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# Absolute eosinophil count predicts clinical outcomes and toxicity in non-small cell lung cancer patients treated with immunotherapy

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ABSTRACT

*Objectives:* Immune checkpoint inhibitors (ICIs) have led to a paradigm shift in non-small cell lung cancer (NSCLC) treatment. We investigated absolute eosinophil count (AEC) as a predictor of clinical outcomes and toxicity in NSCLC patients receiving ICIs.

*Materials and Methods:* AEC was retrospectively collected at baseline and during treatment from 158 advanced NSCLC patients treated with single agent anti-PD1/anti-PDL1 monoclonal antibody in first or subsequent line of therapy at Medical Oncology Unit, Careggi University Hospital, Florence (Italy), between January 2016 to October 2020.

*Results:* We found a significant association between high baseline AEC (≥130/μL) and better clinical outcomes. The response rates were 64.4% and 35.6% for patients with high and low AEC, respectively ($p = 0.009$). The high-AEC group showed a significantly longer PFS and OS than the low-AEC group (mPFS = 7.0 months, 95% CI 5.0–10.0 vs 2.5 months, 95% CI 2.0–4.0, $p = 0.007$ and mOS = 9.0 months, CI 95% 7.0–15.0 vs 5.5 months, 95% CI 4.0–8.0, $p = 0.009$, respectively). An increased risk of immune-related adverse events (irAEs) was reported in the high-AEC group ($p = 0.133$). IrAEs resulted an independent prognostic factor for both better outcomes (mPFS = 8.0 months, 95% CI 7.0–12.0 vs 2.0 months, 95% CI 2.0–3.0, $p<0.001$; mOS = 13.0 months 95% CI 9.0–19.0 vs 4.0 months 95% CI 3.0–6–0, $p<0.001$) and response to ICIs (response rate = 33.8% vs 14.9%, disease control rate = 72.0% vs 32.1%, $p<0.001$).

*Conclusion:* High baseline AEC value (≥130/μL) is a predictive biomarker of clinical benefit and irAEs occurrence in NSCLC patients treated with ICIs.

## 1. Introduction

Immunotherapy has been a major breakthrough in cancer treatment in the last decades. [1,2] The introduction of immune checkpoint inhibitors (ICIs), targeting the programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) axis, into clinical practice has remarkably changed the treatment perspective in several types of cancers, including non-small cell lung cancer (NSCLC). [3,4] Though NSCLC patients can achieve a significant improvement in clinical outcomes from ICIs, both in first and subsequent lines of treatments, the benefit of

*Abbreviations:* ICIs, immune checkpoint inhibitors; PD1/PD-L1, programmed death-1/programmed death-ligand 1; NSCLC, non-small cell lung cancer; irAEs, immune-related adverse events; TILs, tumor-infiltrating lymphocytes; TATE, tumor-associated tissue eosinophilia; AEC, absolute eosinophil count; CR, complete response; PR, partial response; SD, stable disease; PD, progression disease; ORR, Objective Response Rate; DCR, Disease Control Rate; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; TME, tumor microenvironment; TAMs, tumor-associated macrophages.

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immunotherapy is still limited to a subgroup of patients. [4,5] Moreover, patients treated with ICIs may develop a peculiar spectrum of toxicities, namely immune-related adverse events (irAEs) which may lead to discontinuation of treatment and increase of mortality. [6,7] No well-defined clinical and biological markers of response to ICIs or associated with the development of irAEs have been identified so far. Therefore, the study of routinely available biomarkers that may predict response and toxicity to immunotherapeutic agents is a very expanding area of active research. [4,8,9]

Among several biomarkers evaluated as predictor of response to ICIs, the greatest focus has been on PD-L1 expression, both in pre-clinical and clinical settings. PD-L1 expression on tumor cells and tumor-infiltrating immune cells has been shown to correlate with the efficacy of anti-PD-1/PD-L1 agents. [10,11] However, only a limited number of patients with PD-L1–expressing tumors obtain durable response and also patients without PD-L1 staining can achieve clinical benefit from ICI treatment. [12] Moreover, a universal method for detecting and measuring the PD-L1 status has not yet been standardized. [13] In the past few years, new data on potentially predictive biomarkers of response to immunotherapy have emerged, pointing out the existence of additional biological factors, which may define the subgroup of patients who are most likely to benefit from ICIs. Examples include tumor mutational or neoantigen burden, immune gene signatures, tumor-infiltrating lymphocytes (TILs) and peripheral blood markers. [9] On the other hand, the development of predictive factors is also necessary to minimize the risk of immune-toxicities in patients receiving immunotherapy. Recent clinical observations have suggested that peripheral blood cell counts can also be used as biomarkers to predict emergence of irAEs during treatment with ICIs. [8,14]

Eosinophil count is a reliable and available peripheral blood-based parameter as part of the routine comprehensive blood examination performed for all patients in daily clinical practice prior and during cancer treatments, including immunotherapy. The accumulation of eosinophils both in the peripheral blood and in the tumor tissue, defined tumor-associated tissue eosinophilia (TATE), were reported to be prognostic markers for a better clinical outcome for cancer patients treated with ICIs. [15] The role of eosinophilia as independent predictor of favorable outcomes in cancer patients, has initially emerged from studies in patients with melanoma. The development of eosinophilia during the course of the disease is associated with a longer survival in melanoma patients, independent of the therapy. [16,17] An increase in absolute eosinophil count (AEC) in peripheral blood of melanoma patients treated with immunotherapy is a predictor of response to ICIs and also correlates with significantly prolonged overall survival (OS). [16,18–21] Recent evidence has shown that as well as being evaluated as a potential biomarker of response, eosinophil count can be linked also to the development of irAEs in different solid tumors. [14,22–24]

To date, few studies have investigated the role of AEC as a potential marker for clinical outcome and immune-related toxicities development in patients with NSCLC treated with ICIs. [23,25,26] The aim of the present study was to determine the correlation between peripheral blood AEC and disease clinical outcomes and emergence of irAEs, in a real-world population of patients with advanced NSCLC treated with ICIs. In addition, by monitoring changes in AEC during treatment, we analyzed the association between eosinophils longitudinal trend and response to therapy.

