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Eosinophils in non-small cell lung cancer

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It always seems impossible until it’s done.
Nelson Mandela

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List of abbreviations

AAPC: average annual percental change

AEC: absolute eosinophil count

ALC: absolute lymphocyte count

ALK: anaplastic lymphoma kinase

AMC: absolute monocyte count

ANC: absolute neutrophil count

ASR: age-standardized rate

AUC: area under the receiver operating characteristic (ROC) curve

B-Eos: blood eosinophils

BCSS: breast cancer-specific survival

BSC: best supportive care

C/EBP: CAAT/enhancer binding protein

CD: cluster of differentiation

CI: confidence interval

COPD: chronic obstructive pulmonary disease

CRC: colorectal cancer

CRT: chemoradiation therapy

CT: chemotherapy

CT scan: computed tomography scan

CTCAE: common terminology criteria for adverse events

CTLA-4: Cytotoxic T Lymphocyte Antigen-4

DAMP: danger-associated molecular pattern

DFS: disease-free survival

DoR: duration of response

DNA: deoxyribonucleic acid

DNLR: delta NLR

ECOG: Eastern Cooperative Oncology Group

EGFR: epidermal growth factor receptor

EPO: eosinophil peroxidase

ERCC1: excision repair cross-complementation group 1

EPX: eosinophil peroxidase

FOG: friend of GATA1

GM-CSF: granulocyte-macrophage colony stimulating factor

HE: hematoxilin-eosin

ICI: immune checkpoint inhibitor

IFN: interferon

IHC: immunohistochemistry

IL: interleukin

ILC2: type 2 innate lymphoid cells

irAE: immune-related adverse event(s)

IRF8: interferon regulatory factor 8

IT: immunotherapy

KI: Karnofski index

KRAS: Kirsten rat sarcoma

LC: lung cancer

MBP: major basic protein

Mut/Mb: mutation/Megabase

NER: nucleotide excision repair

NLR: neutrophil-to-lymphocyte ratio

NSCLC: non-small cell lung cancer

ORR: objective response rate

OS: overall survival

PAMP: pathogen-associated molecular pattern

PD-(L)1: programmed death-(ligand) 1

PFS: progression-free survival

PS: performance status

PU.1: transcription factor binding to purine-rich box 1

RECIST: Response criteria in solid tumors

REC: relative eosinophil count

RLC: relative lymphocyte count

RNA: ribonucleic acid

RNC: relative neutrophil count

ROC: receiver operating characteristic

SCLC: small cell lung cancer

SoC: standard of care

Sc: single cell

Seq: sequencing

TATE: tumor-associated tissue eosinophils

T-Eos: tissue eosinophils

TKI: tyrosine kinase inhibitor

TILs: tumor infiltrating lymphocytes

TMB: tumor mutation burden

TME: tumor microenvironment

TNM: tumor, node and metastasis

pTNM: pathological TNM

cTNM: clinical TNM

WBC: white blood cell

PREAMBLE

For many years lung cancer has been and, today, still remains one of the principal cancer types across the world. It is also the leading cause of cancer-related death. For those and other reasons detailed in the next section, the burden of lung cancer is even increasing, explaining intensive clinical, translational, and fundamental research efforts.

The advent of immunotherapy has led to a paradigm shift in cancer treatment with its fundamentally different approach to cancer. Since its advent, cure and long-term survivorship can be seen among patients treated with this class of medication for their disease, even in an advanced stage. Particularly since the introduction of immune checkpoint inhibitors, the most commonly used type of immunotherapy, clinicians have reported an observed relationship between blood eosinophilia and clinical outcomes.

Along with these developments in oncology, eosinophils and eosinophil-related cytokines, e.g., interleukin-5 proved to be meaningful targets in the treatment of severe asthma and, more recently, in subsets of patients with chronic obstructive pulmonary disease.

With this work, we aimed at expanding the clinical data on blood eosinophils in patients suffering from the most frequent subtype of lung cancer, i.e., non-small cell lung cancer. First, we studied blood eosinophilia in the context of immune checkpoint inhibitors used for advanced stages of disease. Then, we focused on tissue eosinophils in early stage, resected non-small cell lung cancer.

INTRODUCTION

Epidemiology

Lung cancer (LC) is the second most frequent cancer type worldwide, the first in men and the third in women (Globocan, 2020). Overall, the estimated incidence in 2020 was 2.206.771 cases for both genders, representing the leading cause of cancer-related death with an estimated 1.796.144 deaths in 2020. Importantly, Western Europe ranks 6th in the list of LC incidence (32.7 cases/100.000 persons, age-standardized rate (ASR)) and 4th in mortality (23.8 deaths/100.000 persons, ASR). Estimated worldwide incidence rates for 2025 and 2040 are 2.519.186 and 3.503.377 new LC cases, respectively, representing a 14% (2025) and 58% (2040) increase. Aging and, in low-income countries, rising tobacco consumption are the main reasons for the projected increase in lung cancer incidence (Bray & Weiderpass, 2010).

