CD4 by TILs (x400), e/ expression of CD3 by TILs (x400), f/ expression of PD-L1 by the tumor cells (x400)

- Tumor immunity in the micro-environment: The assessment of the tumor immunity microenvironment was performed according to Gennen et al. (16). The tumors were categorized into: type I (PD-L1 - with no TILs indicating immune ignorance), type II (PD-L1 + with TILs implying adaptive immune resistance), type III (PD-L1-

with TILs suggesting the role of other suppressors), and type IV (PD-L1+ with no TILs indicating intrinsic induction).

- CD8, CD3, CD4, and CD20 quantification: The authors chose the method used by Dirican et al. (17). For every slide, positive cells into the stroma were counted into the most representing 5 high power fields (x400) (Figure 1). The reported density was the mean of the total number of positive cells. The percentage of positive TILs compared to the total number of nucleated cells in stromal compartments was assessed using the cut-off values published by Choe et al. as follows: low density ( $\leq 25\%$ ), intermediate density ( $>25\%$  to 50), and high density ( $>50\%$ ) (18).

- FoxP3 ratio assessment: The Fox-P3 ratio was assessed according to Giatromanolaki et al. method (15). The percentages of Fox-P3 expressing lymphocytes among the TILs were assessed in 5 high representative fields, in x40 optical fields and the mean score was used to define the Fox-P3 ratio for each case (Figure 1).

- FIL-score: As the Fox-P3 ratio doesn't take into account the distribution of Fox-P3+ lymphocytes, FIL-score was performed according to the method published by Giatromanolaki et al. (15). FIL-score was the product of TIL-score x Fox-P-3 ratio.

For every specimen, the ratios CD8+/CD4+, Fox-P3+/CD8+, Fox-P3+/CD4+, and Fox-P3+/PD-L1 were assessed.

### Statistical analysis

For the qualitative variables, absolute and relative frequencies were recorded. For quantitative variables, the means, medians, and standard deviations were recorded. Overall survival was defined as the period in the month between the date of diagnosis and the date of last news or death (the point date was 31 December 2022). The recurrence free survival (ReFS) was defined as the length of time between the date of the surgical resection and the date of progression. The progression of the disease was defined according to RECIST radiological criteria. All

patients who lost visits were contacted by phone. The analyzed factors consisted of: age, gender, adenocarcinoma subtypes, the TIL score, TIL grade, PD-L1 ratio, PD-L1 grade, H score, TIM, the CD3 TILs amount, the CD4 TILs amount, the CD20 TILs amount, the CD8 TILs amount, the Fox-P3 TILs amount, the Fox-P3 ratio, the FIL score, the ratios Fox-P3/CD8, Fox-P3/CD4, Fox-P3/PD-L1, and CD8/CD4 ratios.

The survival data was performed using the Kaplan-Meier method. Univariate analysis consisted in comparing survival curves using Log rank test. A multivariate logistic regression was used to identify the factors that were independently associated to the survival. After performing a univariate analysis, we assessed all factors with a significant degree inferior to 0.2 using a multivariate analysis. Statistical significance was considered only for  $p$  inferior to 0,05. We included factors with no significant importance ( $p < 0.2$ ) in the multivariate analysis in order to assess the maximum of factors, especially those which showed a prognostic tendency.

### Ethical consideration

Ethic approval was obtained from the ethic committee of the authors' hospital (ref:08/2022). Besides, all sensitive data were pseudonymized to respect the personal data. All the authors declare that they have no conflict of interest.

## RESULTS

### Patients' and samples' characteristics

During the period of study, 1900 non-small cell lung carcinomas (NSCLC) were diagnosed on biopsies and surgical specimen and 50 localized stage I or IIa adenocarcinomas were included according to our inclusion criteria. Six cases were excluded because of incomplete data. The 44 patients included consisted of 36 men (81.8%) and 8 women (18.2%). The sex ratio M/F reached 4.5. The mean age of the patients accounted for 63.32 years (median: 64, SD: 8,4). Surgical resection was performed in all cases.

The adenocarcinomas were classified as 17 (38.6%) solid adenocarcinomas, 9 (20.5%) papillary adenocarcinomas, 17 (38.6%) acinar adenocarcinomas, and 1 (2.3%) mucinous adenocarcinomas.

