CD8⁺PD-1⁺ to CD4⁺PD-1⁺ ratio (PERLS) is associated with prognosis of patients with advanced NSCLC treated with PD-(L)1 blockers

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ABSTRACT

Background Programmed cell death protein-1 (PD-1) expression has been associated with activation and exhaustion of both the CD4 and CD8 populations in advanced non-small cell lung cancer (aNSCLC). Nevertheless, the impact of the balance between circulating CD8⁺PD-1⁺ and CD4⁺PD-1⁺ in patients treated with immune checkpoint blockers (ICB) is unknown.
Methods The CD8⁺PD-1⁺ to CD4⁺PD-1⁺ ratio (PD-1-Expressing Ratio on Lymphocytes in a Systemic blood sample, or 'PERLS') was determined by cytometry in fresh whole blood from patients with aNSCLC before treatment with single-agent ICB targeting PD-1 or programmed cell death-ligand 1 (PD-L1 (discovery cohort). A PERLS cut-off was identified by log-rank maximization. Patients treated with ICB (validation cohort) or polychemotherapy (control cohort) were classified as PERLS+/− (above/below cut-off). Circulating immune cell phenotype and function were correlated with PERLS. A composite score (good, intermediate and poor) was determined using the combination of PERLS and senescent immune phenotype as previously described in aNSCLC.
Results In the discovery cohort (N=75), the PERLS cut-off was 1.91, and 11% of patients were PERLS+. PERLS + correlated significantly with median progression-free survival (PFS) of 9.63 months (95% CI 7.82 to not reached (NR)) versus 2.69 months (95% CI 1.81 to 5.52; p=0.03). In an independent validation cohort (N=36), median PFS was NR (95% CI 7.9 to NR) versus 2.00 months (95% CI 1.3 to 4.5; p=0.04) for PERLS + and PERLS−, respectively; overall survival (OS) followed a similar but non-significant trend. In the pooled cohort (N=111), PERLS + correlated significantly with PFS and OS. PERLS did not correlate with outcome in the polychemotherapy cohort. PERLS did not correlate with clinical characteristics but was significantly associated with baseline circulating naïve CD4⁺ T cells and the increase of memory T cells post-ICB treatment. Accumulation of memory T cells during treatment was linked to CD4⁺ T cell polyfunctionality. The composite score was evaluated in the pooled cohort (N=68). The median OS for good, intermediate and poor composite scores was NR (95% CI NR to NR), 8.54 months (95% CI 4.96 to NR) and

2.42 months (95% CI 1.97 to 15.5; p=0.001), respectively. The median PFS was 12.60 months (95% CI 9.63 to NR), 2.58 months (95% CI 1.74 to 7.29) and 1.76 months (95% CI 1.31 to 4.57; p<0.0001), respectively.
Conclusions Elevated PERLS, determined from a blood sample before immunotherapy, was correlated with benefit from PD-(L)1 blockers in aNSCLC.

INTRODUCTION

Despite recent major advances in therapeutic including targeted therapy and immunotherapy lung cancer has a particularly poor prognosis.[1] For patients with advanced non-small cell lung cancer (aNSCLC), programmed cell death-ligand 1 (PD-L1)/programmed cell death protein-1 (PD-1) blockers administered alone or in association with chemotherapy are now the backbone of treatment in most clinical settings.[2] PD-L1 expression on biopsy tumorous cells is the only biomarker currently used to select patients for potential sensitivity to immune checkpoint inhibitors (ICB). Nevertheless these biomarkers suffer several well-known limitations, including result variability related to technical issues,[3] the need for tumor biopsy tissue,[4] and the lack of host immune status evaluation.

Several circulating biomarkers have been studied as potential biomarkers.[5] Among them, PD-1 expression on circulating immune T cells is thought to reflect the activation of the PD-1/PD-L1 axis and consequently the patient's sensitivity to ICB. Studies showed promising results using circulating lymphocytes as biomarkers in patients with aNSCLC. Baseline CD8⁺PD-1⁺ T cells have been described as being associated with good prognosis with ICB.[6] Similarly, early CD8⁺PD-1⁺

T cell proliferation under ICB has been associated with good prognosis.[7 8] On the other hand, CD4$^+$PD-1$^+$ has been correlated with worse outcome of treatment naïve patients eligible for standard of care chemotherapy or tyrosine kinase inhibitors[9] and with poor clinical outcome after anti-PD-L1 treatment.[10] However, these evaluations are not consistently reported, with the absence of a validation and control groups.[11]

We hypothesized that a combination of PD-1 expression on both circulating CD4$^+$ and CD8$^+$ T cells before ICB treatment could be able to guide therapy for patients with aNSCLC. Thus, we explored the CD8$^+$PD-1$^+$ to CD4$^+$PD-1$^+$ ratio (PERLS) as a predictive biomarker of response to PD-(L)1 blockers.

MATERIALS & METHODS
Patients and treatments
Data were collected from consecutive patients with aNSCLC enrolled in three prospective studies: CEC-CTC (NCT02666612, (discovery ICB cohort)) between March 2017 and June 2018, MSN (NCT02105168, polychemotherapy (PCT) control cohort) between September 2017 and July 2018, and PREMIS (NCT03984318) (validation ICB cohort) between July 2019 and March 2020. Fresh whole blood samples were analyzed (maximum 10 samples over 3 years for each patient). The ethics committee approved these studies. All patients provided written informed consent.

In the ICB discovery and validation cohorts, patients with aNSCLC were treated with single-agent ICB (anti-PD-1 or anti-PD-L1). In the control cohort, treatment-naïve patients with aNSCLC received PCT. To be eligible, patients had to be 18 years or older, with histologically or cytologically confirmed stage III or IV NSCLC and have available fresh blood samples collected immediately prior to PCT and/or single agent ICB. The pooled cohort combined patients from the discovery and the validation cohorts.

All radiological evaluations were centrally reviewed by a senior radiologist. Tumor response was assessed according to Response Evaluation Criteria in Solid Tumors V.1.1.[12] Objective response rate (ORR) was the sum of complete response (CR) and partial response (PR), disease control rate (DCR) was the sum of CR, PR and stable disease (SD). Durable clinical benefit (DCB) was defined as CR and PR, plus SD of more than 6 months otherwise patients were considered as no-DCB.