In Belgium 8874 new cases of LC were registered in 2020 (*Belgian Cancer Registry — Cancer Fact Sheets*, n.d.). This type of cancer is the second most frequent in men after prostate cancer and the third in women after breast and colorectal cancer. The incidence in our country peaks at 75 years in men and at 70 years in women, with a significant rise from 45 years on for both genders. While the number of new LC cases over the last 17 years declined in men (-1.6 average annual percental change (AAPC)), a clear rise was seen in women (+3.2 AAPC). This trend is also seen in other European countries and is the consequence of an increased tobacco consumption as well as to a longer life expectancy in women (Bray & Weiderpass, 2010; Jakobsen et al., 2021). Based on mortality data between 2016 and 2020, the 5-year overall survival (OS) of Belgian LC patients was 22.8% in men and 31.4% in women. Non-small cell lung cancer (NSCLC) represents the most frequent type of LC (71% in 2018), small cell lung cancer (SCLC) accounting for 15% of the total number of LC cases, leaving 14% for other, rare, and undetermined subtypes (Belgian Cancer Registry — Cancer Fact Sheets, n.d.).

Taken together these data underline the high incidence of LC in all parts of the world, including Belgium. The burden of this common disease in terms of public health policy is expected to increase even further due to earlier disease detection (screening) and longer survival of LC patients treated with more effective agents.

Treatments in thoracic oncology

The treatment choice in LC is based on multiple factors, mainly patient- and tumor-dependent (Hendriks et al., 2023; *NCCN Guidelines on Non-Small Cell Lung Cancer*, n.d.; Postmus et al., 2017). Determinant patient-related factors are the functional status, the comorbidities, and the willingness to undergo treatment. The clinician can rate a patient's functional or performance status (PS) on two scales: the Eastern Cooperative Oncology Group (ECOG) PS (ECOG PS) and the Karnofsky Index (KI) (Oken, Creech, & Davis, 1982) (**Table 1**). Each scale expresses the burden of disease on a patient's ability to maintain his/her daily activities. The comorbidities that can hamper treatment administration are diverse. For early-stage LC, cardiac and pulmonary fitness are the two cardinal functions to check before allocating a treatment (Postmus et al., 2017); in later stages of disease where immunotherapy (IT) is an option, preexisting autoimmune disease will be taken into the equation of any therapeutic proposal (Hendriks et al., 2023). The tumor itself will also dictate which options are considered: the stage of disease, defined according to the Tumor, Node and Metastasis (TNM) classification (see biomarker section), the histology, the biomolecular (presence of an oncogenic alteration) and immune (Programmed death (PD)-Ligand (L)1 expression level) characteristics, and, in case of early-stage disease, the resectability (Hendriks et al., 2023; *NCCN Guidelines on Non-Small Cell Lung Cancer*, n.d.; Postmus et al., 2017). Finally, country-specific situations regarding market access will also play a role in treatment allocation. This factor, although rarely debated in the oncology community because of local specificities, seems to have an increasing weight on the speed at which new, meaningful treatment possibilities, can be implemented (Piccart et al., 2023). In this opinion paper, we outline alternative evaluation tools and factors to objectively evaluate the value of innovative oncology drugs and to guarantee their access at a decent speed.

Table 1. Functional status scales used in oncology.

Increasing disease severity

KI	%	Grade	ECOG PS
Able to carry on normal activities. Minor signs or symptoms of disease.	90	0	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work.
Normal activity with effort	80	1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work.
Care for self. Unable to carry on normal activity or to do active work.	70	1	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours.
Requires occasional assistance, but able to care for most of his needs.	60	2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours.
Requires considerable assistance and frequent medical care.	50	2	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours.
Disabled. Requires special care and assistance.	40	3	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours.
Severly disabled. Hospitalisation indicated though death nonimminent.	30	3	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair.
Very sick. Hospitalisation necessary. Active supportive treatment necessary.	20	4	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair.
Moribund	10	4	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair.
Dead	0	5	Dead

Performance scales used in clinical oncology. KI: Karnosfy Index scale: a lower figure indicates a poorer PS. ECOG PS: Eastern Cooperative Oncology Group Performance Status: a higher figure indicates a poorer PS. Adapted from (Oken, Creech, Tormey, et al., 1982).

The standard of care for early-stage, resectable NSCLC, is surgery (*NCCN Guidelines on Non-Small Cell Lung Cancer*, n.d.; Postmus et al., 2017). In small, peripheral lesions, lobectomy, the standard of care for many years, has recently been challenged by two randomized trials (Altorki et al., 2023; Saji et al., 2022). In those, it was demonstrated that, for tumors ≤2 cm and pathologically staged "N0", wedge resection or segmentectomy yielded an equivalent disease-free survival (DFS) and overall survival (OS) as lobectomy. Inoperable patients or patients refusing surgery should be offered radical intent radiotherapy, preferably stereotactic ablative body radiation (SABR) at a biologically equivalent dose of ≥100 Gray. The use of perioperative systemic treatments and the specific situation of locally advanced NSCLC will be discussed further in this section.

In advanced stages of disease, the treatment landscape for LC has dramatically evolved over the last decades moving from a platinum-based chemotherapy (CT), one-size-fits-all strategy to a personalised therapeutic approach, including tyrosine kinase inhibitors (TKIs) targeting molecular alterations and, not the least, IT (Thai et al., 2021; M. Wang et al., 2021).