### Follow-up

The mean follow-up period reached 17.18 months (median: 64 months, SD: 8.4). Two patients died during the period of study. A tumor progression was noticed in 6 patients after a follow-up period of respectively 6, 49, 1, 12, 2, and 14 months.

### Survival

The overall mortality accounted for 4.5%. The mean ReFS was 61.76 months with extremes ranging from 43.41 to 80.109 months, 95%CI (43.4, 80.1).

### Infiltrating lymphocytes (TILs) assessment

TIL score: TIL score varied from 2 to 370/5HMF with a mean of 95.36 (SD=80).

TIL grade: Two cases (4.5%) were classified as grade 0, 20 (45.5%) cases were classified as grade 1, 14 (31.8) cases were classified as grade 2, and 8 cases (18.2%) were classified as grade 3.

CD3 expression: The mean of CD3 expression accounted for 98.15 (SD=66.88) with extremes ranging between 24 and 293/5HMF.

CD4 expression: CD4 expression by TILS varied from 0 to 406/5HMF with a mean of 72.5 (SD=74).

CD8 expression: The mean expression of CD8 by TILS reached 56.75 with extremes ranging from 0 to 406 (SD=35.6).

CD20 expression: The mean of CD20 expression accounted for 83.72 (SD=95.2) with extremes ranging from 0 to 470.

Fox-P3 expression: Fox-P3+ lymphocytes' amount varied from 0 to 260 lymphocytes/5 HMF with a mean of 31.65 (SD=52.16).

Fox-P3 ratio: The Fox-P3 ratio reached 19.94 (SD=21.33) and extremes ranging from 0 to 80.

FIL score: The mean FIL score reached 1929.22 (SD=893) with extremes ranging from 0 to 16458.

CD8/CD4 ratio varied from 0 to 49 with a mean of 2.07(SD=7.27).

Fox-P3/CD8 ratio varied from 0 to 5.39 with a mean of 0.63 (SD=1.15).

Fox-P3/CD4 ratio varied from 0 to 14 with a mean of 0.88(SD=2.24).

Fox-P3/PDL1 ratio mean reached 2.3 (SD=11.09) with extremes ranging from 0 to 72.

The comparison of the different means according to the histologic subtype revealed a statistically significant difference in Fox-P3 (p=0.018) ratio and FIL score (p=0.036)

### PDL-1 expression by tumor cells

PD-L1 grade 0 (<1%) was observed in 33 cases (75%). PD-L1 grade 1 (1-5%) was observed in 3 cases (6.8%), PD-L1 grade 2 (>5%) was observed in 8 cases (18.2%).

### TIM score

The team score was rated 1 (PD-L1 - with no TILs indicating immune ignorance) in 2 cases (4.5%), 2 (PD-L1 + with TILs implying adaptive immune resistance) in 11 cases (25%), and 3 (PD-L1- with TILs suggesting the role of other suppressors) in 31 cases (70.5%). No cases were scored 4 (PD-L1+ with no TILs indicating intrinsic induction).

### Prognostic study

Univariate analysis was performed to assess the prognostic impact of age, gender, histologic subtype, TIL score, TIL grade, PDL1 ratio, PDL1 grade, TIM, CD3 ratio, CD4 ratio, CD8 ratio, CD20 ratio, Fox-P3 expression, FIL score, CD8/CD4 ratio, Fox-P3/CD8 ratio, Fox-P3/CD4 ratio, and Fox-P3/PD-L1 ratio on the OS and the ReFS. A multivariate analysis was performed to assess prognostic factors revealed in the univariate study and those with a probability inferior to 0.2. The details of the univariate and multivariate analysis for the different factors are represented in Table 2.

In univariate analysis, the prognostic factors influencing the OS consisted in gender (p=0.038) (Figure 2a), adenocarcinoma subtype (p=0.05), TIL score (p=0.00) (Figure 2b), TIL grade (p=0.049) (Figure 2c), PDL1 expression (p=0.03) (Figure 2d), PDL1 grade (p=0.03) (Figure 2e), TIM (p=0.08), CD3 expression (p=0.00) (Figure 2f), CD4 expression (p=0.00) (Figure 2g), CD8 expression (p=0.00) (Figure 2h), CD20 expression (p=0.00) (Figure 3a), Fox-P3 expression (p=0.00) (Figure 3b), Fox-P3 ratio (p=0.00), FIL score (p=0.00) (Figure 3c), CD8/CD4 ratio (p=0.00) (Figure 3d), Fox-P3/CD8 ratio (p=0.00) (Figure

3e), Fox-P3/CD4 ratio (p=0.00) (Figure 3f), and Fox-P3/PDL1 ratio (p=0.03) (Figure 3g).