## 2. Materials and methods

### 2.1. Patients

In this single-center observational study we retrospectively collected data from medical records of patients with advanced or recurrent NSCLC (stage IIIB-IV disease, according to the VII edition of the TNM staging system), who were treated with single agent anti-PD1/PD-L1 monoclonal antibody at Medical Oncology Unit, Careggi University Hospital, Florence (Italy), between January 2016 to October 2020. All patients enrolled in the analysis received anti-PD1/PD-L1 agent (i.e. pembrolizumab, nivolumab and atezolizumab) as monotherapy in the first or subsequent lines of treatment as per clinical practice. Treatment was continued until disease progression, intolerable toxicity, clinical decision or patient refusal.

Patients' responses to ICIs were assessed with radiological imaging every three months, according to clinical practice. The radiological complete response (CR), partial response (PR), stable disease (SD), and progression disease (PD) were defined in accordance with the Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1. The measured clinical outcomes were the following: Objective Response Rate (ORR, defined as CR plus PR) and Disease Control Rate (DCR, defined as the sum of patients who have achieved CR or PR or SD), the Progression-Free Survival (PFS) and the Overall Survival (OS). The cut-off date for follow-up data was 30 April 2021.

### 2.2. Data collection

The following characteristics were reviewed from each patient's medical record: a) clinical-demographic data, i.e. age, sex, Eastern Cooperative Oncology Group Performance Status (ECOG PS), smoking status, NSCLC histological subtype, mutational status and PD-L1 expression levels in tumoral tissue samples; b) information about treatment: type of ICIs, therapy line in which ICI was administered, treatment duration, best response and clinical outcomes; c) data about irAEs: type of irAE developed and severity of toxicity, according to CTCAE (Common Terminology Criteria for Adverse Events) version 4.03; and d) data about AEC evaluated in the peripheral blood of patients. In detail, peripheral-blood AEC (n° of cells/μL) was recorded for each patient at baseline (prior to immunotherapy treatment) after the first and the second administration of ICIs and at week 12 and week 24 of treatment. In addition, AEC was collected at the PD (when available) and at occurrence of any irAE. The median AEC (mAEC) value at baseline of 130/μL (0.130 cells $\times$ $10^9$/L) was selected as the cutoff value to divide patients into high and low AEC groups.

### 2.3. PD-L1 analysis

PD-L1 expression was analysed at the Histopathology and Molecular Pathology Units at Careggi University Hospital, Florence. Analysis was performed using the VENTANA PD-L1 (SP263) Assay. Expression of PD-L1 was assessed by tumor proportion score (TPS) that was defined as the percentage of at least 100 viable tumor cells with complete or partial membrane staining.

### 2.4. Statistical analysis

Patient characteristics were presented by descriptive statistics. Continuous variables were presented as median and range and categorical data as counts and percentages. The clinical and demographic data of the patients were statistically compared with the Pearson $\chi 2$ test for categorical variables and with independent-samples *t*-test for continuous variables. Association between baseline AEC and clinical outcomes for patients treated with ICIs was analysed according to the treatment received. Better responses to treatment according to baseline AEC were compared by the $\chi 2$ test. Statistically significant difference was considered for p-values $<0.05$.

PFS was defined as the time from the first dose of immunotherapy until disease progression or death from any cause, while OS was defined as the time interval from ICI initiation until death or last follow-up. The Kaplan-Meier analysis was used to estimate PFS and OS, and log-rank test was applied to test for statistical significance. Cox proportional hazards model analysis was used to generate point estimates of hazard ratio (HR) and corresponding 95% Confidence Interval (CI) to estimate the risk of each individual AEC with outcome.

### 2.5. Ethics and regulatory considerations

The present study was approved by the Regional Ethics Committee for Clinical Trials of the Tuscany Region (protocol code "17,332_oss"). All informed consent documents are in compliance with the International Conference on Harmonization (ICH) guideline on good clinical practice (GCP). The study protocol is performed in accordance to the principles of the Declaration of Helsinki and in compliance with GCP and the applicable laws and regulations. Each patient will be identified by a code instead of the patient's name in order to protect the patient's identity when reporting study-related data.