Chemotherapy (CT), compared to best supportive care (BSC) only, was the first class of anticancer drugs to provide proof of efficacy in terms of objective response rates (ORR) as defined by the Response Criteria In Solid Tumors (RECIST) and in terms of OS, despite a high rate of high-grade toxicity (Rapp et al., 1988) (**Table 2**). Of note, the median OS was barely 4.2 months for the BSC group and 6.2 to 8.5 months for the treatment groups. These statistically significant differences led to the introduction of platinum-based doublet CT as the standard first-line treatment for patients suffering from advanced NSCLC (Schiller et al., 2002). Next, landmark studies showed a benefit with the addition of the anti-angiogenic agent bevacizumab to this regimen (Reck et al., 2010; Sandler et al., 2006). Due to the meager benefit and to toxicity concerns, this drug regimen was not approved uniformly across Europe. At the same time, Scagliotti and colleagues demonstrated a small additional gain in OS with histology-tailored CT (Scagliotti et al., 2008). In Europe, platinum-pemetrexed became the standard doublet for non-squamous NSCLC while platinum-gemcitabine or carboplatin-paclitaxel stayed the backbone regimen for squamous NSCLC.

Table 2. Pioneer trials on chemotherapy for advanced stage NSCLC.

First author	**Rapp et al. 1988**	**Schiller et al. 2002**	**Scagliotti et al. 2008**	**Sandler et al. 2006**	**Reck et al. 2009**
Design	*A:* BSC vs CAP vs VP *B:* CAP vs VP	PaP vs PG vs PD vs CPa	Ppem vs PG	CPa vs CPaB	PG+Placebo vs PG+B (HD vs LD)
Primary objective	ORR	OS	OS	OS	PFS
ORR (%)	*B:* 15.3 vs 25.3 *NS*	19% (all*)	NR	35 vs 15	20.1 vs 34.1 (HD) 20.1 vs 30.4 (LD)
OS, median (mo.)	6.2 (CAP) vs 4.3 (BSC) 8.2 (VP) vs 4.3 (BSC)	7.9 (all**)	10.3 vs 10.3 (All) 12.6 vs 10.9 (NSq.) 10.4 vs 6.7 (LCNE)	12.3 vs 10.3 HR=0.79 (95% CI 0.67-0.92)	13.7 vs 14.5 (HD) vs 14.1 (LD) *NS*
PFS, median (mo.)	NR	3.6 (all***)	4.8 vs 5.1 (All) 5.3 vs 4.7 (NSq.)	6.2 vs 4.5	6.1 vs 6.5 (HD) HR=0.82 (95% CI 0.68-0.98) 6.1 vs 6.7 (LD) HR=0.75 (95% CI 0.62-0.91)

All trials were randomized. BSC: best supportive care. CAP: cyclophosphamide, doxorubicin, cisplatin. VP: vindesin, cisplatin. PP: paclitaxel, cisplatin. PG: cisplatin, gemcitabine. PD: ciplatin, docetaxel. CP: carboplatin, paclitaxel. Ppem: cisplatin, pemetrexed. CPB: carboplatin, paclitaxel, bevacizumab (+ maintenance bevacizumab). B: bevacizumab. HD: high dose, i.e., 15 mg/kg. LD: low dose, i.e., 7.5 mg/kg. *NS*: non statistically significant. NR: not reported. 1 EP: primary endpoint. * PaP 21%, PG 22%, PD 17%, CPa 17%. **: PaP 7.8 mo, PG 8.1 mo, PD 7.4 mo, CPa 8.1 mo. ***: PaP 3.4 mo, PG 4.2 mo, PD 3.7 mo, CPa 3.1 mo. NSq.: non-squamous carcinoma. LCNE: large cell neuroendocrine carcinoma.

Despite this statistical and clinically meaningful advance with CT, the studies did not bring the median OS of patients treated for advanced NSCLC much further than one year. Contrasting with this, the discovery of activating Epidermal Growth Factor Receptor (EGFR) mutations and later of multiple other oncogenic drivers brought the second revolution in the treatment landscape of this patient population (Lynch et al., 2004; Paez et al., 2004). Firstly, studies with TKIs showed the superiority of these drugs as compared to CT in progression-free survival (PFS), ORR and quality of life (T. S. Mok et al., 2009; Peters et al., 2017; Rosell et al., 2012; Solomon et al., 2014; Soria et al., 2017). Secondly, a benefit in OS was demonstrated, establishing their place as a standard of care (SoC) for patients with an advanced NSCLC harboring an oncogenic driver mutation (Camidge et al., 2021; T. Mok et al., 2020; Ramalingam et al., 2020; Solomon et al., 2018) (**Tables 3A & 3B**). Newer generations of TKIs

brought substantial improvement in response rates, especially in the brain, and in OS (T. Mok et al., 2020; Ramalingam et al., 2020; Solomon et al., 2023).

Table 3A. EGFR TKI in NSCLC.

Molecule	Gefinitib	Erlotinib	Afatinib	Dacomitinib	Osimertinib
Generation	1	1	2	2	3
Comparator	CT	CT	CT	1G TKI	1G TKI
PFS	5.7 vs 5.8 (HR=0.74)*	9.7 vs 5.2 (HR=0.54)	11.1 vs 6.9 (HR=0.58)	14.7 vs 9.2 (HR=0.59)	18.9 vs 10.2 (HR=0.46)
ORR	43 vs 32.2**	58 vs 15	56 vs 23	75 vs 72	80 vs 76
DoR	NR	NR	11.1 vs 5.5	14.8 vs 8.3	17.2 vs 8.5
OS	18.6 vs 17.3	19.3 vs 19.5	16.6 vs 14.2#	NR	38.6 vs 31.8##
Grade 3+ toxicity	28.7 vs 61	NR*	49 vs 48	63 vs 41	34 vs 45
Phase III trial	IPASS Mok et al. 2009	EURTAC Rosell et al. 2012	LUX-Lung3 Seqvist et al. 2013	ARCHER 1050 Wu et al. 2017	FLAURA Soria et al. 2018

