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Clinical and molecular parameters associated to pneumonitis development in non-small-cell lung cancer patients receiving chemoimmunotherapy from NADIM trial

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ABSTRACT

Background Pneumonitis (Pn) is one of the main immune-related adverse effects, having a special importance in lung cancer, since they share affected tissue. Despite its clinical relevance, Pn development remains an unpredictable treatment adverse effect, whose mechanisms are mainly unknown, being even more obscure when it is associated to chemoimmunotherapy.

Methods In order to identify parameters associated to treatment related Pn, we analyzed clinical variables and molecular parameters from 46 patients with potentially resectable stage IIIA non-small-cell lung cancer treated with neoadjuvant chemoimmunotherapy included in the NADIM clinical trial (NCT03081689). Pn was defined as clinical or radiographic evidence of lung inflammation without alternative diagnoses, from treatment initiation to 180 days.

Results Among 46 patients, 12 developed Pn (26.1%). Sex, age, smoking status, packs-year, histological subtype, clinical or pathological response, progression-free survival, overall survival and number of nivolumab cycles, were not associated to Pn development. Regarding molecular parameters at diagnosis, Pn development was not associated to programmed death ligand 1, TPS, T cell receptor repertoire parameters, or tumor mutational burden. However, patients who developed Pn had statistically significant lower blood median levels of platelet to monocyte ratio (p=0.012) and teratocarcinoma-derived growth factor 1 (p=0.013; area under the curve (AUC) 0.801), but higher median percentages of natural killers (NKs) (p=0.019; AUC 0.786), monocytes (p=0.017; AUC 0.791), MSP (p=0.006; AUC 0.838), PARN (p=0.017; AUC 0.790), and E-Cadherin (p=0.022; AUC 0.788). In addition, the immune scenario of Pn after neoadjuvant treatment involves: high levels of neutrophils and NK cells, but low levels of B and T cells in peripheral blood; increased clonality of intratumoral T cells; and elevated plasma levels of several growth factors (EGF, HGF, VEGF, ANG-1, PDGF, NGF, and NT4) and inflammatory cytokines (MIF, CCL16, neutrophil gelatinase-associated lipocalin, BMP-4, and u-PAR).

Conclusions Although statistically underpowered, our results shed light on the possible mechanisms behind Pn development, involving innate and adaptative immunity, and open the possibility to predict patients at high risk. If confirmed, this may allow the personalization of both, the surveillance strategy and the therapeutic approaches to manage Pn in patients receiving chemoimmunotherapy.

INTRODUCTION

The programmed death-1 (PD1)/PD ligand 1 (PD-L1) pathway is one of the several mechanisms that tumor cells use to repress natural antitumor immune activity.[1] Development of antibodies that specifically target these molecules has permitted weaponizing the host immune system against cancer, boosting the immune response against the tumor.[2]

Nevertheless, these molecules are also involved in the maintenance of immunologic homeostasis and self-molecules immunotolerance, preventing excessive autoimmunity throughout life. Thus, its inhibition may as well lead to autoimmune-like adverse events, by disturbing the normal immunoregulation of the body. These immune-relates adverse effects (irAEs) have a significant impact on patients, leading in some cases to disruption of treatments and to life-threatening situations.[3]

The combination of immune checkpoint inhibitors with chemotherapy, has not only increased survival, but toxicity as well, making it an arising issue as chemoimmunotherapy is being positioned in the frontline treatment of some tumors, and in particular, in locally or metastatic non-small-cell lung cancer (NSCLC).[4 5]

One of the main irAEs in terms of incidence and fatality rate is pneumonitis (Pn).[6] Patients with lung cancer have the highest rates of Pn,[7] specially addressed in advanced NSCLC, where its documented incidence using combination of anti PD-(L)1 with chemotherapy oscillates from 1.3% to 6.5%, with a fatal rate between 0% and 0.4%. However, rates of reported dyspnea oscillate from 13.6% to 23.4%, which may suggest that the real rate of Pn may be underestimated.[8–12] Pn incidence of chemoimmunotherapy is still less known in the NSCLC perioperative scenario, where few studies have reported efficacy results, with no specific data reported on Pn incidence.[13–16]

In addition, although carboplatin or paclitaxel have a reasonably safe pulmonary toxicity profile, it has been described that chemotherapy alone can produce Pn and fibrosis, being the incidence higher when these treatments are combined.[17 18] Thus, potentially modifying the possible mechanisms behind anti-PD(L)1 Pn and contributing to increased pulmonary toxicity of chemoimmunotherapy.

Some risk factors have been proposed, such as prior lung disease, prior lung radiotherapy, combination with anti-CTLA-4,[19] or the levels of some immune-related molecular parameters. However, the scarcity of data on the biological mechanisms, has resulted in a lack of reliable biomarkers to identify patients at risk and limited understanding on how to best treat them.[20 21]

The aim of this study is to identify potential clinical and molecular biomarkers associated to Pn development after neoadjuvant chemoimmunotherapy in NSCLC patients.

METHODS

Study population and design

We analyzed treatment related adverse events and clinical variables of 46 patients with potentially resectable stage IIIA NSCLC in the neoadjuvant setting with chemoimmunotherapy included in the NADIM clinical trial (NCT03081689), an open-label, multicenter, single-arm phase II trial done at 18 hospitals in Spain.

Patients included in the study were treated with nivolumab (360 mg), paclitaxel (200 mg/m^2) and carboplatin (area under the curve (AUC) 6; 6 mg/mL per min), on day 1 of each 21-day cycle, for three cycles before surgical resection.[13] Main exclusion criteria were the presence of active autoimmune or infectious disease, ongoing systemic corticosteroid or other immunosuppressive therapy, history of symptomatic grade 3 or 4 interstitial lung disease, clinically significant concurrent malignancies and previous treatment with checkpoint inhibitors.

Adverse events and abnormal laboratory findings were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events V.4.0. Investigators determined whether adverse events were treatment related according to the study protocol and standard regulatory requirements.

A central committee confirmed all Pn cases and considered adverse events related with neoadjuvant treatment those developed within 180 days after the administration of first dose of neoadjuvant nivolumab. Patients with Pn were considered to be those who had clinical and/or radiological findings of Pn of any grade (such as dyspnea and pleural pain), once other clinical entities had been ruled out (namely infectious diseases, pulmonary thromboembolism, or tumor progression, among others).

Clinical variables as well as molecular parameters (at diagnosis and after neoadjuvant treatment, along with their treatment variation) were compared between patients who developed Pn as an adverse effect to treatment and patients who did not, in order to search for markers associated with this irAE.

PD-L1 TPS, tumor mutational burden and specific mutations

Basal PD-L1 tumor proportion score (TPS), tumor mutational burden (TMB) and specific mutations were retrieved from.[13] Briefly, PD-L1 immunohistochemistry assay (22C3 pharmaDx, Code SK006; Dako, Glostrup, Denmark) was used to assess diagnostic PD-L1 TPS following manufacturer's instructions.