## 3. Results

### 3.1. Patients characteristics

A total of 158 patients with advanced or recurrent NSCLC treated with ICIs during the study period were included in the analysis. The clinical baseline characteristics of enrolled patients are summarized in Table 1. The median age at the time of diagnosis was 70 years (range 41–83 years), 102 were males (64.6%) and 56 were females (35.4%), most of patients (91.1%) were smokers (56.3% former and 34.8% current) while 14 patients (8.9%) had never been exposed to smoke. At enrolling time, the ECOG PS was $\leq$ 1 in most of patients (84.8%). All patients received single-agent anti-PD1/PD-L1 inhibitor; ICIs were used as first-line treatment in 71 patients (44.9%) and as second- or subsequent-line treatment in 87 patients (55.1%). 72 (45.6%) and 68 (43.0%) patients were treated with the anti-PD1 pembrolizumab and nivolumab, respectively; the anti-PD-L1 atezolizumab was administered in 18 patients (11.4%). PD-L1 expression levels were as follows: 0% in 24 patients (15.2%), 1 to 49% in 18 patients (11.4%), $\geq$50% in 73 patients (46.2%), while data was unknown for 43 patients (27.2%).

**Table 1**
Patients' baseline characteristics.

| **Patients ($n$ = 158)** | |
|---|---|
| Age | |
| Median (range) – years | 70 (41–83) |
| Sex – no. (%) | |
| Male | 102 (64.6) |
| Female | 56 (35.4) |
| Smoking Status – no. (%) | |
| Never | 14 (8.9) |
| Current | 55 (34.8) |
| Former | 89 (56.3) |
| ECOG Performance Status – no. (%) | |
| 0 | 47 (29.7) |
| 1 | 87 (55.1) |
| $\geq$ 2 | 24 (15.2) |
| Histology – no. (%) | |
| Adenocarcinoma | 104 (65.8) |
| Squamous Cell Carcinoma | 46 (29.1) |
| NOS Carcinoma | 8 (5.1) |
| Mutational status – no. (%) | |
| Positive for EGFR mutation | 6 (3.8) |
| Positive for KRAS mutation | 22 (13.9) |
| PD-L1 tumor proportion score – no. (%) | |
| < 1% | 24 (15.2) |
| 1–49% | 18 (11.4) |
| $\geq$ 50% | 73 (46.2) |
| Not available | 43 (27.2) |
| Baseline Eosinophil Count – no. (%) | |
| < 130 / μL | 76 (48.1) |
| $\geq$ 130 / μL | 82 (51.9) |
| Type of ICI treatment – no. (%) | |
| Pembrolizumab | 72 (45.6) |
| Nivolumab | 68 (43.0) |
| Atezolizumab | 18 (11.4) |
| Line of treatment – no. (%) | |
| 1st line | 71 (44.9) |
| $\geq$ 2nd line | 87 (55.1) |
| Best response to treatment – no. (%) | |
| Complete Response | 6 (3.8) |
| Partial Response | 30 (18.9) |
| Stable Disease | 41 (25.9) |
| Progression Disease | 78 (49.4) |

In the overall population, 6 patients achieved CR (3.8%), 30 PR (18.9%), 41 SD (25.9%), while 78 experienced PD as best response (49.4%). For 3 patients best response to ICIs could not be assessed, as treatment was early discontinued due to serious AEs.

### 3.2. Association analysis between baseline AEC and clinicopathological characteristics

We considered 130 cells/μL as the cutoff value for eosinophils count, which corresponds to the median AEC value at baseline of the patients. Patients were divided into high and low AEC groups according to this value. In the 115 patients for whom the PD-L1 status was available, a statistically significant association has emerged between high baseline AEC $\geq$130/μL and PD-L1 expression $\geq$50% ($p = 0.018$) (Fig. 1.A), while no significant association has emerged from the analysis between high baseline AEC and other clinicopathological characteristics of patients, including age, sex, smoking status, ECOG PS and histological subtype.

### 3.3. Association between baseline AEC and clinical outcomes

In the overall population, the median PFS (mPFS) and median OS (mOS) were 4.0 months (95% CI 3.0–6.0) and 7.0 months (95% CI 6.0–8.0), respectively. Patients with baseline AEC $\geq$130/μL showed a statistically significant longer PFS than patients with baseline AEC <130/μL (mPFS = 7.0 months, 95% CI 5.0–10.0 versus 2.5 months, 95% CI 2.0–4.0; $p = 0.007$) (Fig. 1.B). Consistently, a significantly better OS was reported in the high AEC group than in the low AEC group (mOS = 9.0 months, CI 95% 7.0–15.0 vs 5.5 months, 95% CI 4.0–8.0; $p = 0.009$) (Fig. 1.C).

In the whole cohort of patients, the ORR was 22.8%, while the DCR was 48.7%, respectively. Of note, our results showed a significant association between high baseline AEC group and best response to treatment ($p<0.001$). In detail, clinical-radiological response to immunotherapy was described in 64.4% of patients with baseline AEC $\geq$130/μL, while patients in the low AEC group showed a response rate of 35.6% ($p = 0.009$) (Fig. 1.D).

### 3.4. Longitudinal trend of AEC during treatment

In addition to baseline AEC, we analyzed the longitudinal trend of AEC during ICI therapy by collecting eosinophil value after the first and second administration of ICI, at week 12 and week 24 of treatment and, when available, at PD. In comparison to baseline, we found a significant increase of AEC after the first and second administration of therapy (mAEC = 150/μL vs 130/μL after first cycle, $p = 0.004$; and mAEC = 160/μL vs 130/μL after second cycle, $p = 0.016$, respectively). mAEC values reported at week 12 and 24 were 160/μL and 185/μL, respectively, with no significant variation compared to baseline, while a decreasing trend in mAEC was registered at PD (110/μL, $p = 0.756$) (Fig. 1.E).