EGFR: epidermal growth factor receptor. TKI: tyrosine kinase inhibitor. PFS: progression-free survival (median; months). ORR: objective response rate, according to the RECIST 1.1 (%). DoR: duration of response (months); OS : overall survival (median; months). Grade 3^{+} toxicity: according to the CTCAE. CT : chemotherapy. 1G: first generation. HR : hazard ratio. NR : not reached. NR* : Not reported * HR progression/death for EGFR mutated patients=0.48 with gefitinib. ** ORR=71.2% vs 43.7% for EGFR mutated patients with gefitinib vs CT. # p=0.60. ## p=0.046, (Ramalingam et al., 2020). IPASS study : non-inferiority trials of gefitinib compared to CT; enriched population (Asians, female gender, adenocarcinoma, non smokers), not preselected upon EGFR mutation status.

Table 3B. ALK inhibitors in NSCLC.

Molecule	Crizotinib	Ceritinib	Alectinib	Brigatinib	Lorlatinib
Generation	1	2	2	2	3
Comparator	CT	CT	Crizotinib	Crizotinib	Crizotinib
PFS (mo.)	10.9 vs 7.0 HR=0.45 (95% CI 0.65-0.85)	16.6 vs 8.1 HR=0.55 (95% CI 0.42-0.73)	34.8 vs 10.9 HR=0.43 (95% CI 0.32-0.58)	24.0 vs 11.1 HR=0.48 (95% CI 0.35-0.66)	NR vs 9.3 3y PFS=64 vs 19
ORR (%)	74.0 vs 45.0	72.5 vs 26.7	82.9 vs 75.5	74 vs 62	77 vs 59
DoR (mo.)	11.3 vs 5.3	23.9 vs 11.1	33.1 vs 11.1	33.2 vs 13.8	NR vs 9.6
ICR (%)	NR*	72.7 vs 27.3	76.6 vs 65.5	78 vs 26	65 vs 18
OS (mo.)	NR vs 47.5	immature (HR=0.73) (95% CI 0.50-1.08)	immature (HR=0.67) (95% CI 0.46-0.98)	NR vs NR 3y OS=71 vs 68 HR =0.81 (95% CI 0.53-1.22)	NR
Grade 3^+ toxicity (%)	50.3 vs 53.3	65 vs 40	41 vs 50	73 vs 61	76 vs 57
Phase III trial	PROFILE 1014 Solomon et al. 2018	ASCEND-4 Soria et al. 2017	ALEX Mok et al. 2020	ALTA-1L Camidge et al. 2021	CROWN Solomon et al. 2023

ALK: anaplastic lymphoma kinase. TKI: tyrosine kinase inhibitor. PFS: progression-free survival (median; months). ORR: objective response rate, according to the RECIST 1.1 (%). DoR: duration of response (months). ICR: intracranial response (%). OS : overall survival (median; months). Grade 3^+ toxicity: according to the CTCAE. CT: chemotherapy. HR : hazard ratio. CI: confidence interval. NR: not reached. NR*: not reported.

More recently, a substantial benefit in relapse-free survival but also in OS was demonstrated for EGFR mutant, completely resected (stages II & III TNM 8th edition) NSCLC using adjuvant osimertinib after standard adjuvant CT (Herbst et al., 2023; Tsuboi et al., 2023). With this, the hope of curing more patients suffering from LC rises again. Despite these exciting advances, one must acknowledge that most of LC patients do not harbor an oncogenic-driven tumor. To date, routinely targetable genomic alterations are predominantly found in adenocarcinoma (Hendriks et al., 2023). Histology, gender, age, smoking status for some alterations and geographical localisation impact the frequency of these mutations in LC patients (Kosaka et al., 2004; Kuo et al., 2000; Shigematsu et al., 2005). In a large French cohort, half of the pre-treatment samples from patients suffering from NSCLC showed no actionable driver mutation, 12% an EGFR mutation, 32% a KRAS mutation and 5% an ALK rearrangement (Barlesi et al., 2016). The figures at the CHU de Liège are even lower for adenocarcinomas: 8.7% EGFR mutations and 1.6% ALK rearrangements, and slightly higher figures than French data for the KRAS mutation (37.4%) with 13.6% of NSCLC showing a KRAS G12C mutation (Sibille et al., 2021) (**Figure 1**).