TMB Library generation and sequencing of samples was performed on an Ion Chef System and S5 Sequencer. Specifically, 20 ng of extracted DNA was treated with heat labile uracil-DNA glycosylase and used for library preparation using the Oncomine Tumor Mutation Load Assay. Eight samples were loaded at 50pM and sequenced onto an Ion 540 chip. Reads were aligned to hg19 using Torrent Suite 5.12 and BAM files were transferred to Ion Reporter 5.12 for variant calling. TMB was computed using the TMB filter chain and the TMB algorithm 3.0. Germline variants were filtered out using a germline filter-chain based on population databases. Additionally, specific mutations using the Oncomine Variants 5.12 filter whose variant allele frequency was greater than 5%, reached ≥60 of coverage and a p≤0.05 were reported.

Blood counts

Patients had laboratory blood tests before each 21-day treatment cycle to monitor complete blood cell counts and biochemical parameters. Hemoglobin, leucocytes, neutrophils, eosinophils, lymphocytes, monocytes, platelets and LDH levels were retrieved from laboratory blood tests at two timepoints: prior treatment initiation and after neoadjuvant treatment but before surgery. Additionally, Lung Immune Prognostic Index (LIPI), neutrophil to lymphocyte ratio (NLR), monocyte to lymphocyte ratio (MLR), platelet to lymphocyte ratio (PLR), derived NLR (dNLR), and platelet to monocyte ratio (PMR) were calculated. dNLR was defined as neutrophils/(leucocytes-neutrophils). LIPI was estimated as the sum of two factors, dNLR greater than three and lactate dehydrogenase

(LDH) greater than 333 IU/L, defining three groups (good, 0 factors; intermediate, 1 factor; poor, 2 factors).[22]

Immunophenotyping and cytokine levels

Cell and plasma isolation, immunophenotyping of peripheral blood mononuclear cells (PBMCs) and immunoassays for detecting cytokines in plasma were obtained and processed as previously described [23] . Briefly, 29 samples of peripheral blood mononuclear cells (PBMCs) at baseline or after neoadjuvant treatment, but presurgery, were isolated by lymphoprep gradient centrifugation and cryopreserved with freezing medium until use for flow cytometry analysis. Immunophenotyping of general and specific subpopulations of circulating natural killer (NK) cells (CD3-CD56+), T cells (CD3+), cytotoxic T cells (CD3 +CD8+), helper T cells (CD3 +CD4+), T-NK like cells (CD3 +CD56+), B cells (CD3-CD19+) and monocytes (CD14+) with different activation and checkpoint markers was determined by multicolor panels on a MACS Quant 10 cytometer (Miltenyi Biotec) and analyzed with the FLOWJO software, as previously described.[23] When data from different antibody panels were available for the same cell population median value among them was used. Information derived from anti-PD-1 clone PD1.3.1.3 labeling in posttreatment samples was not included in the analysis due to nivolumab binding competition to PD-1 receptor.

Plasma fraction of 30 patients at baseline and 34 patients after neoadjuvant treatment was collected after gradient centrifugation and stored diluted 1:2 with RPMI at −80°C. Levels of 200 cytokines related to cancer biomarkers were measured using G-Series Human Cytokine Antibody Array 4000 (RayBiotech) following manufacturer protocols.

T cell receptor repertoire

Tissue and blood samples were used for the analysis of the T cell receptor (TCR) repertoire, both at diagnosis and after neoadjuvant treatment. TCR Library preparation and sequencing was carried out as previously described.[24] Briefly, RNA input for PBMCs-derived libraries using the Oncomine TCR Beta–LR Assay was 25 ng, whereas for formalin-fixed, paraffin-embedded (FFPE) derived libraries using Oncomine TCR Beta–SR Assay, was 100 ng. For PBMCs-derived libraries, equal volumes from eight samples at 25 pM were pooled for sequencing on an Ion 530 chip. For FFPE-derived libraries, equal volumes from up to 32 samples at 25 pM were combined for sequencing on an Ion 540 chip.

Once the libraries were templated on an Ion Chef System, they were sequenced in the Ion GeneStudio S5 Series and analysis was done via Ion Reporter V.5.12. Convergence, Shannon's diversity index and evenness were provided by Ion Reporter Software as a standard output. Dynamics of expanded and contracted clones in blood and tissue were calculated as percentages and clonal space of clones that increased or decreased their frequency after treatment, relative to pretreatment repertoire.

Statistical analysis

To test for clinical and molecular data categorical associations with Pn development, we performed a Fisher's exact test, in variables such as sex, histology, smoking status, pathological and clinical response, number of cycles, specific mutations and LIPI score. Non-parametric Mann-Whitney U test was performed for clinical variable analysis (age and packs-year) and quantitative molecular parameters, such as blood counts, cytokines, TCR repertoire and immune populations. To analyze changes related to neoadjuvant treatment specific of Pn development, differences between paired postsamples and presamples were calculated for each patient group and Mann-Whitney U test was performed. Kaplan-Meier survival analysis was performed using the log-rank test in order to identify difference between patients who developed pneumonitis and those who did not. The receiver operating characteristic (ROC) curve analysis was calculated to predict the potential value of the parameters as predictors of pneumonitis development, and AUC values over 0.75 were considered good enough predictors to be included in this study. Since the research was designed as a discovery study, p values were not adjusted in order to maximize the finding of new biomarkers and the generation of new hypothesis. $P<0.05$ was considered statistically significant for all tests. Finally, the post hoc nature of the analyses, together with the large number of variables analyzed and the small number of patients, requires confirmation of the biomarkers found in independent cohorts.

RESULTS

Clinical characteristics

The cohort of patients included 46 patients with NSCLC stage IIIA, according to American Joint Committee on Cancer seventh edition criteria, recruited across 18 centers in Spain between April 26, 2017 and Aug 25, 2018. All of them were treated with neoadjuvant chemoimmunotherapy according to protocol described in Material and Methods.

At the time of the data cut-off, with a median follow-up of 24.0 months (IQR 21.4–28.1), 12/46 patients (26.1%) developed a clinical and/or radiological treatment related Pn within 180 days after receiving the first dose of nivolumab. The median to Pn development was 109.5 day (IQR 58–130).