### 3.5. Association between baseline AEC and irAEs development

The toxicities described in our cohort study population were as expected for patients treated with ICIs. In detail, 69 patients (43.7%) experienced at least one irAE during immunotherapy treatment. The reported immune-related toxicities and corresponding mAEC values at event occurrence were as follows: dermatological in 30 patients (18.9%), mAEC = 145/μL; endocrine in 21 patients (13.3%), mAEC = 160/μL; hepatic in 16 patients (10.1%), mAEC = 215/μL; gastrointestinal in 13 patients (8.2%), mAEC = 125/μL; pulmonary in 12 patients (7.6%), mAEC = 80/μL; rheumatological in 10 patients (6.3%),

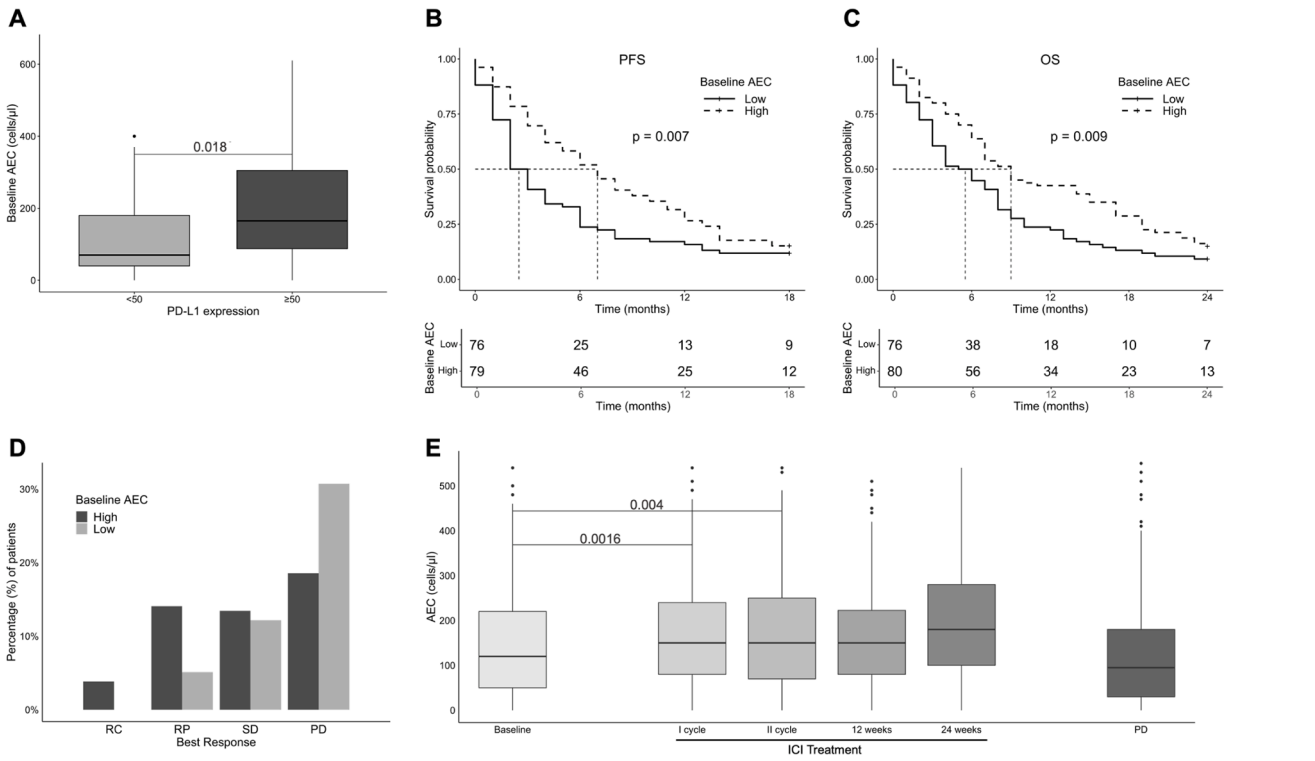


**Fig. 1.** **(A)** Association between baseline absolute eosinophil count (AEC) and PD-L1 expression ≥50% ($p = 0.018$). The data refer to the 115 patients for whom the PD-L1 status was available. **(B)** Progression free survival (PFS) and **(C)** Overall survival (OS) in the high- vs low-AEC group; high group: baseline AEC ≥130/μL; low group: baseline AEC <130/μL. **(D)** Association between AEC groups and best response to therapy in the study population. **(E)** Longitudinal trend of AEC collected during ICI treatment. Eosinophils were valued at baseline, after first and second administration of ICI, at week 12 and week 24 of treatment and, when available, at progressive disease. (CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ICI, immune-checkpoint). The p-values are indicated in the figure when they resulted statistically significant.

mAEC = 185/μL (Fig. 2.A). Grade 3 or 4 (G3-G4) irAEs were reported in 13 patients (8.2%) and the most common serious AE was pulmonary toxicity ($n = 7$ patients, 4.4%). No treatment-related deaths were reported. In the study population, a non-significant trend for an increased risk of developing irAEs during treatment was reported in patients with high baseline AEC (≥130/μL) compared to the group with low baseline AEC ($p = 0.133$) (Fig. 2.B).