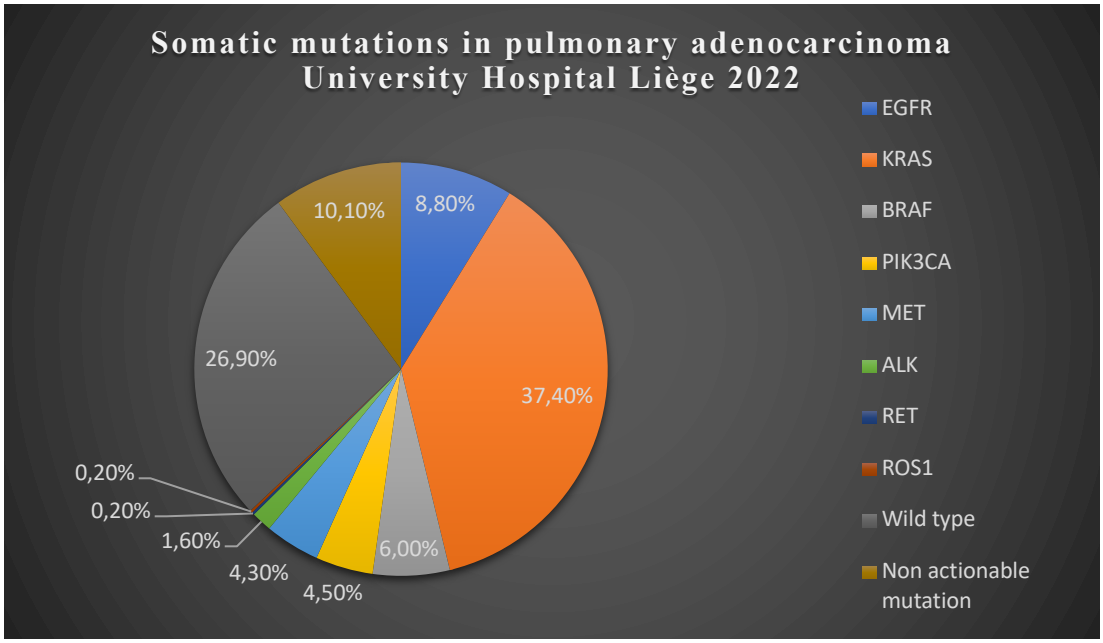

Figure 1. Somatic mutations in pulmonary adenocarcinoma tested at the CHU de Liège in 2022.
N=416. KRAS: Kirsten RAt Sarcoma; 37.4% of the total number of mutations were actionable and 13.6% were KRAS G12C mutations. EGFR: Epidermal growth factor receptor; 8.02% of the total number of mutations were actionable and 3.5% were exon 19 deletions or exon 21 insertions. BRAF: 0.8% of the total number of mutations were V600E. MET mutations: 1.6% of the total number of mutations were exon 14 skipping mutations. ALK: anaplastic lymphoma kinase. RET: Rearranged during Transfection; 0%. Wild type: no somatic mutation found. Non-actionable mutation: mutation of unknown significance. Actionable/non-actionable: according to OncoKB definition https://www.oncokb.org/gene.

The next revolution in LC treatment came with the use of immune checkpoint inhibitors (ICI) and applies to most patients with advanced, non-oncogenic driven NSCLC. Immune checkpoints are control mechanisms that downregulate immune activation pathways. They are physiologically present to avoid autoimmune phenomena but can also, via overexpression either of their receptor or ligand, lead to the escape from anticancer immunity. Cytotoxic T Lymphocyte-associated Antigen (CTLA)-4 (on $CD4^+$ and $CD8^+$ T cells) and Programmed cell Death (PD)-1 (on $CD8^+$ T cells, B cells and natural killer (NK) cells) are two of these checkpoints involved in NSCLC. Binding of CTLA-4 and PD-1 to their ligands inhibits immune cell activation, leading to uncontrolled tumor growth (Chambers et al., 1997; Qureshi et al., 2011; Tivol et al., 1995; Waterhouse et al., 1995). Based on encouraging phase I study results with PD-1 inhibitors (ORR, PFS), these drugs were further developed in NSCLC (Antonia et al., 2019; Garon et al., 2015; Herbst et al., 2014; Topalian et al., 2012). Their use in unselected patients with advanced, pre-treated NSCLC showed a statistically and clinically meaningful improvement of OS (~3 months) with low toxicity rates compared to CT (~10% vs 50% grade 3 or higher) (Borghaei et al., 2015; Brahmer et al., 2015; Fehrenbacher et al., 2016; Herbst et al., 2016). Notably, responses were more durable than what was observed with CT. Meanwhile, Garon and colleagues noted a higher rate of and more durable responses in patients with higher PD-L1 expression levels (Garon et al., 2015). Following this, first line treatment with CT was challenged by anti-PD-(L)1 monotherapies in several phase III trials in PD-L1 high tumors, i.e., ≥50% (Carbone et al., 2017; Herbst et al., 2020; Reck et al., 2016; N. A. Rizvi et al., 2020; Sezer et al., 2021). In lower PD-L1 categories, combination therapies successfully challenged CT alone (Gandhi et al., 2018; Gogishvili et al., 2022; Hellmann et al., 2019; Paz-Ares et al., 2018; Reck et al., 2021; Socinski et al., 2018). Nowadays, combinatorial approaches are the SoC for this patient population: IT monotherapy + CT, double IT alone (not reimbursed in Europe) or double IT with a shorter course (2 cycles) of CT (Hendriks et al., 2023).

In a similar way as for EGFR mutant NSCLC, important milestones were reached for earlier stages of non-oncogenic driven (wild type) NSCLC. This represents an important group of patients knowing that approximately half of the patients are diagnosed with stage I-III disease according to the TNM classification at first presentation (*Belgian Cancer Registry — Cancer Fact Sheets*, n.d.). Although theoretically curable, many of these patients will have recurrent disease after radical treatment (Goldstraw et al., 2016). Until 2017 stage III unresectable NSCLC was classically treated with chemoradiation (CRT) in a concurrent fashion when

clinically tolerable or, alternatively, in a sequential schema (Postmus et al., 2017). With concurrent CRT median PFS reached 8.1 months and a median OS of 21 months, reflecting an 11.6% 5-year PFS rate and a 15.1% 5-year OS rate (Ahn et al., 2015; Aupérin et al., 2010). The PACIFIC trial demonstrated a statistically and clinically meaningful benefit (PFS, OS) for patients receiving consolidation IT with durvalumab after concurrent CRT when they had not progressed on that regimen (Antonia et al., 2017, 2018; Spigel et al., 2022). This approach led to an increase in median PFS to 16.9 months (estimated 5-year PFS: 33.1%) and a median OS of 47.5 months (estimated 5-year OS: 42.9%).