Flow cytometry
The procedure to perform blood immune phenotyping on fresh whole blood samples has been described elsewhere.[13] Flow cytometry antibodies and fluorochromes used are described in the online supplemental methods and table S1. Two PD-1 clones were used; PD-1.3 from Beckman (first part; N=35) and MIH4 from eBioscience (second part; N=40) which recognize different epitopes of PD-1. As patients were treated with PD-1 inhibitor,

follow-up with the PD-1.3 antibody was not possible due to competition with the therapeutic antibody. In the second part of the cohort, the MIH4 clone was selected as it does not compete with therapeutic PD-1 inhibitors, thereby allowing follow-up. Result of ratio was reproducible in patients with both antibodies.

Senescent immune phenotype (SIP) was defined as positive (SIP+) when the percentage of CD28$^-$CD57$^+$KLRG1$^+$ among CD8$^+$ T cells was over 39.5%, as published elsewhere.[14]

A differentiation panel allowed phenotypic characterization of CD4$^+$ T-helper and CD8$^+$ T-cytotoxic cell subsets particularly their differentiation status (naïve (TN; CD45RA$^+$CCR7$^+$), effector memory (TEM; CD45RA$^-$CCR7$^-$), central memory (TCM; CD45RA$^-$CCR7$^+$) and terminally differentiated (TEMRA; CD45RA$^+$CCR7$^-$) T cells). CD4$^+$CD127$^{lo/-}$CD25^{high} defined regulatory CD4$^+$ T cells (T$_{reg}$) otherwise CD4$^+$ T cells were considered as conventional T cells (T$_{conv}$). These flow cytometry panels as well as their gating strategies has already been published.[15]

Intracellular staining of cytokines to determine the polyfunctionality of T cells was realized. Interferon (IFN) γ, tumor necrosis factor (TNF)α, interleukin (IL)-9, IL-4 and IL-17A were monitored. Frozen peripheral blood mononuclear cells (PBMC) were used from the discovery cohort prior to ICB administration (N=25) and after 2 months of treatment when the sample was received (N=18). Polyfunctionality was defined as the ability for a lymphocyte to secrete more than one cytokine (two or above). DurActive and DuraClone IF T Helper Cell Tubes (Beckman Coulter) were used for cytokine assessment. DurActive tubes are ready-to-use cell activation mixture containing the phorbol ester, PMA (phorbol 12-myristate 13-acetate) and a calcium ionophore (Ionomycin). DuraClone IF T Helper Cell Tubes are preformulated, dried antibody panels of six markers in fluorochrome combinations that provide robust population identification, including CD3-AF750, CD4-APC, IL-4-PC7, IL-17A-PB, IFNγ–FITC. A drop of IL-9-PE (BioLegend) and TNFα-AA700 (Beckman Coulter) was added in the DuraClone IF T Helper Cell Tubes. For the cytokine staining of PBMCs, 50 µL suspension of PBMCs (containing ~5×10^5 cells) were directly introduce into the DurActive tube, vortexed for 6–8 s and incubated at 37°C for 3 hours. After the 3 hours incubation, 25 µL of buffer R1 (PerFix-nc fixative reagent, Beckman Coulter) was added and incubate for 15 min at room temperature. After washing with 2 mL 1× phosphate-buffered saline, 25 µL of fetal bovine serum and 300 µL of buffer R2 (PerFix-nc permeabilizing reagent) were added and transferred to the DuraClone IF T Helper Cell Tube. Tubes were incubated 45 min at room temperature and 3 mL of R3 (final solution 1× in water) (PerFix-nc wash reagent, Beckman Coulter) was added. After centrifugation and aspiration of the supernatant, 500 µL of R3 (final solution 1× in water) was added. After vortex, samples were ready for FACS acquisition. Flow cytometry data were analyzed using Kaluza

Flow Cytometry Software (Beckman Coulter) by a single operator, blinded to the patients' clinical data. Graphs for flow cytometry data were produced using GraphPad Prism V.7.0 or V.8.0 (GraphPad Software, San Diego, California, USA). Online supplemental figure S1 illustrates dot plots data and gating strategies of immune cell populations.

Statistical analysis

Median values (IQR) and frequencies (percentage) were calculated for descriptions of continuous and categorical variables, respectively. Means and proportions were compared using the Student's t-test and χ^2 test (or Fisher's exact test, if appropriate), respectively. Overall survival (OS) was defined as the time between ICB initiation and death from any cause. Progression-free survival (PFS) was defined as the time between ICB initiation and progression or death, whichever occurred first. OS and PFS were estimated using the Kaplan-Meier method and described using median values with their 95% CIs. Follow-up was calculated using the reverse Kaplan-Meier method. To better stratify PFS risk, an optimal cut-off for the PD-1$^+$ Expression Ratio on Lymphocyte on Systemic sample, or 'PERLS' (CD8$^+$PD-1$^+$/CD4$^+$PD-1$^+$) was obtained with the log-rank maximization method.[16] The ratio CD8$^+$PD-1$^+$/CD4$^+$ PD-1$^+$, expressed as a percentage of total CD8$^+$ or total CD4,$^+$ respectively, was assessed by flow cytometry in blood and patients were classified as having a high or low PERLS ratio (PERLS+ or PERLS−). Analyses were reported according to REMARK guidelines for prognostic studies.[17] A composite PERLS score was determined based on PERLS and SIP; as SIP$^+$ is a marker for non-responders, responders were selected by applying PERLS to SIP$^-$ patients. Patients were categorized as good composite score if they were PERLS+ and SIP$^-$, as poor composite score if they were PERLS− and SIP$^+$, and as intermediate composite score otherwise. The composite score was evaluated in patients from the discovery and exploratory cohorts with available data for both variables. The discrimination of the Cox models was assessed with the c-index. Statistical analyses were performed with RStudio, V.2.15.2. P values<0.05 were considered statistically significant and all tests were two-sided.

RESULTS
Patient characteristics

PERLS ratio analyses were performed prior to treatment in 84 patients with aNSCLC in the discovery cohort. Of them, 76 then received at least one ICB infusion, had available cytometry data, and 75 were evaluable for response. Patient characteristics for the discovery cohort are presented in table 1. Median follow-up was 20.1 months (95% CI 16.9 to 31.2). Among 75 patients evaluable for response, the ORR was 25.3% (19/75), and the DCR was 53.3% (40/75), with 35 patients (46.7%) having progressive disease as a best response. Median PFS and OS were 3.68 months (95% CI 1.87 to 5.68), 9.40 months (95% CI 6.77 to 18.96), respectively.

In the PCT cohort, PERLS analysis was performed in 39 treatment-naïve patients with aNSCLC eligible for PCT. After a median follow-up of 6.0 months (95% CI 4.6 to 6.8), the ORR was 33.3% (13/39), and the DCR was 85.0% (33/39). Median PFS and OS were 4.99 months (95% CI 4.17 to not reached (NR)) and 7.10 months (95% CI 6.96 to NR), respectively.