### 3.6. Association between irAEs and clinical outcome

Overall, PFS and OS were significantly longer in patients who experienced irAEs than in patients who did not: mPFS was 8.0 months (95% CI 7.0–12.0) versus 2.0 months (95% CI 2.0–3.0) ($p<0.001$) and mOS was 13.0 months (95% CI 9.0–19.0) versus 4.0 months (95% CI 3.0–6–0) ($p<0.001$), respectively (Fig. 2.C and 2.D). Consistent with these results, a statistically significant longer PFS was described in patients who developed pulmonary ($p<0.01$), endocrine ($p = 0.006$) and dermatological ($p<0.001$) irAEs, compared to patients who did not experience toxicity. A non-significant trend for a longer PFS was also reported in the group of patients who developed hepatic toxicity ($p = 0.180$). Moreover, a significant better response to ICIs was described in patients who developed irAEs during treatment. In particular, ORR and DCR were respectively 33.8% vs 14.9% and 72.0% vs 32.1%, in patients who developed irAEs compared to patients who did not reported immune-toxicity ($p<0.001$).

### 3.7. Univariate and Multivariate analysis of clinical and pathological factors for clinical outcomes

Results of univariate and multivariate analysis of clinicopathological and biomolecular variables related to PFS and OS are shown in table 2.A and 2.B.

In the univariate analysis, high-AEC group had significantly longer PFS and OS than the low-AEC group (PFS HR = 1.70, 95% CI 1.12–2.58, $p = 0.012$; OS HR = 1.56, 95% CI 1.11–2.18, $p = 0.010$). A significant positive impact both in PFS and OS was also reported in the univariate analysis for irAEs (PFS HR = 0.36, 95% CI 0.23–0.55, $p<0.001$; OS HR = 0.31, 95% CI 0.22–0.45, $p<0.001$), PD-L1 ≥50% (PFS HR = 0.57, 95% CI 0.38–0.86, $p = 0.007$; OS HR = 0.65, 95% CI 0.44–0–97, $p = 0.037$) and PS ECOG ≤1 (PFS HR = 1.86, 95% CI 1.19–2.93, $p = 0.007$; OS HR = 2.34, 95% CI 1.36–4.02, $p = 0.002$).

We performed multivariate analysis including parameters found to have a $p$ value less than 0.05 in the univariate analysis, to identify independent prognostic factors related to survival outcomes.. After adjustment for confounding factors, a high AEC at baseline was associated with longer PFS and OS (PFS HR = 1.77, 95% CI 1.10–2.86, $p = 0.019$; OS HR = 1.46, 95% CI 1.02–2.10, $p = 0.040$). Patients who experienced irAEs still tended to have significant longer OS (HR = 0.51, 95% CI 0.30–0.86, $p = 0.011$), but no statistical difference in PFS, while pulmonary toxicity was associated with both better PFS (HR = 0.23, 95% CI 0.07–0.79, $p = 0.019$) and OS (HR = 0.37, 95% CI 0.16–0.85, $p = 0.019$). Finally, also PS ECOG ≤1 and PD-L1 ≥50% resulted to be independent prognostic factors for longer survival outcomes (table 2.A).

## 4. Discussion

In our study, we evaluated a real-world population of NSCLC patients treated with single agent ICI as first or subsequent line of therapy to assess the correlation between the readily available peripheral-blood biomarker AEC with clinical outcomes and the development of irAEs. We found a significant correlation between high baseline AEC and PD-L1 expression ≥50% ($p = 0.018$), while other clinic-pathological features