More recently, several trials demonstrated efficacy of ICI in the perioperative setting upon or after CT (Forde et al., 2022; Heymach et al., 2023; Lu et al., 2023; O'Brien et al., 2022; Wakelee et al., 2023). Although the optimal strategy (neoadjuvant vs adjuvant vs both) and, above all, the individualised treatment plan for a patient still need to be defined, the rates of pathological complete or major responses (surrogate endpoints for OS) and recurrence-free survival suggest that the use of ICI in this setting will benefit certain patients. However, longer follow up of the patients included in these studies with mature OS data, definition of selection criteria for a personalised approach, long-term toxicity reports and, ideally, head-to-head comparisons of different IT strategies are eagerly awaited.

Further developments in the field of IT are ongoing. Chimeric antigen receptor (CAR)-T cells, therapeutic vaccines, and engineered tumor infiltrating lymphocytes (TILs) are examples of what is currently being developed and set to proof within clinical trials (García-Pardo et al., 2022; Solomon et al., 2020; Xiao et al., 2021).

Along with these therapeutic advances, one must acknowledge that untailored treatments yield generally poor results in terms of efficacy. The common denominator to successful therapies in cancer remains the preselection of a patient for a given treatment based on his/her cancer characteristics, i.e., for now, a somatic mutation or the PD-L1 expression level. These characteristics are called biomarkers. A biomarker means a "defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention" (Silver Spring (MD): Food and Drug Administration (US); Bethesda (MD): National Institutes of Health (US), 2016). Several types of biomarkers can be defined, among others: diagnostic, monitoring, response, predictive and prognostic biomarkers. Of particular interest for clinical practice are the two latest. A predictive biomarker is a

characteristic that is linked to the (positive or negative) response to a given intervention or environmental exposure. A prognostic biomarker is a characteristic that indicates the likelihood of a clinical event such as disease progression or death.

Biomarkers in thoracic oncology

Prognostic biomarkers in LC have already been identified long ago and remain globally unchanged. The strongest survival predictors are the patient's PS and the anatomical extent of disease (Gospodarowicz & O'Sullivan, 2003). As already mentioned, the PS is rated on the ECOG PS or on the KI scale. The reference tool for the anatomical extent of disease is the Tumor, Node and Metastasis (TNM) classification of which several editions exist, the newest bringing refinements to older ones, yet keeping 4 classes of disease stage, from early (I) to metastatic stage (IV) as illustrated in **Table 4**. Finally, histology remains an important prognostic biomarker, discerning better prognosis in patients suffering from NSCLC as compared to small-cell lung cancer (SCLC).

Table 4. Tumor, node, and metastasis (TNM) classification, 8th edition.

T: Primary tumor	
Tx	Primary tumor cannot be assessed or tumor proven by presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor ≤3 cm in greatest dimension surrounded by lung or visceral pleura without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus)[a]
T1a(mi)	**Minimally invasive adenocarcinoma**[b]
T1a	**Tumor ≤1 cm in greatest dimension**[a]
T1b	**Tumor >1 cm but ≤2 cm in greatest dimension**[a]
T1c	**Tumor >2 cm but ≤3 cm in greatest dimension**[a]
T2	Tumor >3 cm **but ≤5 cm** or tumor with any of the following features[c]: - **Involves main bronchus regardless of distance from the carina but without involvement of the carina** - Invades visceral pleura - **Associated with atelectasis or obstructive pneumonitis that extends to the hilar region, involving part or all of the lung**
T2a	**Tumor >3 cm but ≤4 cm in greatest dimension**
T2b	**Tumor >4 cm but ≤5 cm in greatest dimension**
T3	**Tumor >5 cm but ≤7 cm in greatest dimension** or associated with separate tumor nodule(s) in the same lobe as the primary tumor or directly invades any of the following structures: chest wall (including the parietal pleura and superior sulcus tumors), phrenic nerve, parietal pericardium
T4	**Tumor >7 cm in greatest dimension** or associated with separate tumor nodule(s) in a different ipsilateral lobe than that of the primary tumor or invades any of the following structures: **diaphragm,** mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, and carina
N: Regional lymph node involvement	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)
M: Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis present
M1a	Separate tumor nodule(s) in a contralateral lobe; tumor with pleural or pericardial nodule(s) or malignant pleural or pericardial effusion[d]
M1b	**Single extrathoracic metastasis**[e]
M1c	**Multiple extrathoracic metastases in one or more organs**

Note: Changes to the seventh edition are in bold.
[a]The uncommon superficial spreading tumor of any size with its invasive component limited to the bronchial wall, which may extend proximal to the main bronchus, is also classified as T1a.
[b]Solitary adenocarcinoma, ≤ 3cm with a predominately lepidic pattern and ≤ 5mm invasion in any one focus.
[c]T2 tumors with these features are classified as T2a if ≤4 cm in greatest dimension or if size cannot be determined, and T2b if >4 cm but ≤5 cm in greatest dimension.
[d]Most pleural (pericardial) effusions with lung cancer are due to tumor. In a few patients, however, multiple microscopic examinations of pleural (pericardial) fluid are negative for tumor and the fluid is nonbloody and not an exudate. When these elements and clinical judgment dictate that the effusion is not related to the tumor, the effusion should be excluded as a staging descriptor.
[e]This includes involvement of a single distant (nonregional) lymph node.