In the validation cohort, PERLS analysis was performed in 36 treatment-naïve patients with aNSCLC eligible for ICB. Patient characteristics of this cohort are reported in online supplemental table S2. Median follow-up was 17.3 months (15.1–20.8), ORR was 25.0% (9/36), and DCR was 33.3% (12/36). Median PFS and OS were 2.5 months (95% CI 1.8 to 4.7) and 8.6 months (95% CI 3.2 to NR), respectively.

PERLS cut-off, outcome and clinical characteristics
Discovery cohort

As both CD8$^+$PD-1$^+$ and CD4$^+$PD-1$^+$ have been implicated in outcome with ICB in published data, we focused on the CD8$^+$PD-1$^+$/CD4$^+$PD-1$^+$ ratio to define PERLS. In the ICB discovery cohort (n=75), the median PERLS value was 1.18. The cut-off for survival risk computed by log-rank maximization method was 1.91. In this cohort, 11% (8/75) of patients had PERLS >1.91 and were classified as high PERLS (PERLS$^+$).

Patients with DCB had significantly higher PERLS than those without (mean 1.42 vs 0.89 p=0.0022) (online supplemental figure S2A). No significant difference was seen with the CD8$^+$/CD4$^+$ ratio (ie, not incorporating PD-1 expression) for either DCB or no-DCB (median 0.62 vs 0.71; p=0.66) (online supplemental figure S2B). PERLS$^+$ patients had significantly better median PFS (9.63 months 95% CI 7.82 to NR; p=0.03) compared with PERLS$^-$ patients (2.69 months; 95% CI 1.81 to 5.52) (online supplemental figure S2C). High PERLS was also associated with better OS without reaching significance, with median OS NR (95% CI 9.6 to NR) versus 8.11 months (95% CI 6.31 to 18.00).

Validation, pooled and control cohorts

In the ICB validation cohort, PERLS was available for 36 patients. Median PERLS value was 0.93 (0.77–1.21). Using the cut-off of 1.91, patients with a high PERLS value had a median PFS of NR (95% CI 7.9 to NR) versus 2.0 months (95% CI 1.30 to 4.50; p=0.04) compared with patients with a low PERLS value. The same trend was found for median OS which was NR (95% CI NR to NR) versus 7.1 months (95% CI 3.1 to NR; p=0.09).

We then evaluated PERLS in the pooled cohort, discovery and validation cohorts (N=111). Patients with DCB had significantly higher PERLS values than patients with no-DCB (mean 1.43 vs 1.06; p=0.002) (figure 1A). This difference was not found for the CD8$^+$/CD4$^+$ ratio (mean ratio was 0.69 DCB vs 0.74 no-DCB; p=0.79) (figure 1B). PERLS + patients had significantly longer median PFS compared with PERLS− patients (9.63 months, 95% CI 7.82 to NR vs 2.20 months, 95% CI 1.81 to 4.50; p=0.004)

Table 1 Patient characteristics in the discovery cohort

		All patients (N=75)	Low PERLS (N=67)	High PERLS (N=8)	P value
Age in years, median (IQR)		61 (55.5; 70.5)	61 (56; 71.5)	55.5 (52.75; 61.5)	0.974
Sex, n (%)	Female	35 (46.7)	32 (47.8)	3 (37.5)	*0.716*
	Male	40 (53.3)	35 (52.2)	5 (62.5)	
Smoking history, n (%)	Never	8 (10.8)	6 (9.1)	2 (25)	*0.396*
	Former	39 (52.7)	35 (53.0)	4 (50)	
	Active	27 (36.5)	25 (37.9)	2 (25)	
	Missing	*	*	0	
Histology, n (%)	Adenocarcinoma	61 (81.3)	53 (79.1)	8 (100)	*0.535*
	Squamous	11 (14.7)	11 (16.4)	0 (0)	
	Other*	3 (4)	3 (4.5)	0 (0)	
TNM†, n (%)	III	13 (17.3)	12 (17.9)	1 (12.5)	*
	IV	62 (82.7)	55 (82.1)	7 (87.5)	
Tumorous PD-L1 (% tumorous cells), n (%)	<1	10 (25)	8 (22.9)	2 (40)	*0.654*
	1%–49	6 (15)	6 (17.1)	0 (0)	
	>49	24 (60)	21 (60)	3 (60)	
	Missing	35	32	‡	
Main molecular alteration, n (%)	K-RAS	16 (37.2)	16 (37.2)	0 (0)	*
	Wild-type‡	18 (41.9)	18 (41.9)	0 (0)	
	Targetable§	9 (20.9)	9 (20.9)	0 (0)	
	Missing	32	24	8	
N metastatic sites, n (%)	≤2	54 (72)	49 (73.1)	5 (62.5)	*0.679*
	>2	21 (28)	18 (26.9)	3 (37.5)	
ECOG PS, n (%)	0–1	65 (86.7)	57 (85.1)	8 (100)	*0.587*
	†	10 (13.3)	10 (14.9)	0 (0)	
Lines of prior therapy, n (%)	0	6 (8)	4 (6.0)	2 (25)	*0.121*
	≥1	69 (92)	63 (94.0)	6 (75)	
Prior chemotherapy, n (%)	No	6 (8)	4 (6.0)	2 (25)	*0.121*
	Yes	69 (92)	63 (94.0)	6 (75)	
Prior radiotherapy, n (%)	No	51 (68)	45 (67.2)	6 (75)	*
	Yes	24 (32)	22 (32.8)	2 (25)	
dNLR ≥3, n (%)	No	36 (60)	30 (57.7)	6 (75)	*0.457*
	Yes	24 (40)	22 (42.3)	2 (25)	
	Missing	15	15	0	
IrAEs (grade ≥3 or requiring CS)¶	Yes	5 (67)	4 (6)	1 (13)	*0.437*
	No	70 (33)	63 (94)	7 (87)	
LDH≥ULN, n (%)	No	20 (47.6)	17 (46.0)	3 (60)	*0.656*
	Yes	22 (52.4)	20 (54.1)	2 (40)	
	Missing	33	30	‡	
LIPI score**, n (%)	Good	10 (24.4)	8 (22.2)	2 (40)	*0.482*
	Intermediate	22 (53.7)	19 (52.8)	3 (60)	
	Poor	9 (22.0)	9 (25)	0 (0)	
	NA	34	31	‡	