We found no difference between patients who developed Pn and those who did not regarding sex (p=1.000), age (p=0.910), smoking status (p=0.749), packs-year (p=0.196), histological subtype (p=0.316), or clinical response (p=0.836). No significant differences were found between patients who had complete pathological response (CPR) and those who had not (p=0.480) or those who had CPR or major pathological response and those with incomplete pathological response (p=0.398). We found no differences in progression-free survival (p=0.761) or overall survival (p=0.726) between patients who developed Pn and those who did not. No association

Table 1 Clinical characteristics of patients and statistical significance related to pneumonitis development

Characteristic	Pneumonitis (n=12)	Non-pneumonitis (n=34)	P value
Age (years)—median (range)	61.0 (47.0–76.0)	63.5 (41.0–76.0)	0.910
Sex—n (%)			1.000
Male	9 (75.0)	25 (73.5)	
Female	3 (25.0)	9 (26.5)	
Histology—n (%)*			0.316
Adenocarcinoma	9 (75.0)	17 (50.0)	
Squamous	3 (25.0)	13 (38.2)	
No otherwise specified	0 (0.0)	4 (11.8)	
Smoking status—n (%)			0.749
Former	6 (50.0)	19 (55.9)	
Current	6 (50.0)	15 (44.1)	
Packs-year—median (range)	57.0 (20.0–100.0)	45.0 (22.0–114.0)	0.196
Clinical response—n (%)			0.836
Complete	0 (0.0)	2 (5.9)	
Partial	10 (83.3)	23 (67.6)	
Stable	2 (16.7)	9 (26.5)	
Pathological response—n (%)†			0.480‡, 0.398§
Complete	9 (75.0)	17 (58.6)	
Major	0 (0.0)	8 (27.6)	
Incomplete	3 (25.0)	4 (13.8)	
PFS (months)—median (95% CI)	NR	NR	0.761
OS (months)—median (95% CI)	NR	NR	0.726
No cycles—N (%)			0.409
≤3 cycles	1 (8.3)	8 (23.5)	
>3 cycles	11 (91.7)	26 (76.5)	

*Four patients had no specified histology
†Five patients did not undergo surgery
‡Complete responses versus other
§Incomplete responses versus other
NR, not reported; OS, overall survival; PFS, progression-free survival.

between number of nivolumab cycles received and Pn development was found (p=0.409) (table 1).

We also wanted to investigate any relationship between Pn development and molecular characteristics of the tumors, specifically, PD-L1 TPS, TMB or specific baseline mutations. 28 of 46 patients had valid data for PD-L1 TPS (six developed Pn), and 29 had valid data for baseline mutation analysis (five developed Pn).

No association between PD-L1 TPS (Pn 45%, IQR 0%–100%; Non-Pn 32.5%, IQR 0%–75%; p=0.491) or TMB (Pn 7.6, IQR 4.2–55.8; Non-Pn 5.9, IQR 3.6–9.0; p=0.298) and Pn development were observed (figure 1A). Mutations in *KEAP1*, *ARID1A*, *RB1*, *HNF1A*, *TP53* or *KRAS* genes did not show significant association to Pn development. However, despite the low number of cases, the presence of *ARID1A* mutations showed a trend to be associated to Pn development (p=0.068). Out of three patients who presented the mutation, two developed Pn (66.7%), while only three cases of Pn were described in the 26 non-mutated patients (11.5%) (figure 1B and table 2).

Other less frequent mutations found in our cohort of patients (n<3 cases) were *STK11* (one mutated patient who did not develop Pn), *EGFR* (one mutated patient who did not develop Pn) and BRAF (one mutated patient who developed Pn). No mutations were found in RET, ROS, MET, or ALK.

We further sought to identify Pn associated biomarkers in patient cell blood counts at baseline (n=46) or postneoadjuvant treatment (n=45). Different standard parameters in the hemograms from all patients were analyzed, as well as the ratios and scores derived from this data.

No significant differences were observed for hemoglobin, platelets, eosinophils, basophils, lymphocytes and LDH. (online supplemental figure S1A and online supplemental table 1). At diagnosis, only a trend to

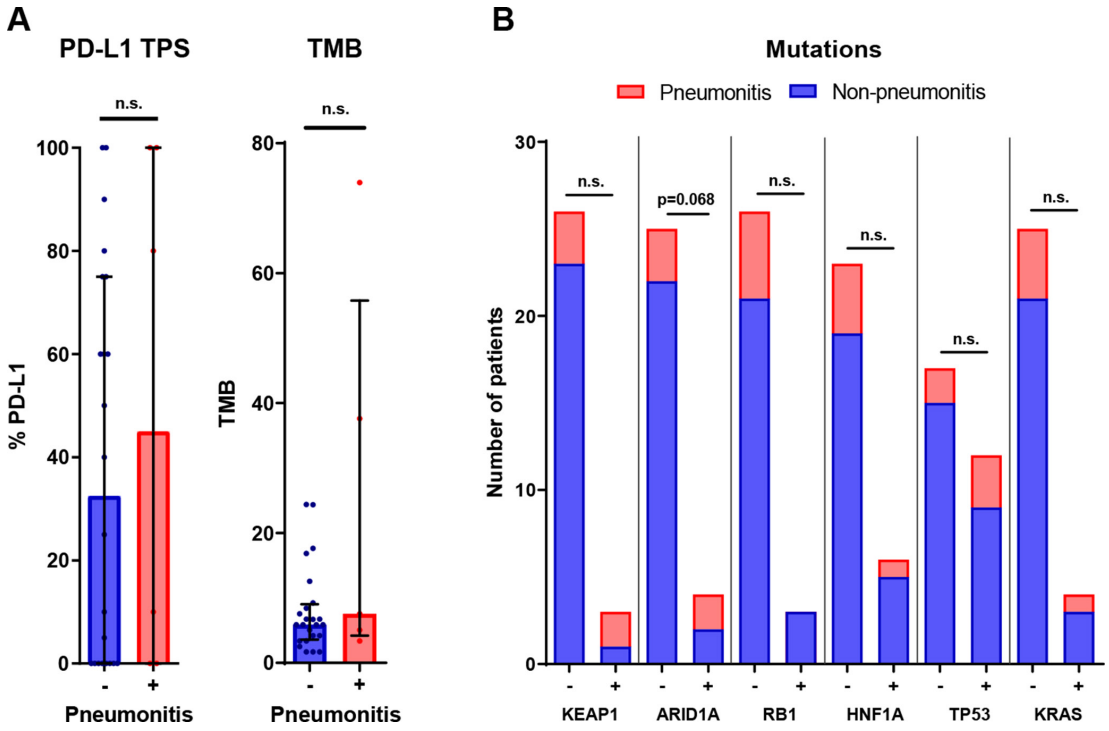

Figure 1 PD-L1, TMB and specific mutations. PD-L1 TPS levels (n=28; p=0.491), TMB (n=29; p=0.298) and specific mutations (n=29; p=0.127 for *KEAP1;* p=0.068 for *ARID1A*; p=1 for *RB1; p=1 for HNF1A*; p=0.622 for *TP53* and p=0.553 for *KRAS*). *P<0.05; **p<0.01; ***p<0.001. n.s., not significant; PD-L1, programmed death ligand 1; TMB, tumor mutational burden.

higher monocyte counts in patients who develop Pn, was observed (Pn 0.75, IQR 0.63–1.15; non-Pn 0.70, IQR 0.50–0.84; p=0.079) (figure 2A). However, in postneoadjuvant treatment samples, patients who developed Pn showed increased levels of neutrophils (Pn 4.31, IQR 3.38–4.84; non-Pn 3.1, IQR 2.56–4.15; p=0.041), as well as, a trend to higher leukocyte counts (Pn 7.61, IQR 6.16–8.27; non-Pn 5.95, IQR 4.8–7.11; p=0.063) (figure 2A).