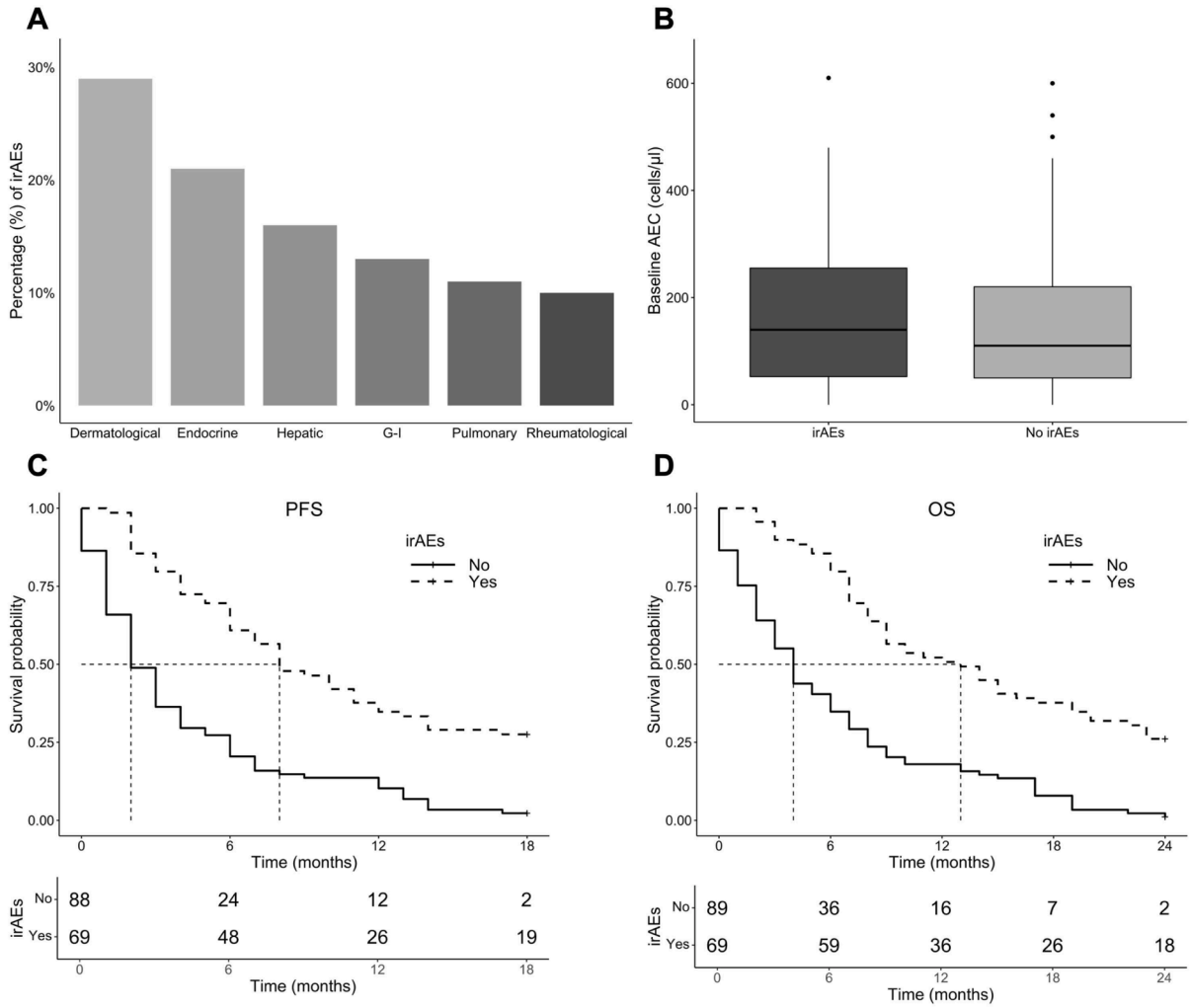


**Fig. 2.** **(A)** Types and percentage of immune-related adverse events (irAEs) reported in the study population (G-I, gastro-intestinal). **(B)** Association between baseline absolute eosinophil count (AEC) and irAEs developed during therapy ($p = 0.133$). **(C)** Progression free survival (PFS) and **(D)** Overall Survival (OS) in patients who experienced irAEs during therapy compared to patients who did not experience toxicity.

were not associated with eosinophil count. Overall, our results suggest that baseline AEC $\geq$130/µL and the development of irAEs during therapy were significantly associated with longer PFS and OS. Moreover, patients with higher AEC and patients who experienced irAEs of any grade had a significant better response to immunotherapy. Interestingly, we found an increase in AEC at the beginning of immunotherapy, whereas a decreasing trend was described at disease progression. Of note, the results of multivariate analysis suggest that higher AEC, PS ECOG $\leq$1, irAEs developed during therapy and pulmonary toxicity were independent prognostic factors for longer survival.

Although growing evidence has emerged on the efficacy of anti-PD1/PD-L1 agents in the treatment of several type of cancer, including NSCLC, [27] there is still a lack of available biomarkers that could predict clinical response and toxicity to immunotherapeutic agents in daily clinical practice. [8,9] Peripheral blood biomarkers are an attractive option compared to tumor tissue due to easy access and availability for routine monitoring during immunotherapy. AEC is a widely available parameter as part of the complete blood examination routinely performed for cancer patients before and during therapy. Eosinophils are a subtype of granulocytic leukocytes that can influence homeostasis maintenance and integrity of tissues and are involved in numerous immune functions. [28]

Eosinophilia, defined as a peripheral blood AEC greater than 0.5 $\times$ $10^9$/L, and eosinophils accumulation in the tissues are associated with different pathologic conditions, including allergic and chronic inflammatory disorders (e.g. asthma and inflammatory bowel diseases), parasitic helminth and viral infections, tissue injury and neoplastic diseases. [28,29] However, the specific role of eosinophils in cancer is still controversial: whether eosinophils play an active role or are bystanders cells in tumor immune surveillance remains unclear. Hence, the relationship between eosinophils and other immune cells known to be involved in tumor immune surveillance is of considerable interest. Moreover, recent data suggest a divergent roles of eosinophils depending on the tumor histotype; although in different type of malignancies (e.g. melanoma, gastric, colorectal and oral squamous cancer) eosinophils have an anti-tumoral activity, in others (e.g. cervical carcinoma and Hodgkin's lymphoma) they are associated with a poor prognosis. [15,30–32] Recently, in melanoma patients treated with ICIs has been reported that eosinophils exert a potentially predictive role for response to immunotherapy and a significant association with prolonged survival, [16,33,34] thus, eosinophilia has emerged as a novel on-treatment biomarker. Conversely, the potential correlation of eosinophils count with clinical outcomes in NSCLC patients has been far less investigated.