The prognostic factors influencing the ReFS consisted in the gender (p=0.027), adenocarcinoma subtype (p=0.105), TIL score (p=0.27), TIL grade (p=0.1), PDL1 (p=0.24), PD-L1 grade (p=0.024), TIM (p=0.24), CD8 expression (p=0.00), CD20 expression (p=0.00), FIL score (p=0.00), Fox-P3/CD8 ratio (p=0.00), Fox-P3/CD4 ratio (p=0.00), and Fox-P3/PD-L1 ratio (p=0.07). Multivariate analysis didn't reveal a prognostic factor associated with the ReFS or the overall survival.

**Table 2.** The univariate and multivariate analyses of the different factors assessed.

	Overall survival		Recurrence-free survival	
	Univariate analysis	Multivariate analysis	Univariate analysis	Multivariate analysis
Age	p=0.25	-	p=0.35	-
Gender	p=0.038	p>0.05	p=0.027	p=0.896
Histologic subtype	p=0.05	p>0.05	p=0.105	p=0.74
TIL score	p=0.00	p>0.05	p=0.2	p=0.64
TIL grade	p=0.049	p>0.05	p=0.1	p=0.85
PD-L1 expression	p=0.03	p>0.05	p=0.2	p=0.37
PD-L1 grade	p=0.03	p>0.05	p=0.024	p=0.55
TIM	p=0.08	p>0.05	p=0.2	p=0.9
CD3 ratio	p=0.00	p>0.05	p=0.35	-
CD4 ratio	p=0.00	p>0.05	p=0.354	-
CD8 ratio	p=0.00	p>0.05	p=0.00	p=0.96
CD20 ratio	p=0.00	p>0.05	p=0.00	p=1.2
Fox-P3 expression	p=0.00	p>0.05	p=0.38	-
FIL score	p=0.00	p>0.05	p=0.00	p=0.98
CD8/CD4	p=0.00	p>0.05	p=0.36	-
Fox-P3/CD8	p=0.00	p>0.05	p=0.00	p=0.8
Fox-P3/CD4	p=0.00	p>0.05	p=0.00	p=0.77
Fox-P3/PD-L1	p=0.03	p>0.05	p=0.07	p=0.823

Factors with no significant importance (p<0.2) in the multivariate analysis were included in order to assess the maximum of factors, especially those which showed a prognostic tendency.