Table 2 Association of specific mutations and pneumonitis development using statistical Fisher's exact values

Gene	Non-pneumonitis (n=24)	Pneumonitis (n=5)	Total (n=29)	P value
KEAP1—n (%)				0.127
Wt	22 (88)	3 (12)	25	
Mut	2 (50)	2 (50)	4	
ARID1A—n (%)				0.068
Wt	23 (88.5)	3 (11.5)	26	
Mut	1 (33.3)	2 (66.7)	3	
RB1—n (%)				1
Wt	21 (80.8)	5 (19.2)	26	
Mut	3 (100)	0 (0)	3	
HNF1A—n (%)				1
Wt	19 (82.6)	4 (17.4)	23	
Mut	5 (83.3)	1 (16.7)	6	
TP53—n (%)				0.622
Wt	15 (88.2)	2 (11.8)	17	
Mut	9 (75)	3 (25)	12	
KRAS—n (%)				0.553
Wt	21 (84)	4 (16)	25	
Mut	3 (75)	1 (25)	4	

Mut, mutated; Wt, wild-type.

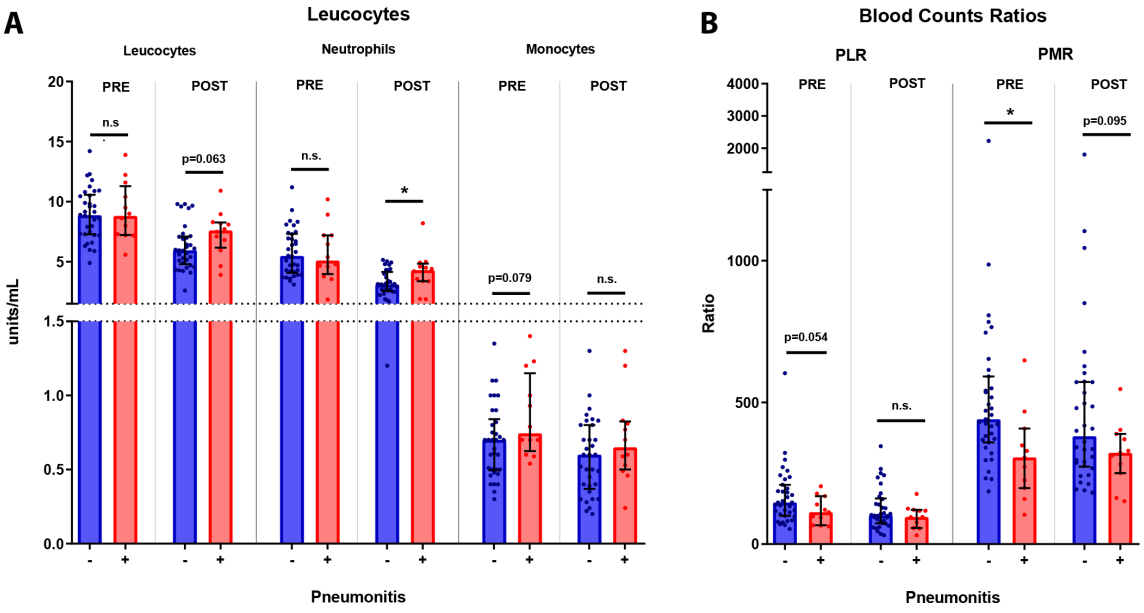

Figure 2 Total blood counts and blood ratios. (A) Total blood count parameters (n=46 in pretreatment and n=45 in postneoadjuvant treatment samples; p=0.940 and p=0.063 for leucocytes; p=0.881 and p=0.041 for neutrophils; and p=0.079 and p=0.275 for monocytes; in preneoadjuvant and postneoadjuvant samples, respectively). (B) Ratios derived from hemograms (n=46 in pretreatment and n=45 in postneoadjuvant treatment samples; p=0.054 and p=0.270 for PLR, and p=0.012 and p=0.095 for PMR). *P<0.05; **p<0.01; ***p<0.001. n.s., not significant; PLR, platelet to lymphocyte ratio; PMR, platelet to monocyte ratio.

Related to blood counts ratios, there were no significant differences in NLR, dNLR, MLR, and LIPI between patients with and without Pn (online supplemental figure S1B). However, patients who developed Pn showed a trend to lower platelet-to-lymphocyte ratio (PLR; Pn 105.3, IQR 70.8–161.8; non-Pn 146.9, IQR 103.8–214.2; p=0.054) and statistically significant lower platelet-to-monocyte ratio, at baseline(PMR; Pn 317.3, IQR 204.6–443.8; non-Pn 436.0, IQR 353.9–597.0; p=0.012). These differences were reduced after neo-adjuvant treatment (figure 2B).

Immunophenotyping and TCR repertoire

To identify candidate immune populations to serve as peripheral blood biomarkers, we performed flow cytometry analysis in preneoadjuvant and postneoadjuvant samples from 29 patients, of which 8 developed Pn. We characterized general monocytes, NK, T and B cells as well as additional immune subpopulations (online supplemental table 2).

Similarly, as described above for blood counts, we saw higher percentage of monocytes in pretreatment samples, characterized by CD14 expression detected by flow cytometry, in patients who had developed Pn (Pn 36.6, IQR 26.6–43.4; non-Pn 25.7, IQR 17.4–35.7; p=0.017) (figure 3A). The percentage of CD14 + population at diagnosis could serve as a good predictive biomarker since its area under the ROC curve value was 0.791 (95% CI 0.613 to 0.970) (figure 3A). Nevertheless, deepening in the different monocyte subsets, we did not see significant differences between classical (CD14 +CD16−; Pn 84.0, IQR 60.7–88.6; non-Pn 78.9, IQR 57.7–88.1; p=0.591), intermediate (CD14 +CD16+; Pn 2.8, IQR 1.7–5.7; non-Pn 2.0, IQR 1.2–4.6; p=0.283) and non-classical (CD14-CD16+; Pn 2.1, IQR 1.0–6.2; non-Pn 1.5, IQR 0.6–2.9; p=0.317) monocytes (online supplemental figure S2A). In postneoadjuvant treatment samples, there was no statistical significance, neither in CD14 + general monocytes (figure 3A), nor in their different subpopulations (online supplemental figure S2A).

Regarding B cells, Pn patients had a trend to lower percentage of CD3-CD19+B cell population in pretreatment samples (Pn 12.6, IQR 7.9–23.7; non-Pn 22.5, IQR 15.1–38.8; p=0.064), that reached statistical significance after neoadjuvant treatment (Pn 5.44, IQR 3.43–11.94; non-Pn 24, IQR 14.15–35.15; p=0.002) (figure 3B). However, we did not see any differences regarding to CD25 + and CTLA4 + B cells subpopulations in both pretreatment and posttreatment samples (online supplemental figure S2B).