*Large cell lung cancer, non-small cell lung cancer, not otherwise specified.
†TNM stage eighth edition.
‡Absence of *EGFR* mutations, *KRAS*, *ALK*, *ROS1* rearrangements.
§*EGFR* mutations, *ALK*, *ROS1* rearrangement, *HER2* mutations, *MET* alterations, *BRAF* mutations. Radiotherapy (including stereotactic radiotherapy) on any site (including bone or central nervous system).
¶Two patients with grade 3 colitis, one with grade 3 pneumonitis and two patients with grade 2 pneumonitis requiring cortico-steroids treatment.
**LIPI high: dNLR ≥3 and LDH ≥ULN; LIPI intermediate: dNLR <3 and LDH ≥ULN or dNLR ≥3 and LDH <ULN; LIPI low: dNLR <3 and LDH <ULN.
dNLR, derived neutrophil to lymphocyte ratio (neutrophils/(leukocytes -neutrophils)); IrAEs, immune related adverse events; LDH, lactate dehydrogenase; LIPI, lung immune prognostic index; PD-L1, programmed cell death-ligand 1 ; PS, performance status; TNM, tumour, node, metastases; ULN, upper limit of normal.
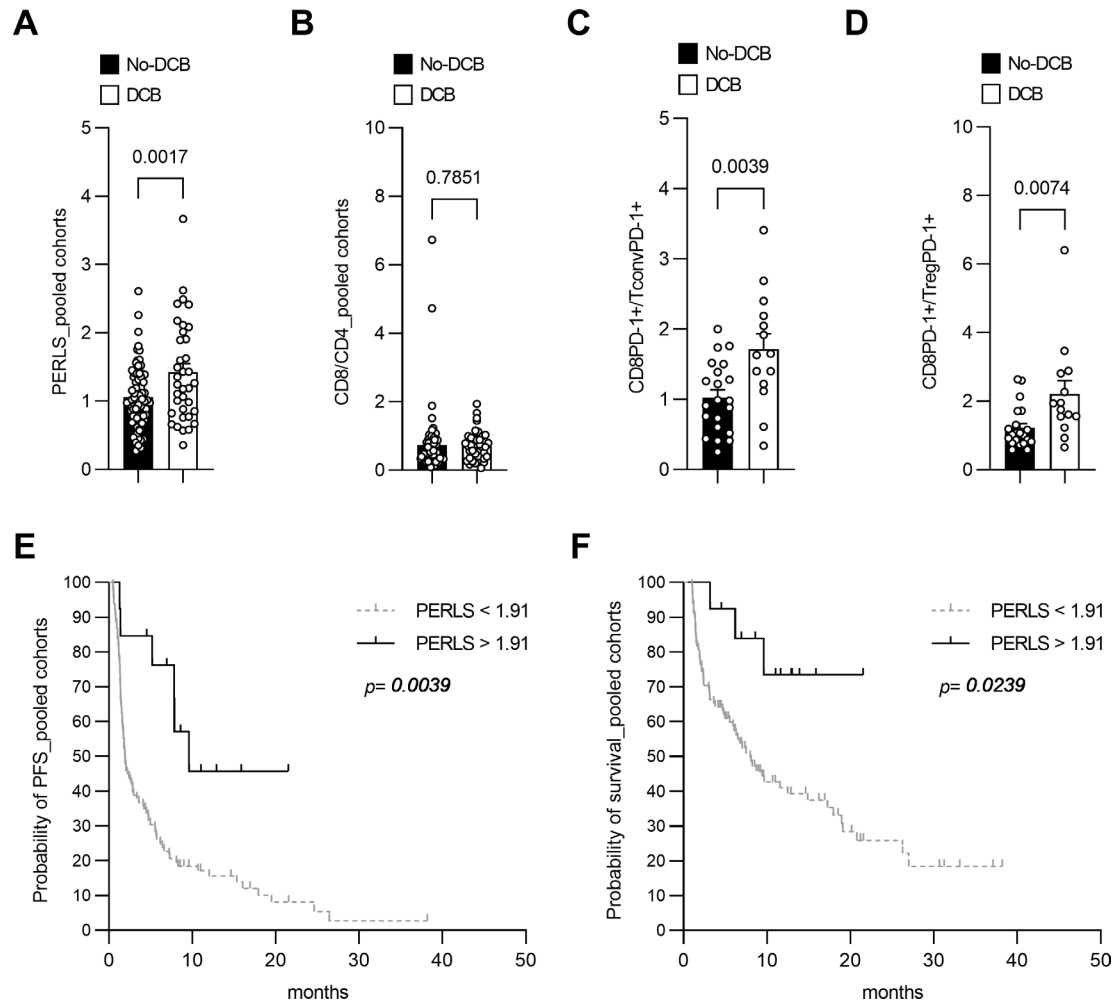
Figure 1 PERLS is associated with disease clinical benefit (DCB) in advanced non-small cell lung cancer treated with immune checkpoint blockers. Blood T cells populations from patients included in the discovery and the validation cohorts, that is, pooled cohort. (A) PERLS ratio from the pooled cohort in patients with no-DCB and DCB. (B) As in (A) but with the CD8+/CD4+ ratio. (C) As in (A) but with CD8+ PD-1+/Tconv PD-1+ ratio. (D) As in (A) but with the CD8+PD-1+/TregPD-1+ ratio. (E) PFS according to PERLS. (F) Overall survival according to PERLS. PD-1, programmed cell death protein-1; PFS, progression-free survival.

(figure 1C). High PERLS was also associated with better OS (NR, 95% CI NR to NR vs 7.98 months, 95% CI 5.95 to 14.90; p=0.02) (figure 1D).

To assess the predictive or prognostic role of PERLS, we evaluated a control cohort of patients treated with PCT without ICB (N=39). There was no significant difference in PFS for patients with high or low PERLS (5.91 months, 95% CI 4.17 to NR vs 4.83 months, 95% CI 3.84 to NR; p=0.20) (online supplemental figure S3).

PERLS and clinical characteristics

Clinical and pathological characteristics of both the discovery and validation cohorts are summarized in table 1 and online supplemental table S2 for the whole cohort and according to PERLS status. No clinical characteristics were associated with the PERLS ratio, notably PD-L1 (by immunohistochemistry), mutational status or tumor burden. Nine patients had an oncogenic addiction, including four patients with BRAF (two patients with V600E mutation), one patient with EGFR activating

mutation, two patients with HER2 mutation, one patient with ROS1, and one patient with RET rearrangement. Patient with EGFR mutation and one patient with BRAF V600E were pretreated with tyrosine kinase inhibitor before ICB. All these patients with oncogenic mutation had a low PERLS.