In addition, eosinophils are commonly found in tumor microenvironment (TME) of many different malignancies as part of the tumor-infiltrating immune cells. [32] In this context, several interactions between tumor cells and host immunity modulate the TME, contributing to tumor rejection or progression which, in turn, influence the prognosis

**Table 2A**
Univariate and multivariate analysis of factors related to PFS, by the Cox's regression model in all patients ($n = 158$).

| Variables | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *p-value* | HR (95% CI) | *p-value* |
| Gender (male vs female) | 1.27 (0.89 - 1.80) | *0.180* | | |
| ECOG PS: | | | | |
| 0 | – | – | – | – |
| 1 | 1.80 (1.08 – 3.01) | *0.024* | 2.19 (1.24 – 3.84) | ***0.007*** |
| 2 | 3.04 (1.55 – 5.99) | *0.001* | 2.62 (1.20 – 5.72) | ***0.016*** |
| Smoking status: | | | | |
| Never | – | – | – | – |
| Former | 0.30 (0.12 – 0.73) | *0.008* | 0.50 (0.19 – 1.33) | *0.167* |
| Current | 0.30 (0.12 – 0.73) | *0.008* | 0.50 (0.20 – 1.24) | *0.133* |
| Histology (squamous vs non-squamous) | 1.08 (0.76 – 1.54) | *0.663* | | |
| PD-L1 expression (<50% vs ≥50%) | 0.57 (0.38 – 0.86) | *0.007* | 0.54 (0.34 – 0.85) | ***0.007*** |
| Baseline AEC vs (<130/μL vs ≥130/μL) | 1.70 (1.12 – 2.58) | *0.012* | 1.77 (1.10 – 2.86) | ***0.019*** |
| irAEs (yes vs no) | 0.36 (0.23 – 0.55) | *<0.001* | 0.63 (0.32 – 1.23) | *0.177* |
| Pulmonary irAEs (yes vs no) | 0.24 (0.07 – 0.75) | *0.014* | 0.23 (0.07 – 0.79) | ***0.019*** |

Variables with *p*-value ≤0.05 in univariate model were analyzed in multivariate analysis model.
HR, hazard ratio; CI, Confidence Interval; ECOG PS, Eastern Cooperative Oncology Group; AEC, absolute eosinophil count; irAEs, immune-related adverse events.

**Table 2B**
Univariate and multivariate analysis of factors related to OS, by the Cox's regression model in all patients ($n = 158$).

| Variables | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *p-value* | HR (95% CI) | *p-value* |
| Gender (male vs female) | 1.11 (0.79 – 1.57) | *0.577* | | |
| ECOG PS: | | | | |
| 0 | – | – | – | – |
| 1 | 1.66 (1.13 – 2.45) | *0.011* | 1.75 (1.17 – 2.62) | ***0.007*** |
| 2 | 3.25 (1.91 – 5.53) | *<0.001* | 2.54 (1.42 – 4.56) | ***0.002*** |
| Smoking status: | | | | |
| Never | – | – | | |
| Former | 0.56 (0.30 – 1.04) | *0.065* | | |
| Current | 0.58 (0.32 – 1.04) | *0.067* | | |
| Histology (squamous vs non-squamous) | 1.15 (0.81 – 1.63) | *0.435* | | |
| PD-L1 expression (<50% vs ≥50%) | 0.65 (0.44 – 0.97) | *0.037* | 0.63 (0.41 – 0.96) | ***0.033*** |
| Baseline AEC vs (<130/μL vs ≥130/μL) | 1.56 (1.11 – 2.18) | *0.010* | 1.46 (1.02 – 2.10) | ***0.040*** |
| irAEs (yes vs no) | 0.31 (0.22 – 0.45) | *<0.001* | 0.51 (0.30 – 0.86) | ***0.011*** |
| Pulmonary irAEs (yes vs no) | 0.33 (0.15 – 0.70) | *0.004* | 0.37 (0.16 – 0.85) | ***0.019*** |

Variables with *p*-value ≤0.05 in univariate model were analyzed in multivariate analysis model.
HR, hazard ratio; CI, Confidence Interval; ECOG PS, Eastern Cooperative Oncology Group; AEC, absolute eosinophil count; irAEs, immune-related adverse events.

and survival outcomes in cancer patients. [35] TATE is related to a good prognosis for cancer patients as reported in different studies. [15,36,37] Activated tumor-infiltrating eosinophils may promote cancer rejection by producing chemokines that recruit tumor specific CD8+ T-cells, enhancing their migration into the tumor [38] and can mediate the anti-tumor response by modulating TME through normalization of tumor vasculature and polarization of tumor-associated macrophages (TAMs) toward M1-like sub-population, which, in turn, facilitate the infiltration and activity of T-cells. In addition, through the release of major basic protein, eosinophils stimulate maturation of dendritic cells and their antigen-presenting ability, which is involved in the anti-tumor immune-response. Finally, eosinophils can play an active role in host immune response against tumor, as they exhibit a direct cytotoxic activity on tumor growth via the release of granule-associated cytotoxic proteins inducing tumor cells apoptosis. [32,38–41] In the meta-analysis of Hu et al. [37] the presence of TATE was significantly associated with improved OS in patients with different solid tumors, such as colorectal, gastric and esophageal cancer, head and neck carcinoma and cervical carcinoma (HR=0.82, 95% CI 0.68–0.99, $p = 0.041$), but not with disease free survival (DFS). Moreover, TATE resulted inversely correlated with lymph node metastasis (OR = 0.59, 95% CI 0.40–0.87, $p = 0.007$), TNM stage (OR = 1.70, 95% CI 1.12–2.58, $p = 0.013$) and lymphatic invasion (OR = 0.58, 95% CI 0.36–0.91, $p = 0.018$). According to stratified analysis based on cancer types, TATE had a markedly positive effect in improving survival in colorectal cancer and esophageal cancer, as reported in this meta-analysis and in previous studies. [42–44]