Considering that the clinician seeks to administer the best (i.e., the most effective) and the less toxic treatment for a given patient, predictive biomarkers are of great interest in daily practice. By guiding the selection of the a priori best treatment option for the patient, the predictive biomarker will save the patient time and fitness under therapy, while saving financial resources for the community. The EGFR mutation perfectly illustrates what a predictive factor means. Initially used in an unselected population, the first-generation EGFR TKI erlotinib and gefitinib demonstrated, in pre-treated patient populations, a (marginal) benefit in terms of survival and ORR (Shepherd et al., 2005; Thatcher et al., 2005). Careful, retrospective analysis of the patient populations identified a subgroup of patients deriving the greatest benefit from those TKI: females, never-smokers, Asians, with adenocarcinoma histology. After the discovery of the EGFR mutation, a clinico-biological correlation was made between those patients and the EGFR mutation (Kosaka et al., 2004; Shigematsu et al., 2005). While the IPASS study confirmed the superiority of gefinitib in a clinically enriched population as described above, multiple studies showed that the superior ORR and survival rates when treated with an EGFR TKI as compared to standard CT was due to the presence of an EGFR mutation (Brugger et al., 2011; Fukuoka et al., 2011; Han et al., 2012; T. S. Mok et al., 2009; Zhou et al., 2011; Zhu et al., 2008). This established the EGFR mutation as a strong predictor of response to EGFR TKI.

Despite its long use in thoracic oncology, no predictive biomarker for CT has been validated so far. Among the candidate biomarkers, three held more promise: the excision repair cross-complementation group-1 (ERCC-1), the ribonucleotide reductase M1 (RRM1) and thymidylate synthase (TS). ERCC-1 has been identified as potentially predicting the efficacy of platinum CT in NSCLC. It belongs to the nucleotide excision repair (NER) pathway, a group of proteins repairing the DNA damage that characterizes tobacco-induced carcinogenesis (Friedberg, 2003; Helleday et al., 2008). Platinum has multiple effects on DNA (double-stranded breaks, cross-links, replication errors and bulky adducts), that can be tackled by the NER pathway, including ERCC-1 (Helleday et al., 2008). Hence, the effect of platinum could be hampered by high ERCC-1 levels. This assumption, however, could not be proved as the studies showed discordant results (Friboulet et al., 2013; Hubner et al., 2011; Malottki et al., 2016).Ribonucleotide reductase (RR) is necessary to balance the level of deoxyribonucleoside triphosphate (dNTP) and, doing so, to prevent DNA damage of the cell (Gautam & Bepler, 2006). When RRM1, the active subunit of RR, is bound to gemcitabine (diphosphate), its function is inhibited. High levels of RRM1 were associated with gemcitabine resistance in multiple retrospective studies but prospective trials failed to confirm this (Bepler et al., 2006,

2008, 2013, 2014; Dong et al., 2014; Mazzoni et al., 2013; Rosell, Danenberg, et al., 2004; Rosell, Felip, et al., 2004). Finally, TS is an enzyme contributing to the synthesis of thymidine, and, thereby, to DNA synthesis and repair. Pemetrexed, as an anti-folate agent, decreases the thymidine pool and the subsequent DNA synthesis. Again, although retrospective analyses showed better OS and ORR in patients with low TS levels treated with pemetrexed, no prospective data confirmed these data (T. Wang et al., 2013). These three examples show common reasons why a potential biomarker for response is mostly not confirmed as such: inadequate sample size and/or heterogeneity of the studied population, heterogeneity in the assays used, variable scoring systems, variable outcome measures, and lack of analytical validation. Consequently, the candidate biomarkers discussed here are not recommended as predictive markers of the response to the specified CT agents.

ICI is now the most frequently applicable treatment for advanced-stage NSCLC without actionable molecular alteration, either alone or in combination with CT. Already in early phase clinical trials using PD-1 or PD-L1 inhibitors, the PD-L1 expression level showed its potential predictive value (Antonia et al., 2019; Fehrenbacher et al., 2016; Garon et al., 2015; Herbst et al., 2014; Topalian et al., 2012). Further development of these ICI confirmed that higher PD-L1 expression levels led to higher ORR and OS for patients receiving ICI as compared to CT (Herbst et al., 2020; Reck et al., 2016; N. A. Rizvi et al., 2020; Sezer et al., 2021). Although this biomarker enriches the responding population, it rapidly appeared to be imperfect: some patients with high PD-L1 expression failed to respond to ICI and some with low or absent expression did respond. And again, different assays, different scoring systems and different cutoff values were used to categorize patients into ‘high’, ‘intermediate’ or ‘low’ expression levels. Finally, a consensus was reached on the comparable performance of the 22C3, SP263 and 28-8 assays, on the definition of high (≥50%) vs intermediate (1-49%) vs low (<1%) expression level and on the scoring system examining the PD-L1 expression on a section with in at least 100 evaluable tumor cells (Hirsch et al., 2017; Tsao et al., 2018). In the search for a more reliable biomarker of response to ICI and with the use of anti-CTLA-4 molecules, the tumor mutation burden (TMB) appeared promising. The TMB reflects the number of somatic mutations per coding area. Tobacco-induced LC is associated with a high TMB, thus a high level of neoantigens and a higher likelihood of immunogenicity, translating into higher response rates with ICI (Goodman et al., 2017; Hellmann et al., 2018; H. Rizvi et al., 2018; N. A. Rizvi et al., 2015). In the Checkmate 227 study authors evaluated the efficacy of the combination nivolumab + ipilimumab in patients with untreated, advanced NSCLC and high