Regarding immune-related adverse events (IrAEs) we focused on the grade ≥3 or those of grade 2 but requiring medical intervention including suspension or stop of ICB and corticosteroids treatment. There was no difference between the low and high PERLS groups in the discovery or validation cohort.

### PERLS correlation with lymphocyte phenotypic profile

At the time of data analysis, a differentiation panel and a Treg panel were available for 75 and 42 patients, respectively, in the discovery cohort. Evaluation after 2 months of ICB treatment (D60) was available in 24 of the 75 patients (32%) for PERLS and in 43 of the 75 patients (57%) for the differentiation panel.

PERLS and Treg

Since a higher CD8$^+$PD-1$^+$/CD4$^+$PD-1$^+$ ratio is associated with better prognosis, we wondered whether increased/decreased immunosuppression could influence this outcome. To answer this question, we monitored Treg in a subcohort of 42 patients and we analyzed T$_{conv}$ (non-T$_{reg}$ CD4$^+$ T cells) and T$_{reg}$ separately. Interestingly, both CD8$^+$PD-1$^+$/T$_{conv}$PD-1$^+$ and CD8$^+$PD-1$^+$/T$_{reg}$PD-1$^+$ ratio were higher in patients with DCB compared with patients with no-DCB (p=0.004 and p=0.007, respectively; figure 1E–F). This suggests that a greater accumulation of CD4$^+$PD-1$^+$ relative to CD8$^+$PD-1$^+$ is deleterious irrespective of the regulatory or non-regulatory phenotype of CD4$^+$ T cells.

PERLS, T cell differentiation and polyfunctionality

PERLS was positively correlated with the proportion of blood naïve CD4$^+$ T cells (CD45RA$^+$CCR7$^+$) at baseline (r=0.60; p<0.001) (figure 2A and B). The proportion of naïve CD4$^+$ T cells was not correlated with DCB (figure 2C), however the negative evolution (decrease) between D60 and D0 of naïve CD4$^+$ T cells was associated with DCB (figure 2D, left panel). Interestingly, only the positive evolution (increase in memory T) between D60 and D0 of memory CD8$^+$ T cells was associated with DCB (figure 2D, right panel). This suggests that differentiation from naïve to memory T cells was associated with clinical benefit. In accordance with this observation, an increase of cytokine production in CD4$^+$ T cells at D60 was
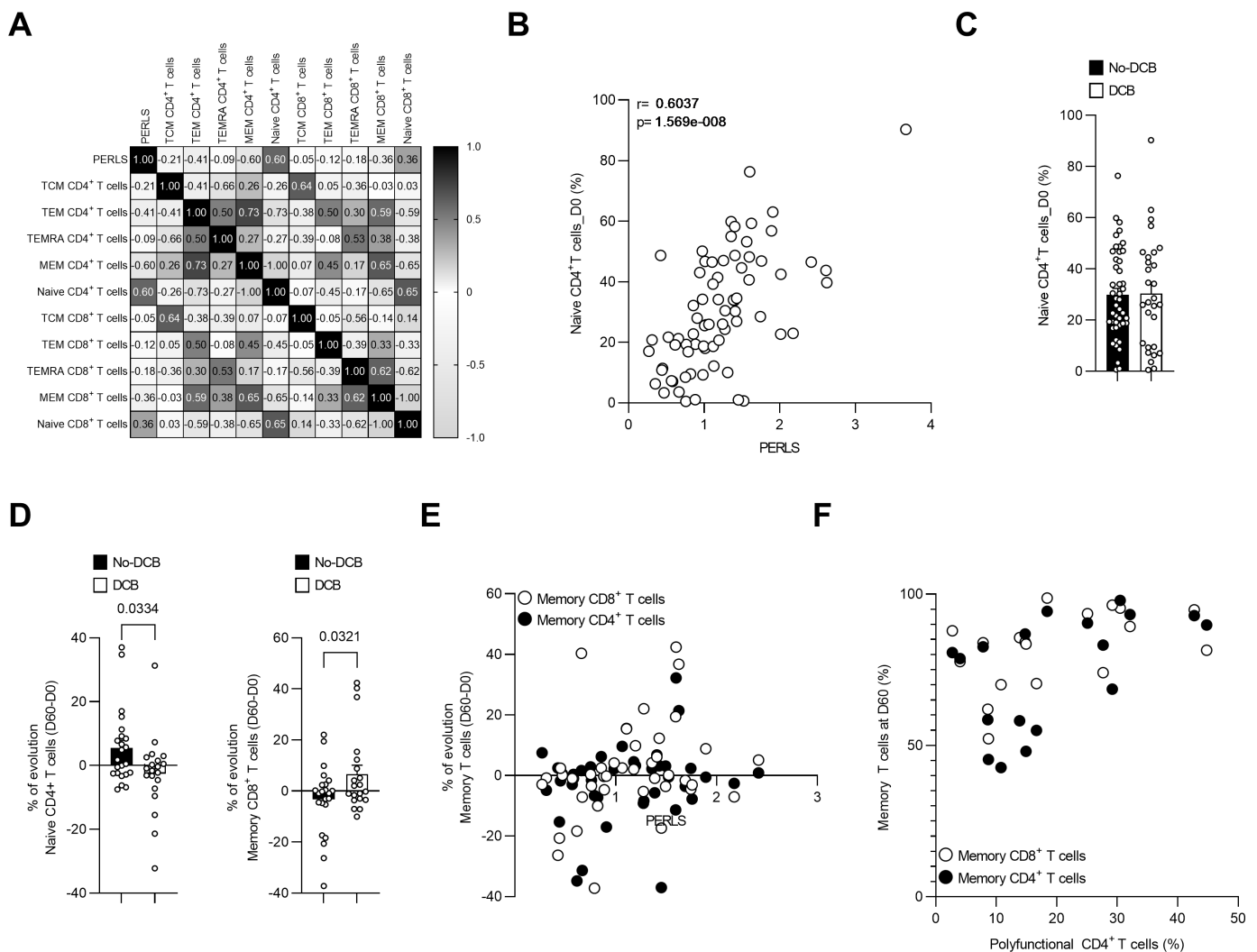
Figure 2 The PERLS ratio correlates with naïve T cells at baseline and induction of memory T cells under ICB treatment. Proportion of naïve (CCR7$^+$CD45RA$^+$), effector memory (TEM; CCR7$^-$CD45RA$^-$), central memory (TCM; CCR7$^+$CD45RA$^-$) and terminally differentiated (TEMRA; CCR7$^-$CD45RA$^+$) among CD4$^+$ (CD3$^+$CD4$^+$) and CD8$^+$ (CD3$^+$CD8$^+$) T cells were assessed at baseline (D0) and post-ICB treatment (D60) in the blood of patients. (A) Correlation matrix of PERLS and T cell subsets at baseline (B) Baseline (D0) correlation of naïve CD4$^+$ T cells and PERLS. (C) Baseline (D0) naïve CD4$^+$ T cells in patients with no-DCB and DCB. (D) Evolution (%) from D60 to D0 (%D60–%D0) of naïve CD4$^+$ T cells (left panel) and evolution (%) from D60 to D0 (%D60–%D0) of memory CD8$^+$ T cells (right panel). (E) Correlation of PERLS and evolution (%) of memory CD4$^+$ (black circles) and CD8$^+$ T (white circles) cells. (F) Correlation of memory CD4$^+$ (black circles) and CD8$^+$ T cells (white circles) at D60 and polyfunctionality of CD4$^+$ T cells at D60. DCB, durable clinical benefit; ICB, immune checkpoint blockers.