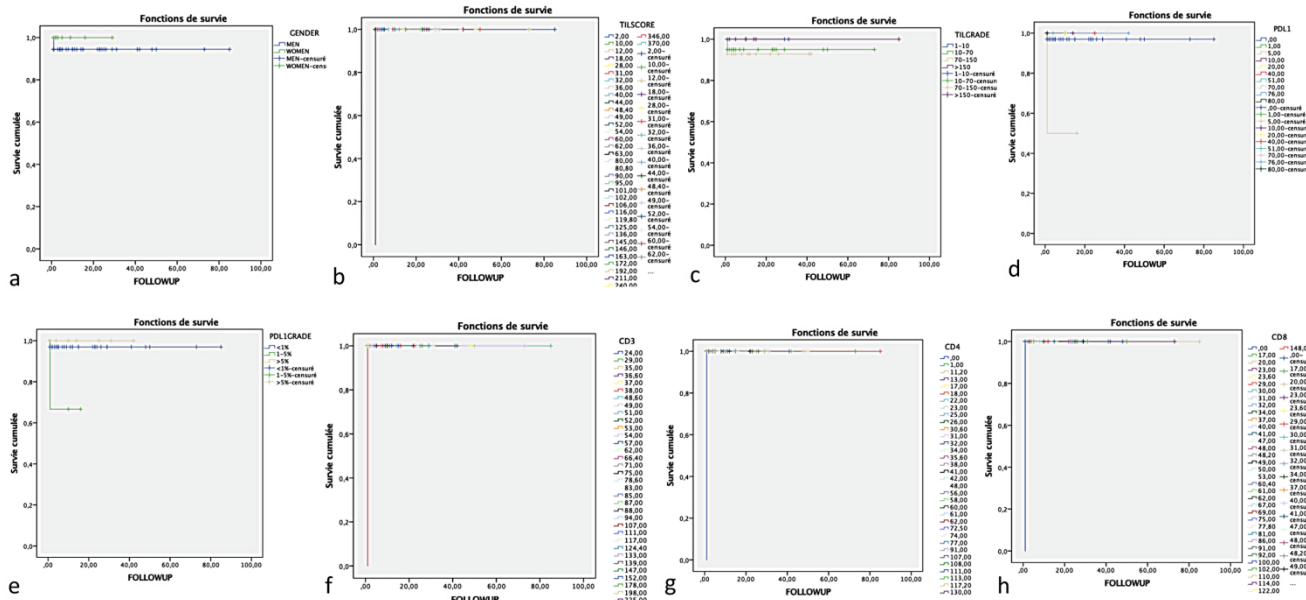


Figure 2. Survival curves of the different significant factors highlighted by the univariate analysis: a/ gender (p=0.038), b/ TIL score (p=0.00), c/ TIL grade (p=0.049), d/ PD-L1 expression (p=0.03), e/ PD-L1 grade (p=0.03), f/ CD3 expression (p=0.00), g/ CD4 expression (p=0.00), h/ CD8 expression (p=0.00)