Our findings were consistent with data previously published in a retrospective study by Tanizaki et al., [25] which enrolled a study population similar to our cohort of patients. Authors retrospectively analyzed the relationship between survival and peripheral blood parameters, including AEC, in 134 patients with metastatic NSCLC treated with the anti-PD1 nivolumab. Similarly to our results, patients with high AEC measured before the start of treatment showed a significant better PFS ($p = 0.02$) and OS ($p = 0.003$) in the multivariate analysis. Likewise, in the retrospective study by Krishnan et al. [45] cancer patients who developed eosinophilia during ICIs therapy were more likely to achieve disease control and there was a non-significant trend towards improved OS in this group of patients. Authors also described an increasing number of patients who developed eosinophilia after the beginning of treatment. In detail, at baseline was present in 3.4% of patients, rising to 8.9% and 17.8% by cycle 2 and week 6, respectively. In addition, authors found that eosinophilia while on treatment was significantly correlated with the development of irAEs of any grade, although there was no association between severe immune-related toxicity and eosinophilia. Similar to these results, we found a significant increase from baseline AEC after the first and second administration of ICI and a trend for an increased risk of developing irAEs during treatment in high-AEC group of patients. However, some substantial differences between the study by Krishnan et al. and our study should be highlighted. Firstly, the different cut off used for AEC as a predictor of response and toxicity; secondly, we focused on baseline AEC, while Krishnan and colleagues evaluated eosinophilia developed during immunotherapy; finally, the patient population in the study by Krishnan et al. was heterogeneous, as it included patients with either NSCLC, melanoma or other solid tumors.

In contrast to our results, in the retrospective analysis of Soyano et al. [46], which evaluated peripheral blood biomarkers that correlate with outcome in NSCLC patients, baseline AEC was not found to have significant association with response and outcomes to immunotherapy in advanced NSCLC patients. Moreover, authors reported no differences in the baseline AEC of patients who developed irAEs and those who did not. Differently from this study, Chu et al. [23] evaluated the association of baseline AEC with ICI-related pneumonitis and clinical outcomes in 300 NSCLC patients. Authors found a higher overall risk of pneumonitis development for patients with AEC ≥125/μL ($p = 0.013$). Moreover the high-AEC group of patients had a higher ORR ($p = 0.029$) and a longer mPFS ($p = 0.038$). These results were consistent with those of our study,

in which AEC ≥130/μL resulted significantly associated with longer PFS and OS and with better ORR to therapy. On the other hand, we did not find a significant correlation between AEC and ICI-pneumonitis ($p$ = 0.488), probably due to the limited number of pulmonary irAEs described in our study. Intriguingly, the threshold of baseline AEC used to divide the study population into high- and low-AEC groups in the Chu et al. study and in ours was similar, although it was identified by two different methods. This supports the reliability and the consistency of our findings on the association between eosinophil count and clinical outcomes.

Finally, in our study population we confirmed the independent predictive role of irAEs experienced during therapy for a longer survival outcome. This result is consistent with a growing body of literature reporting in patients with NSCLC treated with anti-PD1/PD-L1 and experiencing immune-related toxicity during therapy a marked improvement in PFS, OS and ORR. [47–50]

The strengths of our study are the data provided by a real-world cohort of patients with advanced NSCLC treated with ICIs and identified the role of AEC as a routinely available biomarker of response to therapy and a potentially predictor of irAEs development. In addition, we confirmed that patients who experienced immune-related toxicities during treatment had better outcomes.

There are several limitations to the present study. First, its nature as a retrospective and single-center study. Likely, the relatively small study population also led to a lack of power to detect significant association between AEC and different subtypes of irAEs reported. Moreover, PD-L1 expression data were not available for forty-three patients and it was not possible to assess whether mutational status correlated with baseline AEC due to the limited number of patients harboring driver mutations included in the analysis. In addition, as only few patients reported G3-G4 adverse events, it was not possible to separately analyze the association between high AEC at baseline and the risk of developing irAEs in patients who experienced serious or non-serious adverse events, respectively. Another limitation of our study is that other potential confounders such as concomitant use of medications other than corticosteroids at baseline and at the onset of toxicity that could have altered levels of AEC, were not taken into consideration in the analysis, as data were not detailed in patients' medical records.

## 5. Conclusions

Anti-PD1/PD-L1 inhibitors have revolutionized the therapy landscape in NSCLC. However, they are effective only in a percentage of patients and there is an urgent need for available biomarkers predictive of response and toxicity for immunotherapy.

Our findings might help with risk stratification and treatment strategies to avoid unnecessary therapy and related toxicities in patients who are less likely to benefit from treatment. If validated, baseline AEC has the advantage of being easily integrated into clinical practice as a prognostic factor and a potential predictor of response to ICIs in NSCLC patients. In addition, future prospective studies could also assess the longitudinal trend in eosinophil count during immunotherapy and confirm whether an increase in terms of cell count as well as from baseline AEC correlates with clinical outcome and immune-related toxicity and whether a higher AEC is associated with a higher number of eosinophils in the tumor microenvironment.

## CRediT authoship contribution statement

Conceptualization: EC, FM, SP; Collection of data: SF, CO, MRGM, BN; Interpretation of data: EC, SF, DL, SP; Data curation: LP, DL, EG, FS; Writing-original draft preparation: EC; Writing-review and editing: FM, SP, LA; Supervision: LV, CEC, LA. All authors have read and agreed to the published version of the manuscript

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## Declaration of Competing Interest

The authors state that they have no relevant affiliations or financial involvement with any organization or entity with a financial interest or financial conflict with the topics or materials discussed in the present manuscript.

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