observed only in patients benefiting from the treatment (online supplemental figure S4B) and not in patients with no-DCB (online supplemental figure S4A). Moreover, in patients with DCB, polyfunctional CD4$^+$ T cells were significantly more frequent at D60 compared with patients with no-DCB (online supplemental figure S4D). No such difference could be observed at D0 suggesting that polyfunctional T cells were induced by ICB treatment (online supplemental figure S4C). We next evaluated if PERLS could be linked to the induction of memory T cells after ICB treatment. Among the 42 patients where both data were collected, PERLS was positively correlated with the positive evolution (increase) of memory T cells (figure 2E). This correlation was more significant for the evolution of memory CD8$^+$ T cells (r=0.31; p=0.04). Among patients where polyfunctionality of T cells was assessed before treatment (N=25), 18 were also monitored at D60. Among these 18 patients, the proportion of memory CD8$^+$ (r=0.46, p=0.05; N=18) and CD4$^+$ (r=0.58, p=0.01; N=18) T cells at D60 was positively associated with the polyfunctionality of T cells after ICB treatment (figure 2F), no such observation could be depicted at D0 (data not shown).

PERLS and senescence

Based on a previous study showing an association between SIP and worse prognosis,[13] we evaluated our population according to a composite prognostic score using the pooled discovery and validation cohort. PERLS was available for 111 patients, 13 of whom (11.2%) had a high PERLS. Overall, 69 patients had a SIP evaluation, 19 of whom (28.0%) were SIP$^+$. PERLS and SIP were available in 68 patients, with 8 (11.8%), 18 (26.5%) and 42 (61.8%) having good, poor or intermediate composite score, respectively. In the overall population, PERLS alone had a specificity and sensitivity of 38.9% and 93.5%, respectively, to detect responders. SIP and PERLS were inversely correlated (figure 3A). As described in our previous study,[13] in the pooled cohort, SIP$^-$ was associated with longer median PFS of 5.06 months (95% CI 2.0 to 12.0; p<0.0001 for SIP–) versus 1.82 months (95% CI 1.31 to 4.5) and longer median OS of 20.80 (95% CI 7.80 to NR; p=0.002) versus 2.43 months (95% CI 2.17 to 15.5). Considering the composite score, outcomes differed for the good, the intermediate and the poor composite score groups. Indeed, the median PFS was 12.6 months (95% CI 9.63- to NR) versus 2.58 months (95% CI 1.74 to 7.29) and 1.76 months (95% CI 1.31 to 4.57; p<0.0001) (figure 3C) and the median OS was not reach (95% CI NR to NR) versus 8.54 months (95% CI 4.96 to NR) and 2.42 months (95% CI 1.97 to 4.73; p=0.001), respectively, (figure 3D). The c-index of this strategy was 0.627 for PFS and 0.646 for OS. Of note all patients in the good composite score group had a DCB (8/8). Only one patient with poor composite score had a DCB (1/18) (figure 3B). None of the 8 patients in the good composite score group died early (<3 months) compared with 24% in the intermediate composite score group (10/42 patients) and 56% of

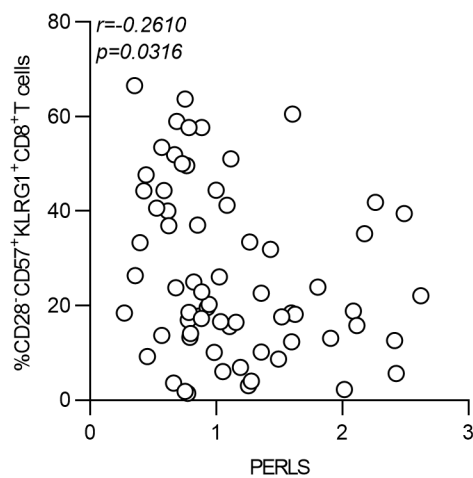the poor composite score group (10/18; p=0.007; online supplemental figure S3).

DISCUSSION

We describe here the predictive value of a preimmunotherapy ratio, CD8$^+$PD-1$^+$/CD4$^+$PD-1$^+$ (PERLS) in blood samples, for patients with aNSCLC and treated with ICB. These results also shows that PERLS is associated with a naïve CD4$^+$ T cells at baseline and suggest that the ability to accumulate memory T cells and polyfunctional CD4$^+$ T cells during treatment is associated with better prognosis. Moreover, PERLS seemed to predict ICB response and not only prognosis since no such observation was made in the chemotherapy cohort.
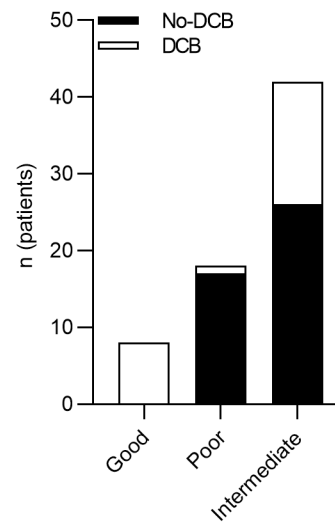
Most of studies have focused on tumor-infiltrating lymphocytes (TILs) and other immune cells in the tumor microenvironment (TME). Many studies have shown that ICB efficacy was dependent on rejuvenation of TILs,[18] CD8$^+$ T cells from the invasive margin of the tumor.[19] CD8$^+$PD-1$^+$ TILs have been shown to be more tumor specific.[20 21] PD-1^{high} TILs have been associated with better prognosis under ICBs.[21] Nevertheless, there is increasing evidence suggesting that expansion and recruitment of peripheral T cells during ICB treatment may have a prominent role to play in disease progression.[22] First, several studies have confirmed that intratumor T lymphocytes have limited expansion ability in melanoma.[23 24] Second, some studies suggested that circulating T cells can be recruited, leading to a 'clonal replacement' rather than the rejuvenation of existing T cell clonotypes.[24] T cell receptor (TCR) clonotype analysis in mice and humans have shown that new clonotypes appear in tumors during treatment and that some of these novel TIL clones can be recruited from peripheral sources.[24] In another study, clonotypic expansion of effector-like T cells in tumor tissue, adjacent tissue and peripheral blood has been associated with better response to PD-(L)1 therapy. A recent study compared both single cell TCR sequencing and RNA sequencing on paired tumor and blood samples.[25] In this study, CD8$^+$ T cells that developed in tumors was preexisting in blood. These data support that in responsive patients, intratumor T cells are replenished with fresh, non-exhausted T cells, that originate from outside the tumor.[25]