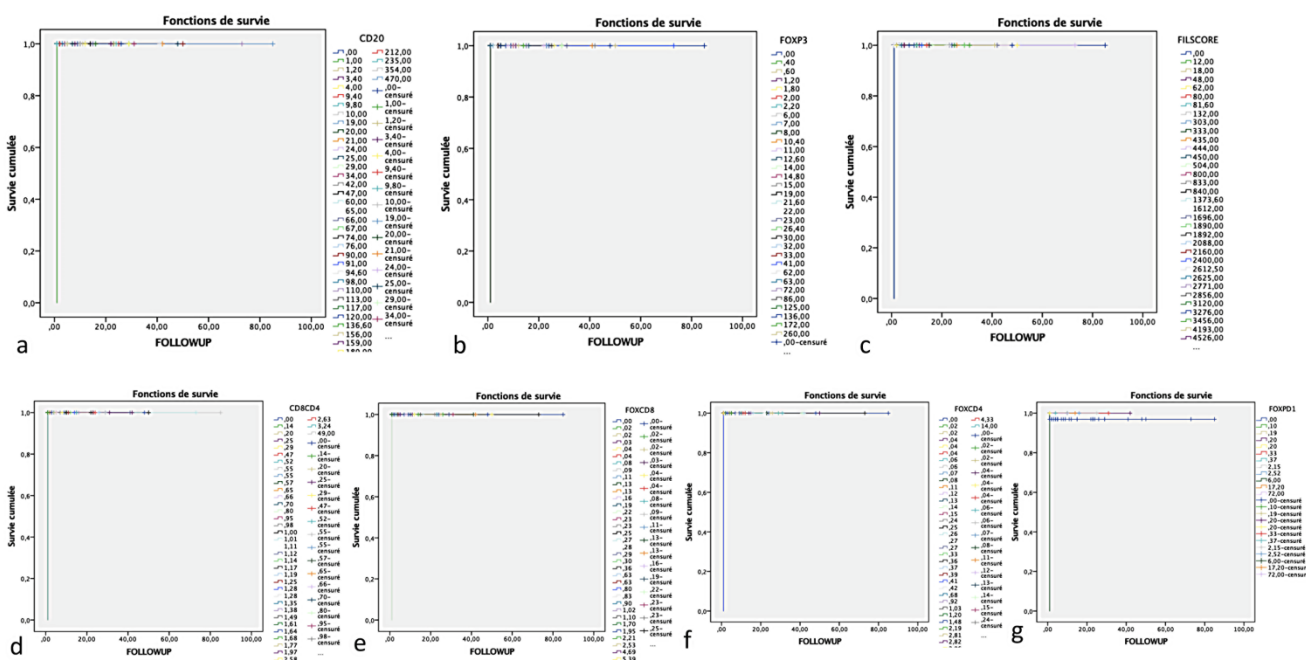


Figure 3. Survival curves of other different significant factors highlighted by the univariate analysis a/ CD20 expression (p=0.00), b/ Fox-P3 expression (p=0.00), c/ Filscore (p=0.00), d/ CD8/CD4 ratio (p=0.00), e/ Fox-P3/CD8 ratio (p=0.00), f/ Fox-P3/CD4 ratio (p=0.00), g/ Fox-P3/PD-L1 ratio (p=0.03).

## DISCUSSION

This was a descriptive retrospective study of 44 localized adenocarcinomas diagnosed in the department of pathology and followed in the department of Thoracic Surgery of the same hospital from 2015 to 2020. We aimed

to assess the prognostic impact of the different subpopulations of lymphocytes and to highlight their potential interactions in localized adenocarcinomas.

In univariate analysis, the prognostic factors influencing the OS consisted of gender, adenocarcinoma

subtype, TIL score, TIL grade, PD-L1 expression, PD-L1 grade, TIM, CD3 expression, CD4 expression, CD8 expression, CD20 expression, Fox-P3 expression, Fox-P3 ratio, FIL score, CD8/CD4 ratio, Fox-P3/CD8 ratio, Fox-P3/CD4 ratio, and Fox-P3/PD-L1 ratio. The prognostic factors influencing the ReFS consisted of the gender, the adenocarcinoma subtype, TIL score, TIL grade, PD-L1, PD-L1 grade, TIM, CD8 expression, CD20 expression, FIL score, Fox-P3/CD8 ratio, Fox-P3/CD4 ratio, and Fox-P3/PD-L1 ratio. Multivariate analysis revealed no independent predictive factors of OS or ReFS.

Mlika et al. performed a retrospective study assessing TILs in lung carcinomas (19). The authors used a quantitative analysis method quite similar to the method used in this study. They included 39 lung cancers consisting of adenocarcinomas (72%), squamous cell carcinomas (23%), typical carcinoid tumor (1 case), and large cell carcinoma (1 case). Thirty-two patients (82%) were in stages III or IV. Univariate analysis revealed complete surgical resection and CD8/CD4 ratio as prognostic factors for OS and complete surgical resection, the Fox-P3/CD8 and Fox-P3/CD4 ratios as prognostic factors for ReFS. In multivariate analysis, age and CD8/CD4 ratio were independent predictors of OS (19). In a patient's group identified as "Lung Adjuvant Cisplatin Evaluation Biomarker", Brambilla and colleagues reported the prognostic impact of an important TILs infiltration on overall survival and ReFS (20). Geng et al. reported a negative correlation between the density of stromal Tregs Fox-P3 positive lymphocytes and the prognosis (5). The prognostic impact on OS of Tregs has been reported by many other authors including Tao et al. (1, 6).

TILs CD8 positive lymphocytes have been reported to be correlated to a better OS (5, 7, 8, 21–23). Because of the prognostic impact of CD8 TILs, especially stromal TILs, some authors advocated the necessity of adjuvant chemotherapy for patients presenting stage I NSCLC tumors with low CD8 TILs (8,13).

CD4 TILs have been reported to play a prognostic role in association with CD8 TILs (9). Zeng et al. reported a

positive correlation between CD4 TILs and the OS (7). Teng et al. reported a better RFS in patients with low Fox-P3 and high CD8 TILs even if they didn't report a prognostic impact of Fox-P3 TILs alone (8).

The assessment of the different ratios of TILs aimed to highlight their collaboration such as the positive correlation between CD8/Fox-P3 and CD4/CD8 ratios reported by Jackute et al. (9) or the correlation of the CD8/Fox-P3 to a good response to cisplatin in advanced NSCLC reported by Liu et al (24).