Patients who had developed Pn showed a significant higher percentage of CD3-CD56+ NK cells at diagnosis (Pn 11.2, IQR 2.8–15.0; non-Pn 3.3, 1.4–6.9; p=0.019), and after neoadjuvant treatment (Pn 6.57, IQR 4.66–15.3; non-Pn 2.74, IQR 1.33–4.79; p=0.005) (figure 3C). Among NK cells, we detected that PD1− percentages were elevated in patients who develop Pn (Pn 76.8, IQR 75.5–80.9; non-Pn 73.6, IQR 66.3–76.7; p=0.032), but no differences in PD1 + cells were observed in these patients (Pn 2.5, IQR 0.8–5.2; non-Pn 1.4, IQR 0.4–3.1; p=0.407) (figure 3C). Their AUC values for ROC curves were 0.786 (95% CI 0.581 to 0.990) for general NK cells and 0.762 (95% CI 0.577 to 0.947) for PD1− subset (figure 3D).

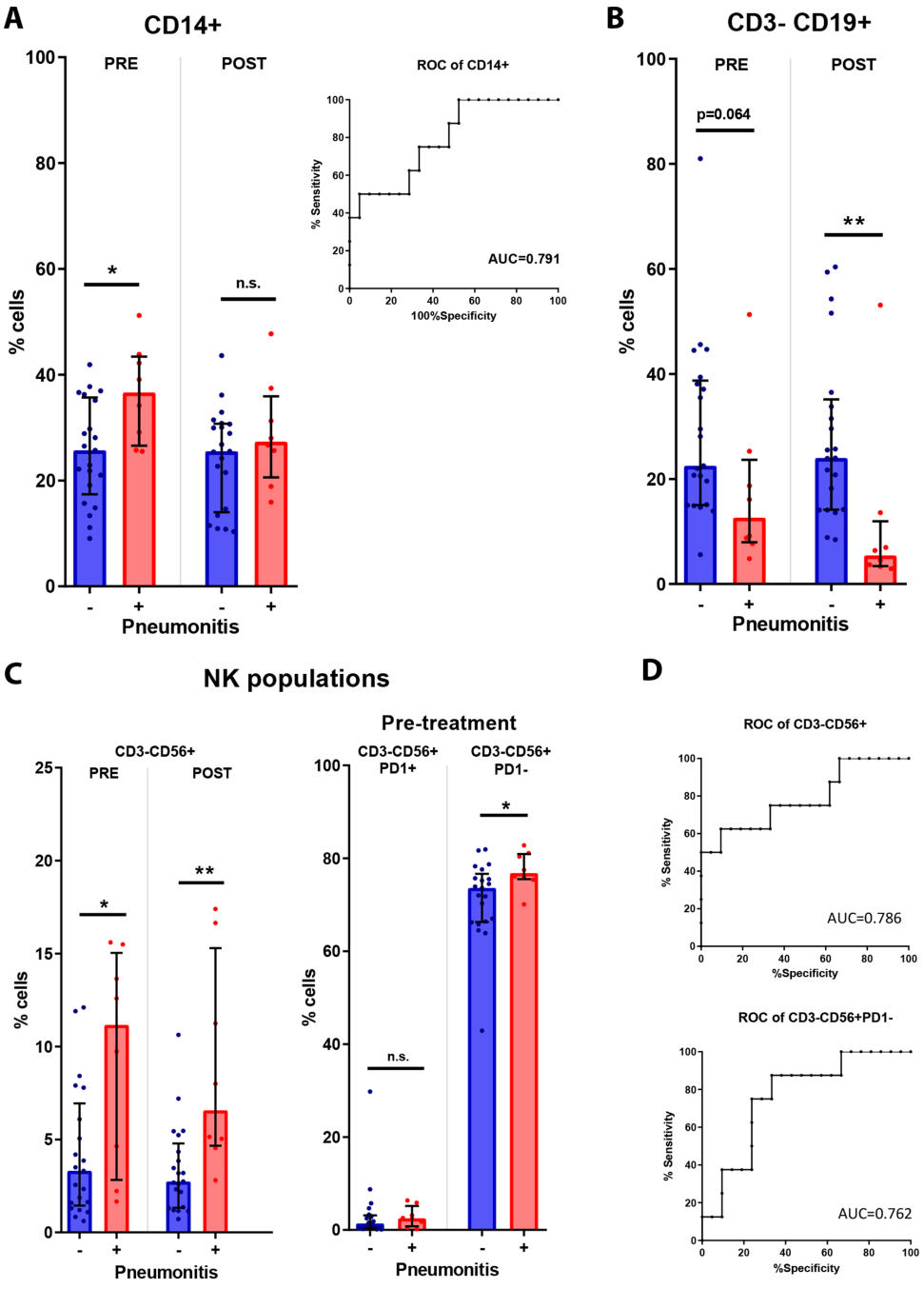

Figure 3 Flow cytometry immunophenotyping of peripheral mononuclear cells (PMBCs). (n=29 in preneoadjuvant and postneoadjuvant treatment samples) (A) CD14 + cells (total monocytes, p=0.017 and p=0.329) and AUC curve to predict pneumonitis at baseline. (B) CD3-CD19+ (total B cells, p=0.064 and p=0.002). (C) CD3-CD56+ (total NK cells, p=0.019 and p=0.005), and positive and negative PD1 subpopulations in pretreatment samples (p=0.407 and p=0.032). (D) AUC curve of total NK and PD1—subpopulation to predict pneumonitis in pretreatment samples. *P<0.05; **p<0.01; ***p<0.001. AUC, area under the curve; NK, natural killer; n.s., not significant; PD1, programmed death-1.

Related to T cells, we found a trend to lower percentage of CD3 + general T cells at diagnosis in patients with Pn (Pn 54.6, IQR 51.0–66.3; non-Pn 67.9, IQR 57.6–74.0; p=0.064), that reached statistical significance after neoadjuvant treatment (Pn 50.3, IQR 38.21–65.10; non-Pn 70.2, IQR 63.45–76.03; p=0.001) (figure 4A). In addition, patients who developed Pn suffered a greater reduction in the percentage of CD3 + T cells with treatment (Pn −8.18, IQR −13.68–5.73; non-Pn 3.9, IQR −4.9–16.63; p=0.045) (figure 4A). Looking deeper in T cells subpopulations, we found no significant differences neither in CD3 +CD4+ helper T cells nor CD3 +CD8+ pretreatment or posttreatment samples. However, in pretreatment samples Pn patients had a trend to lower percentage of CD3 +CD4+PD1+ T helper subpopulation (Pn 1.2, IQR 0.4–4.9; non-Pn 5.2, IQR 1.3–16.6; p=0.088) that was completely absent for CD3 +CD8+PD1+ T cytotoxic subpopulation (Pn 1.8, IQR 0.0–8.0; non-Pn 2.9,