We focused on blood T cells and particularly PD-1$^+$ expressing T lymphocytes. As in TME, circulating PD-1$^+$ T cells have also been reported to be tumor-specific in melanoma.[26] Notably, circulating and tumor infiltrating CD8$^+$PD-1$^+$ shared a similar TCR repertoire suggesting that there is a relationship between circulating and infiltrating PD-1$^+$ T lymphocytes.[26] PD-1$^+$ T cell in tumor-draining lymph nodes were also described as tumor-specific,[27] and can interact closely with dendritic cells. In mice, the targeting of PD-L1-expressing dendritic cells in the tumor draining lymph node induced seeding of the tumor site with T cells, and improved tumor control.[27] In a recent study, deeper characterization of exhausted CD8$^+$ T
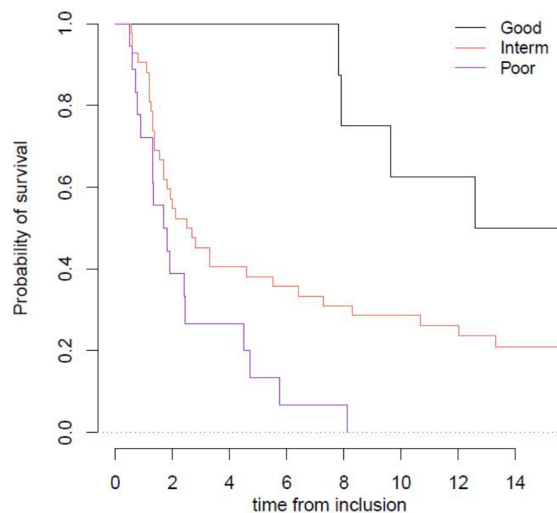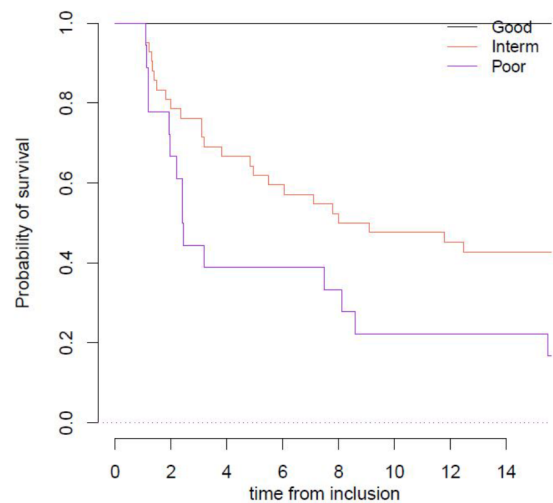
A

B

C

D

Figure 3 SIP and PERLS composite score. Proportion of SIP (CD3$^+$CD8$^+$CD28$^-$CD57$^+$KLRG1$^+$) cells were determined at D0 in the blood of patients treated with immune checkpoint blockers. (A) Correlation of SIP and PERLS. (B) Number of patients with DCB and no-DCB according to the good (SIP– and PERL+), poor (SIP+, PERLS–) and intermediate (SIP+, PERLS+ or SIP–, PERLS–) composite score. (C) Progression-free survival according to the good, intermediate and poor composite score. (D) As in (C) but with overall survival. DCB, durable clinical benefit; SIP, senescent immune phenotype.

cells population was evaluated. This work highlighted the existence of a four-stage developmental hierarchy of exhausted CD8$^+$ T cells.[28] Two populations were progenitor (TCF1^{high}Tox^{high} and TCF1^{int}Tox^{high}), with TCF1^{high}Tox^{high} being quiescent and resident and able to convert to TCF1^{int}Tox^{high}. This conversion was associated with a delocalization from lymphoid residence to blood locations and a proliferation-driven transition to a third subpopulation

TCF1^{neg}T-bet^{high}Tox^{int} that have similarities to circulating 'effector-like' cells. Finally TCF1^{neg}T-bet^{high}Tox^{int} could convert into TCF1^{neg}T-bet^{low}Tox^{high}Eomes^{high} these terminally differentiated exhausted CD8$^+$ T cells are absent from the blood but can accumulate in tumors.[28] Interestingly, intermediate circulating 'effector-like' exhausted CD8$^+$ T cells can be activated and proliferate under PD-(L)1 blockade while terminally exhausted CD8$^+$ T cells

cannot,[28] suggesting a prominent role for these circulating T cells during immunotherapy. These results support that the recruitment of new T cell clones has an important role in the response to treatment and not only expansion/reactivation of intratumor T cells that were already in the tumor site before the initiation of treatment.