The difference in the results observed between our study and those mentioned in the literature may be explained by the low number of localized cases included in these studies in addition to the multiplicity of the microscopic subtypes assessed or the different methods used to assess TILs. A consensus was published concerning the availability of quantification of TILs in hematoxylin and eosin stain slides without a limited cut-off (10). Three main groups of TILs are identified: intra-tumoral, stromal, and peripheral TILs situated in tertiary lymphoid structures. In this study, intra-tumoral and stromal lymphocytes were analyzed. The majority of the authors who reported the prognostic impact of TILs in NSCLC didn't focus on their distribution and the impact of the TILs localization remains to be established (20, 25).

Two methods were mainly used to quantify TILs in NSCLC: a semi-quantitative method (25, 26) with different cut-offs to define low or high TILs levels (10) and a quantitative method, similar to the one we used, with manual or numerical techniques (2–4, 12, 27). Besides, even if our results couldn't rule out the prognostic impact of TILs in localized adenocarcinomas, they highlight the relevance of the tumor stage as a prognostic factor.

The main limits of this study were its retrospective nature and the limited number of patients included and the events assessed.

## CONCLUSION

Our study aimed to assess the prognostic impact of the different TILs subpopulations in localized

adenocarcinomas. Multivariate analysis didn't reveal prognostic factors interfering with OS or ReFS. Despite the limitations of this study, it was revealed that in localized adenocarcinomas, TILs assessment doesn't play a key role as an independent prognostic factor in comparison to the tumor stage.

## REFERENCES

- Hasegawa T, Suzuki H, Yamaura T, Muto S, Okabe N, Osugi J, et al. Prognostic value of peripheral and local forkhead box P3<sup>+</sup> regulatory T cells in patients with non-small-cell lung cancer. *Mol Clin Oncol* 2014;2(5):685-694.
- Ghiringhelli F. Surveillance immune antitumorale et échappement. *Correspondances en Onco-Théragnostie* 2013; 1:6-10.
- Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005;22(3):329-41.
- Sakaguchi S. The origin of FOXP3-expressing CD4<sup>+</sup> regulatory T cells: thymus or periphery. *J Clin Invest* 2003;112(9):1310-2.
- Geng Y, Shao Y, He W, Hu W, Xu Y, Chen J, et al. Prognostic Role of Tumor-Infiltrating Lymphocytes in Lung Cancer: a Meta-Analysis. *Cell Physiol Biochem* 2015;37(4):1560-71.
- Tao H, Mimura Y, Aoe K, Kobayashi S, Yamamoto H, Matsuda E, et al. Prognostic potential of FOXP3 expression in non-small cell lung cancer cells combined with tumor-infiltrating regulatory T cells. *Lung Cancer* 2012;75(1):95-101.
- Zeng DQ, Yu YF, Ou QY, Li XY, Zhong RZ, Xie CM, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes for clinical therapeutic research in patients with non-small cell lung cancer. *Oncotarget* 2016;7(12):13765.
- Teng F, Meng X, Wang X, Yuan J, Liu S, Mu D, et al. Expressions of CD8<sup>+</sup>TILs, PD-L1 and Foxp3<sup>+</sup>TILs in stage I NSCLC guiding adjuvant chemotherapy decisions. *Oncotarget* 2016;7(39):64318-64329.
- Jackute J, Zemaitis M, Pranys D, Sitkauskienė B, Miliauskas S, Bajoriunas V, et al. The prognostic influence of tumor infiltrating Foxp3(+)CD4(+), CD4(+) and CD8(+) T cells in resected non-small cell lung cancer. *J Inflamm (Lond)* 2015;12:63.
- Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, et al. Assessing Tumor-Infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method from the International Immunology Oncology Biomarkers Working Group: Part 2: TILs in Melanoma, Gastrointestinal Tract Carcinomas, Non-Small Cell Lung Carcinoma and Mesothelioma, Endometrial and Ovarian Carcinomas, Squamous Cell Carcinoma of the Head and Neck, Genitourinary Carcinomas, and Primary Brain Tumors. *Adv Anat Pathol* 2017;24(6):311-335.
- Ilie M, Hofman V, Ortholan C, Bonnetaud C, Coëlle C, Mouroux J, et al. Predictive clinical outcome of the intratumoral CD66b-positive neutrophil-to-CD8-positive T-cell ratio in patients with resectable nonsmall cell lung cancer. *Cancer* 2012;118(6):1726-37.
- Alifano M, Mansuet-Lupo A, Lococo F, Roche N, Bobbio A, Canny E, et al. Systemic inflammation, nutritional status and tumor immune microenvironment determine outcome of resected non-small cell lung cancer. *PLoS One* 2014;9(9):e106914.
- Kilvaer TK, Paulsen EE, Andersen S, Rakaee M, Bremnes RM, Busund LR, et al. Digitally quantified CD8<sup>+</sup> cells: the best candidate marker for an immune cell score in non-small cell lung cancer? *Carcinogenesis* 2020;41(12):1671-1681.
- Galon J, Pagès F, Marincola FM, Angell HK, Thurin M, Lugli A, et al. Cancer classification using the Immunoscore: a worldwide task force. *Journal of Translational Medicine* 2012;10:1-0.
- Giatromanolaki A, Banham AH, Harris AL, Koukourakis MI. FOXP3 infiltrating lymphocyte density and PD-L1 expression in operable non-small cell lung carcinoma. *Exp Lung Res* 2019;45(3-4):76-83.
- Gennen K, Käsmann L, Taugner J, Eze C, Karin M, Roengvoraphoj O, et al. Prognostic value of PD-L1 expression on tumor cells combined with CD8<sup>+</sup> TIL density in patients with locally advanced non-small cell lung cancer treated with concurrent chemoradiotherapy. *Radiat Oncol* 2020;15(1):5.