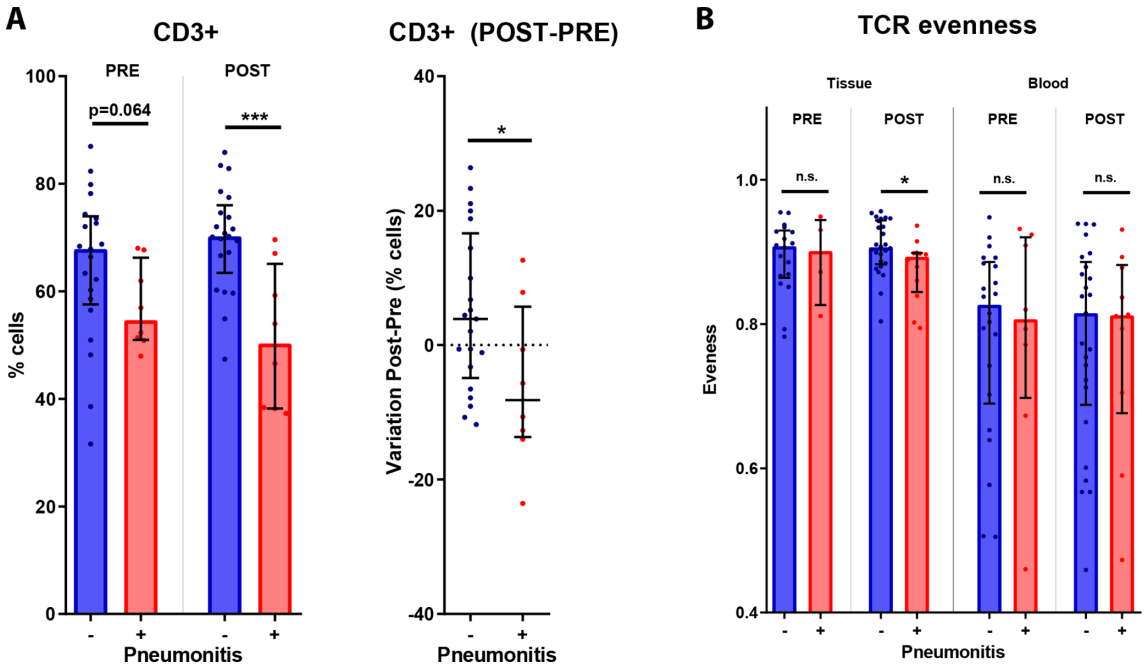

Figure 4 T cells immunophenotyping and TCR repertoire. (A) CD3+ (total T cells, n=29, p=0.064 in pretreatment and p=0.001 in postneoadjuvant treatment samples, respectively) and shift in CD3 + population calculated by POST-PRE differences (p=0.045). (B) T cell receptor repertoire evenness at diagnosis and postneoadjuvant treatment in both tissue (n=22; p=0.865 for pretreatment and n=38; p=0.028 for postneoadjuvant treatment) and blood (n=30; p=0.815 for pretreatment and n=35; p=0.827 for posttreatment samples). *P<0.05; **p<0.01; ***p<0.001. n.s., not significant; TCR, T cell receptor.

IQR 0.3–10.4; p=0.329) (online supplemental figure S3A).

In addition to immunophenotyping, we characterized the T cell repertoire by massive TCR sequencing, in both, tumor (22 samples at diagnosis, 4 Pn; and 38 samples at surgery, 12 Pn) and blood (30 pretreatment samples, 8 Pn; and 35 postneoadjuvant samples, 10 Pn). We found no differences in TCR diversity, convergence, and expanded or contracted clones, between patients who developed Pn and those who did not, for any of the time-points or compartments (online supplemental figure S3B and online supplemental table 3). However, we found significant lower TCR evenness levels in postneoadjuvant tissue samples (Pn 0.89: IQR 0.84–0.90; non-Pn 0.90, IQR 0.88–0.94; p=0.028) (figure 4B).

Cytokine analysis

In order to describe additional blood biomarkers, we performed further protein analysis in baseline (30 patients, 8 developed Pn) and postneoadjuvant (34 patients, 10 developed Pn) plasma samples. Among protein measured (online supplemental table 4), significantly lower baseline levels of teratocarcinoma-derived growth factor 1 (TDGF1) (Pn 582.5, IQR 272.0–1150.4; non-Pn 2174.6, IQR 775.8–3289.8; p=0.013) were found in patients who develop Pn (figure 5A). On the contrary, patients who develop Pn showed significantly higher levels of plasmatic macrophage stimulating protein (MSP) (Pn 1564.1, IQR 1477.2–1599.9; non-Pn 1358.6, IQR 1144.4–1521.2; p=0.006), Poly(A)-specific ribonuclease (PARN) (Pn 774.5, IQR 482.0–1031.3; non-Pn 480.6, IQR 251.0–653.0; p=0.017) and E-cadherin (Pn 1321.5, IQR 843.9–2076.7; non-Pn 683.2, IQR 555.5–947.3; p=0.022) at diagnosis. Also, a trend for higher CCL22 levels, also known as macrophage-derived chemokine (MDC), was found in patients who developed pneumonitis (Pn 2281.8, IQR 1342.3–3168.0; non-Pn 941.7, IQR 612.5–1970.2; p=0.055) (figure 5A). However, these differences were lost after neoadjuvant treatment, only a trend to a lower TDGF1 (Pn 339.4, IQR 81.1–1821.6; non-Pn 1969.2; IQR 691.8–6402.1; p=0.076) and a trend to higher MDC (Pn 1784.3, IQR 1243.9–3051.0; non-Pn 1132.8, IQR 606.1–1778.7; p=0.089) were observed (figure 5A). Referring to postneoadjuvant treatment samples, Pn patients showed significant higher relative levels of proinflammatory cytokines such as angiopoietin-1 (ANG-1), epithelial growth factor (EGF), Chemokine (C-C motif) ligand 16 (CCL16), neutrophil gelatinase-associated lipocalin (NGAL), macrophage migration inhibitory factor (MIF), platelet-derived growth factor (PDGF-AA), bone morphogenetic protein 4 (BMP-4), beta nerve growth factor (b-NGF), hepatocyte growth factor (HGF), neurotrophin-4 (NT-4), urokinase-type plasminogen activator receptor (uPAR) and vascular endothelial growth factor (VEGF) (online supplemental figure S4A). Likewise, a differential impact of treatment on the plasma levels of latency-associated peptide (LAP), chemokine (C-X-C motif) ligand 1, 5, 6 (CXCL1, CXCL5, CXCL6), Cathepsin S, CCL4 (MIP-1b), EGF, and metalloproteinase inhibitor 1 (TIMP-1) was observed between patients with and without Pn (online supplemental figure S5A).