In our study the PD-1^{+} population have a predictive role in patients with aNSCLC treated with ICB. The CD8/CD4 ratio has been associated with chronic activation of the immune system, in particular in the elderly.[29] Nevertheless, in our study elevated CD8/CD4 ratio was not associated with outcomes. As PD-1 is expressed by CD4^{+} and CD8^{+} during activation,[30] the consideration of these two parameters via the PERLS ratio integrates the balance between 'helper' CD4^{+} T cells and 'cytotoxic' CD8^{+} T cells. As already stated, the prognostic value of circulating CD8^{+}PD-1^{+} and CD4^{+}PD-1^{+} in patients with aNSCLC has already been explored. However, while CD8^{+}PD-1^{+} T cells tended to be associated with a favorable clinical outcome,[6] CD4^{+}PD-1^{+} T cells were associated with a poor outcome.[9 10] In our study, high PERLS is associated with good prognosis while low PERLS with poor prognosis. This data demonstrates the importance of both the CD8^{+} T cell and the CD4^{+} T cell compartments. In line with this observation blocking the migration of immune cell from the secondary lymphoid organ impaired response to ICB in murine preclinical models. In this model, peripheral T cells were mandatory for antitumor activity, in particular a subset of activated effector memory Th1 CD4^{+} T cells, expressing low levels of PD-1 and CD127.[31] This work emphasized the need of poorly exhausted CD4^{+} T cells to reach clinical response in hosts treated with ICB. Another work showed that IL-6 could favor PD-1 upregulation on both blood CD8^{+} and CD4^{+} in aNSCLC and that high IL-6 levels was associated with altered T cells functions.[32] In this work, IL-6 increased more PD-1 on CD4^{+} T cells than on CD8^{+} T cells suggesting that a low PERLS could also reflect inflammatory mechanisms associated with resistance during ICB treatment. Thus, the addition of the helper CD4^{+} compartment may improve the accuracy of blood biomarkers and thus help to better identify patients likely to respond to ICB.

ICB have been suspected to favor immunoregulation by activating T regulator (Treg), however the impact of PD-1/PD-L1 axis on Treg remains poorly understood.[33 34] CD8^{+}PD-1^{+}/T_{reg} PD-1^{+} and CD8^{+}PD-1^{+}/T_{conv} PD-1^{+} were both associated with good prognosis under ICB in our study. This data suggests that the PERLS predictive value is not due to the T_{reg} or the T_{conv} compartment but is linked to PD-1 expression among CD4^{+} T cells. Altogether, this suggests that the restriction of CD4^{+} T cells functions may be deleterious for ICB clinical activity.

As PERLS may be associated with response and the ability to mobilize patient immune system we had a focus on IrAEs. We found no association between PERLS and IrAEs but this analysis suffers from possible underreporting of low-grade events and from a low numbers of severe IrAEs.

We next explored the relationship between PERLS and multiple immune blood biomarkers. Only the proportion of naïve T cells at baseline was related to this ratio. Circulating naïve T cells are less frequent in patients with tumors than in healthy individuals.[35] In previous studies, patients with metastatic melanoma treated with ICB and with a higher frequency of naïve CD4^{+} T cells at baseline were shown to better respond to ICB while non-responders have more differentiated CD4 effector memory at baseline.[36] After immunotherapy there was a shift to greater differentiation of CD4^{+} and acquisition of more cytotoxic capacity for CD8^{+} cells in responder patients.[36] This shift was not found after chemotherapy.[36] Another study in patients with melanoma, showed that expansion of CD8^{+} cells with a cytotoxic profile was associated with response during ICB.[37 38] Valpione et al describe the emergence during ICB treatment of a cytotoxic memory effector peripheral T cell subset only in responding-patients with melanoma. Of note, this T cell subset has been associated with effector T cells migrating from lymph nodes to the blood,[37] suggesting that naïve T cell population, able to differentiate during ICB treatment is mandatory for an effective immune response. In line with this previous observation, PERLS was associated with the increase from D0 to D60 of memory T cells, this increase being associated with clinical benefit. This demonstrate that the differentiation from naïve to memory T cells was associated with clinical benefit, PERLS being linked to positive evolution of memory T cells.

Polyfunctional capacity, or the ability to produce several soluble factors at the single cell level, is a key factor of T cell effectiveness. Several studies demonstrated that polyfunctional capacity of T cells correlates with the immune control during viral infections or after vaccination.[39–41] There is growing evidence in cancer-bearing hosts that T cell polyfunctionality may favor immunological control of the tumor. Recently, Imai and colleagues showed in mice that the presence of CD4^{+} T cells was mandatory for the generation of polyfunctional tumor-specific CD8^{+} T cells with long persistence in vivo resulting in enhanced tumor regression.[42] T cell polyfunctionality was associated with clinical response with different approach of immunotherapy such as CAR-T cells[43] or anti–PD-1 after stereotactic body radiation therapy in a mouse model.[44] In patients with melanoma treated by autologous TIL infusion, a subset of tumor-reactive CD8^{+}T cells, expressing PD-1 with polyfunctional features, accumulated over time.[45] Another study has shown that NSCLC-reactive TILs can be polyfunctional.[46] However, in our knowledge no data exist in patients with aNSCLC treated with ICB. In our study, we brought evidence that post-treatment polyfunctional CD4^{+} T cells are more frequent in patients with a favorable outcome. Since, polyfunctional T cells and the proportion of memory T cells after ICB were positively associated and PERLS associated with the induction of memory T cells, we believe that PERLS measured at baseline could reflect that the patient's immune system will be able to shift towards a memory and polyfunctional T cell response.

Our group has recently proposed that SIP may be a biomarker associated with resistance to ICB.[13] PERLS inversely correlated with SIP. This is not surprising since SIP is mainly composed of terminally differentiated T cells. The composite score PERLS and SIP helped to identify a subpopulation of patients admittedly low (11.8%) but in whom the treatment was systematically effective. Thus, we propose that PERLS and SIP could be complementary tools, and together these two circulating biomarkers can accurately select patients benefiting from ICB monotherapy.

Taken together these results may suggest that PERLS that correlates with a naïve T cell, could reflect the capacity of T cells to differentiate and become polyfunctional during treatment, functional properties essential for a clinical benefit in patients treated with therapeutic monoclonal antibodies that target and block PD-(L)1 molecules.

Even though our study has strengths such as performed in a large cohort, with prospective acquisition of fresh whole blood immune data, with control and validation cohorts. Moreover, PERLS is a ratio with a better reliability regardless of the PD-1 clone used for flow cytometry thus easy to implement in routine practice across laboratories. We can also enumerate limitations. First, CD4 PD-1$^+$ and CD8 PD-1$^+$ population may represent a mixture of T cells and a deeper characterization should be carried out. Second, we did not explore their functional capacity or their tumor specificity this is lacking and currently part of our perspectives. We have only studied T cell polyfunctionality in a small number of patients, which makes the exploration of the direct link with the PERLS ratio very difficult.

In conclusion, we propose that PERLS reflects an immunological status necessary for therapeutic response in patients with aNSCLC. This ratio is determined before treatment using an 'untouched' whole blood sample and has few technical requirements. Further studies are needed to better decipher mechanisms underlying our observations, however our finding suggests that the use of therapies that promote CD4$^+$ T cell amplification and activation or limit their exhaustion may be of interest in patients with aNSCLC.

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