17. Dirican N, Karakaya YA, Gunes S, Daloglu FT, Dirican A. Association of intra-tumoral tumour-infiltrating lymphocytes and neutrophil-to-lymphocyte ratio is an independent prognostic factor in non-small cell lung cancer. *Clin Respir J* 2017;11(6):789-796.
18. Choe EA, Cha YJ, Kim JH, Pyo KH, Hong MH, Park SY, et al. Dynamic changes in PD-L1 expression and CD8<sup>+</sup> T cell infiltration in non-small cell lung cancer following chemoradiation therapy. *Lung Cancer* 2019;136:30-36.
19. Mlika M, Saidi A, Mejri N, Abdennadher M, Haddouchi C, Labidi S, et al. Prognostic impact of tumor-infiltrating lymphocytes in non-small cell lung carcinomas. *Asian Cardiovasc Thorac Ann* 2022;30(2):177-184.
20. Brambilla E, Le Teuff G, Marguet S, Lantuejoul S, Dunant A, Graziano S, et al. Prognostic Effect of Tumor Lymphocytic Infiltration in Resectable Non-Small-Cell Lung Cancer. *J Clin Oncol* 2016;34(11):1223-30.
21. Chen B, Li H, Liu C, Xiang X, Wang S, Wu A, et al. Prognostic value of the common tumour-infiltrating lymphocyte subtypes for patients with non-small cell lung cancer: A meta-analysis. *PLoS One* 2020;15(11):e0242173.
22. Hao J, Wang H, Song L, Li S, Che N, Zhang S, et al. Infiltration of CD8<sup>+</sup> FOXP3<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and FOXP3<sup>+</sup> T cells in non-small cell lung cancer microenvironment. *Int J Clin Exp Pathol* 2020;13(5):880-888.
23. Yang Y, Yang X, Wang Y, Xu J, Shen H, Gou H, et al. Combined Consideration of Tumor-Associated Immune Cell Density and Immune Checkpoint Expression in the Peritumoral Microenvironment for Prognostic Stratification of Non-Small-Cell Lung Cancer Patients. *Front Immunol* 2022;13:811007.
24. Liu H, Zhang T, Ye J, Li H, Huang J, Li X, et al. Tumor-infiltrating lymphocytes predict response to chemotherapy in patients with advance non-small cell lung cancer. *Cancer Immunol Immunother* 2012;61(10):1849-56.
25. Donnem T, Kilvaer TK, Andersen S, Richardsen E, Paulsen EE, Hald SM, et al. Strategies for clinical implementation of TNM-Immunoscore in resected nonsmall-cell lung cancer. *Ann Oncol* 2016;27(2):225-32.
26. Al-Shibli K, Al-Saad S, Donnem T, Persson M, Bremnes RM, Busund LT. The prognostic value of intraepithelial and stromal innate immune system cells in non-small cell lung carcinoma. *Histopathology* 2009;55(3):301-12.