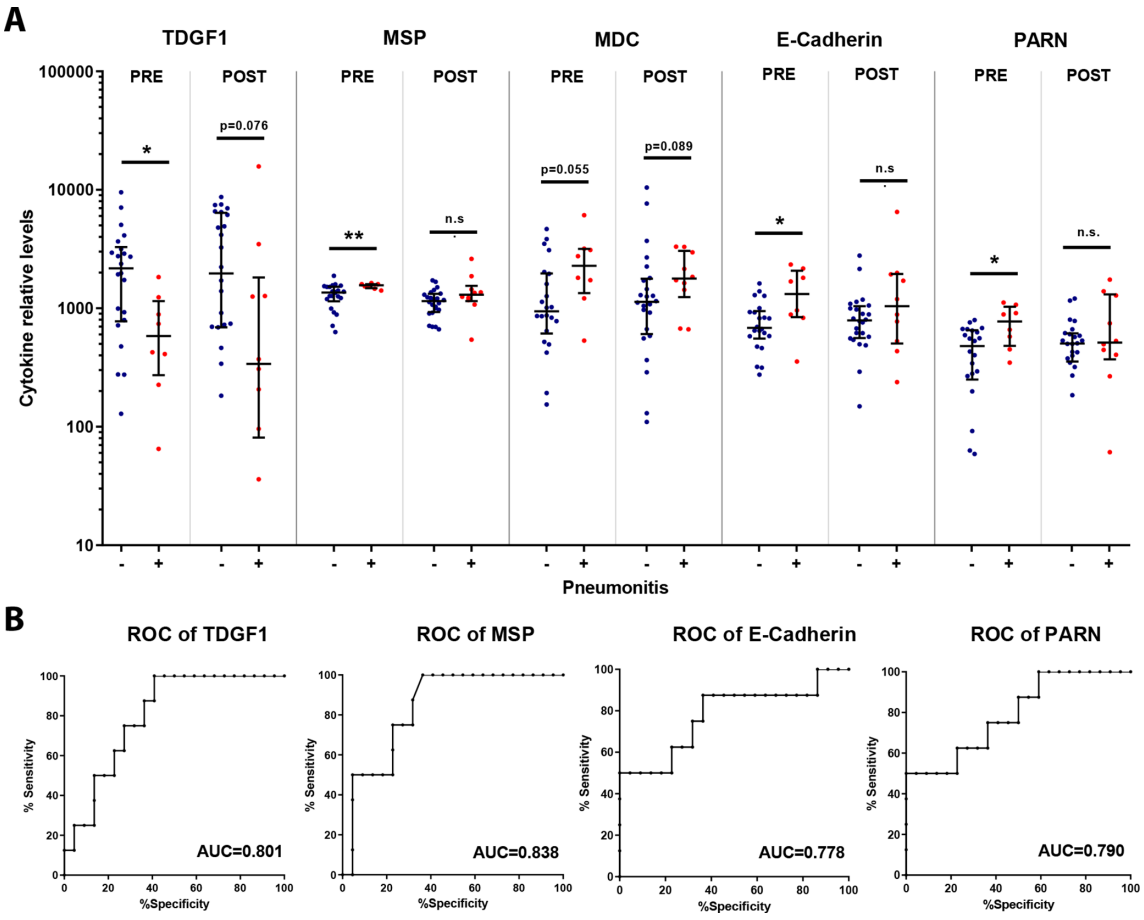

Figure 5 Cytokine levels and pneumonitis development. (n=30 pretreatment; n=34 posttreatment). (A) Relative levels of TDGF1 (p=0.013 and p=0.076), MSP (p=0.006 and p=0.104), MDC (p=0.055 and p=0.089), E-cadherin (p=0.022 and p=0.364) and PARN (Poly(A)-specific ribonuclease, p=0.017 and p=0.496). (B) AUC curves to predict pneumonitis in pretreatment samples. *P<0.05; **p<0.01; ***p<0.001. AUC, area under the curve; MDC, macrophage-derived chemokine; MSP, macrophage stimulating protein; n.s., not significant; PARN, poly(A)-specific ribonuclease; TDGF1, teratocarcinoma-derived growth factor 1.

AUC values for ROC curves to predict Pn in baseline samples were 0.801 (95% CI 0.645 to 0.958) for TDGF1; 0.838 (95% CI 0.695 to 0.982) for MSP; 0.790 (95% CI 0.604 to 0.976) for PARN; and 0.788 (95% CI 0.566 to 0.991) for E-Cadherin (figure 5B).

DISCUSSION

PN development after anti-PD-(L)1 could lead to treatment discontinuation, and in the worst cases, to death.[6] With the increasing use of anti-PD(L)1 and their introduction in the early stage first-line scenario, the absolute burden and mortality of Pn will certainly rise in NSCLC patients. Even more so when the rate of any-grade or high-grade irAEs to immunotherapy is higher in first-line compared with successive lines.[25] Despite its clinical relevance, Pn development remains an unpredictable treatment adverse effect, whose mechanisms are mainly unknown. This is even more true for chemoimmunotherapy, which is associated to higher Pn incidence; and due to its novelty, it completely lacks biomarkers or studies describing the possible mechanisms involved. Probable mechanisms of chemoimmunotherapy Pn include dysregulated immune cells, cytokines levels, and neoantigen cross-reactivity, areas that could also represent a source of predictive biomarkers.

Most hemogram parameters were not associated to Pn development in our cohort, including the NLR ratio that has been associated with anti-PD-(L)1 irAEs in previous studies.[26 27] Nevertheless, two of the platelet-related ratios appear to be relevant in this setting at diagnosis. Platelets are crucial in hemostasis and thrombosis. However, their role as modulators of immune responses has emerged in the last years. In fact, the lungs could be a source of platelet generation, with resident megakaryocytes showing a potential role in lung immunity.[28] Remarkably, the physical interaction of platelets with lymphocytes and monocytes induces these cells to have an anti-inflammatory profile, with higher levels of interleukin 10 (IL-10) and lower levels of TNF, which could explain the observed higher incidence of Pn in our patients with low PMR ratio.[29 30] In addition, it has been observed how lung injury caused by chemotherapy may involve inflammation, T cells, monocytes, and the balance of cytokines such as TNF or MCP1.[17 18] Besides, low levels of PLR ratio

prior to anti PD-(L)1 treatment have been associated to irAEs development in stage III–IV NSCLC patients.[26 27] Concerning PMR, to our knowledge, there are no data regarding its association with irAES to anti PD-(L)1, but a lower PMR has been described in patients with lupus nephritis compared with healthy controls.[31]

Using hemogram data, baseline monocyte levels showed a tendency to be increased in patients who developed Pn. Importantly, this difference in monocyte levels reached statistical significance when monocytes populations were analyzed by flow cytometry using CD14 antibody positivity. Monocytes are important regulators of the immune response, and their levels are increased in several autoimmune diseases.[32] However, no association of basal peripheral monocytes levels to anti-PD(L)1 irAEs development has been reported, although its involvement in the inflammatory component of bronchoalveolar lavage has been described.[33] In addition, we have observed elevated levels of baseline MSP (macrophage stimulating protein) associated with the development of chemoimmunotherapy-related Pn. One possible explanation is that MSP seems to inhibit inducible nitric oxide (NO) synthase, blocking the generation of NO in macrophages.[34] NO is known to induce macrophage apoptosis, downregulate superoxide activity, and generate an immunosuppressive microenvironment recruiting MDSCs and Tregs.[35] Similarly, a trend for higher levels of MDC was observed in patients who developed Pn, and MDC has been associated with lung inflammation in different models, including smoking injure, idiopathic pulmonary fibrosis and acute asthma.[36] Conversely, low baseline levels of the immunosuppressive TDGF1 (teratocarcinoma-derived growth factor 1) were found in patients who developed Pn. Exposure to TDGF1 enriched supernatants to macrophages in vitro, increased their immunosuppressive potential through increased expression of IL-10 and IL1-b.[37] Therefore, a situation with high monocytes, MSP and MDC levels (in addition to low TDGF1 levels), may lead to a basal proinflammatory profile of lungs macrophages that increases the susceptibility to Pn development. Besides, the increased levels of the nuclear protein PARN and the membrane protein E-cadherin, (proteins that are not actively secreted into the bloodstream), in the plasma of patients who developed Pn, may be indirect indicators of increased inflammatory damage at diagnosis in these patients.[38] In this sense, patients who developed Pn had elevated neutrophil levels after neoadjuvant treatment, which may reflect a pro-inflammatory state at systemic level, or even a compensatory response to repair damaged lung tissue.[39 40] Indeed, NGAL, and soluble uPAR, both related to neutrophilic inflammation,[41 42] were higher after treatment in patients with Pn. Similarly, Pn patients showed a differential increase in CXCL1, CXCL5, CXCL6 levels, also related to neutrophil response.[43] Additionally, beyond neutrophils, numerous proinflammatory cytokines and growth factors, related to angiogenesis, epithelial proliferation and wound healing, were elevated in posttreatment plasma of Pn patients, and may indicate inflammation and lung tissue repair in response to injured epithelial cells.[44 45]

On lymphocyte populations, we observed a trend for lower baseline levels of B (CD3–CD19+) and T (CD3+) cells, but statistically higher levels of NKs (CD3–CD56+) cells, in patients developing Pn. Interestingly, these differences were increased after neoadjuvant treatment, which may indicate their involvement in mechanisms maintained during chemoimmunotherapy. Although B cells in tumors and tertiary lymphoid structures are key to the antitumor response,[46] it has been described how B cells at the peripheral level may have an immunosuppressive function through the production of cytokines,[47 48] playing an important role in autoimmune disorders.[49] Thus, low levels of baseline peripheral B cells have been associated with better responses to anti-PD-1 therapy.[48 50]

T cells have been described as pivotal regulators of irAEs.[51] We observed a trend for lower CD3 + levels in Pn patients, with a similar behavior for CD4 + and CD8+ subpopulations. However, we observed a trend for lower PD1 + cells percentage in CD4 + subpopulation in patients with Pn. PD1-deficient mice develop lymphocyte-dependent myocarditis,[52] and showed an increased inflammatory and necrotic response to *Mycobacterium tuberculosis*, indicating an essential role for this coinhibitory receptor in controlling inflammatory responses.[53] Thus, lower levels of PD1 + cells may be associated with a higher frequency of Pn.

Apart from that, there are data supporting that the presence of common antigens between tumor cells and lung tissues could cause T cell dependent damage to the lungs.[54 55] An indirect measure to assess this process can be achieved through the study of the T cell repertoire or the TMB. In fact, both parameters have been associated with the development of irAEs.[51 56 57] We do not see any association between these parameters at baseline and the development of Pn. However, in surgical specimens of patients who developed Pn, we have described a lower TCR repertoire evenness, that is, a higher clonality of T cells, which could reflect a higher activation and proliferation of antitumor clones after neoadjuvant treatment, that could present cross-reactivity with lung tissue.[54 55] Anyhow, a study focused on specific clones with cross-specificities to neoantigens would be necessary, which is beyond the scope of this work. Additionally, we have seen a possible association between ARID1A and the development of Pn, not described to date. ARID1A acts as a tumor suppressor gene controlling DNA damage repair. It has been shown that mutations in ARID1A are associated with better responses to immunotherapy, probably due to a greater presence of neoantigens in these tumors,[58] which could imply a greater predisposition to Pn.

Beyond B and T cells, NK cells have a role stimulating or suppressing autoimmunity.[59] The NKs profile of the lungs is very similar to that found in peripheral blood,[60] and although there are no data on NKs association with irAEs, elevated levels of NKs have been described in the

lungs of patients with hypersensitivity Pn or organizing pneumonitis not related to anti-PD(L)-1 treatment.[61]

To conclude, this is the first study describing parameters associated with the development of Pn after neoadjuvant chemoimmunotherapy treatment. Remarkably, without ruling out the involvement of adaptive immunity in pneumonitis predisposition, our results indicate a relevant role for innate immunity parameters, with several AUC curve values higher than 0.75. Specifically, patients who developed Pn had decreased baseline levels of PMR ratio and TDGF1, but elevated baseline levels of NKs, monocytes, and MSP, E-cadherin and PARN plasma levels. In addition, we have described postneoadjuvant treatment differences that may help to elucidate the inflammatory scenario of chemoimmunotherapy related pneumonitis, involving: high levels of neutrophils and NK cells, but low levels of B and T cells in peripheral blood; increased clonality of intratumoral T cell clones; and elevated plasma levels of several growth factors (EGF, HGF, VEGF, ANG-1, PDGF, NGF, and NT4) and inflammatory cytokines (MIF, CCL16, NGAL, BMP-4, and u-PAR).

This study has several limitations, such as the post hoc nature of the analyses, the reduced number of patients, the elevated number of variables, and the lack of control arms to evaluate the impact of adjuvant treatment or differentiate the effect of chemotherapy or immunotherapy alone. All this makes it necessary to confirm the proposed biomarkers in independent cohorts of patients with appropriate statistical power.

However, our results open the possibility to predict patients at high risk of Pn, allowing the personalization of the surveillance strategy. Moreover, the precise knowledge of the mechanisms involved may allow new personalized therapies to control the harmful effects of chemoimmunotherapy.

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Contributors AC-B and MP conceived and design the study. EN, AI, JM, JC-R, MD, MM, DR-A, AM-M, JDCC, MCo, GLV, EDB, RC, NV, IBA, SV, BM, FF and MP recruited and treated patients. BS-R, AC-B, YG, RB, MCa and AG-G carried out the experiments and analyzed the data. BS-R, AC-B, YG, AR and MP, interpreted the data. All the authors read and contributed to the final version of the manuscript and approve its submission for publication. BS-R and AC-B contributed equally. AC-B and MP are joint senior authors.

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