TMB (i.e., ≥ 10 mut/MB as determined by whole exome sequencing), as compared to CT alone (Hellmann et al., 2018). In the high TMB group the combination ICI was more effective than CT alone, regardless of the PD-L1 expression. However, TMB as a biomarker for ICI efficacy also has limitations: the use of different assays (whole exome versus next-generation sequencing techniques), the lack of a clear cutoff value predicting response, the uncertainty regarding the best sample type (tissue vs blood) and, finally, its cost and turn-around time. Consequently, it has not been adopted as a sole biomarker of efficacy for this class of drugs in NSCLC. Gene expression profiles such as interferon-gamma (IFNγ) and activated T-cells correlated with ORR in the POPLAR and IMpower150 studies but with no further development since (Fehrenbacher et al., 2016; Socinski et al., 2018). TILs were the focus of several studies across different tumor types and a meta-analysis suggested that they are prognostic (and not predictive) in patients treated with ICI for advanced NSCLC (Zeng et al., 2016). Moving a step further, based on the clinical consideration that there is a lack of predictive biomarkers for ICI and reflecting on the cancer immunity cycle described by Chen and Mellman, Karasaki and colleagues developed a personalised immunogram in a small cohort of patients (n=20) (Chen & Mellman, 2013; Karasaki et al., 2017). Through gene expression profiling an 8-axes chart was constructed for each patient, reflecting the presence or absence of different components of the cancer immunity cycle. This led to the identification of three patterns of immunogram: T cell-rich (presence of abundant T cells and of myelosuppressive components, suggesting a dampened antitumor immunity), T cell-poor (absence of T cells and, hence, of antitumor immunity) and intermediate. This work inspired others to further develop, simplify and transpose this initial chart into clinical cohorts and test its predictive and prognostic values (Ghiringhelli et al., 2023). This, as such, has not been endorsed by scientific societies so far.

Beside the candidate biomarkers discussed above, myeloid cells are the subject of intensive research efforts. Indeed, the study of solid tumors has evolved from a tumor-centric perception towards the vision of a more complex interplay of tumor cells with their environment, each element contributing to the persistence and to the development of the tumor (Hanahan & Weinberg, 2011). This paradigm shift established the role of the tumor microenvironment (TME). Intertwined with and surrounding the tumor cells are a range of cells, such as stromal cells (fibroblasts) and myeloid cells, that interact with one another and with cancer cells.

Myeloid cells and cancer

The first and most intensively studies myeloid cells in the TME are macrophages and neutrophils. Transcriptomic analyses have shown that neutrophils and macrophages display functions that are not univocal but rather dictated by their interactions with, in the case of cancer, other TME components (Biswas & Mantovani, 2010; Jaillon et al., 2020). The M1(-like)/M2(-like) macrophage nomenclature refers to macrophages that are polarized into a T helper (T_H)1 - pro-inflammatory, tumor destructive and tissue damaging- or T_H2 - dampening inflammation, tumor permissive and tissue repairing- activation status. This diversity can further evolve over time, as is the case in cancer (Biswas et al., 2008; K. Wu et al., 2020). This dynamic evolution renders their classification as 'pro' or 'anti'-tumoral all the more complex. Paralleling this categorization of macrophages, neutrophils were identified as "N1/N2", depending on their rather immuno-regulatory (N1) vs immunosuppressive (N2) functions, although most of the tumor-associated neutrophils are considered immunosuppressive. As this preclinical evidence grew stronger, some clinicians paid attention to the potential roles of myeloid cells as prognostic or predictive biomarkers in the context of ICI (Delyon et al., 2013a). For advanced-stage NSCLC treated in second or later line with PD(L)-1 inhibitors, several reports ascertained the prognostic role of pre-treatment blood neutrophil and lymphocyte levels (Diem et al., 2017; Mezquita et al., 2018; Park et al., 2018, 2020; Tanizaki et al., 2018). Taken together, data of those uniformly retrospective studies showed a significant higher risk of death in patients with pre-treatment higher neutrophil counts. These were expressed as single markers (ANC $\geq$ or $<$7.5 cells/mL) or as composite biomarkers (neutrophil/lymphocyte ratio (NLR) or deltaNLR (DNLR)). This prognostic value was independent of other known prognostic factors such as ECOG PS, mutation status (EGFR/ALK) or histology (squamous vs non-squamous). Of note, Tanizaki and colleagues studied neutrophils along with lymphocytes and eosinophils and found that a composite marker (absolute neutrophil count (ANC) $<$7.5 cells/mL, absolute lymphocyte count (ALC) $\geq$1.0 cell/mL, absolute eosinophil count (AEC) $\geq$0.15 cells/mL) was associated with OS, PFS and, in their small sample size, with ORR, bringing light upon a potential prognostic or predictive role for eosinophils in NSCLC patients treated with ICI (Tanizaki et al., 2018).

Eosinophils and lung cancer

Eosinophils are rare leucocytes (normal range: <5% of the total white blood cell count and <0.5 cells/mL) whose growth, expansion, tissue migration and survival depend on interleukin (IL)-5 and, in inflammatory conditions, also on IL-33 (Mack & Pear, 2020). Paul Ehrlich first described this myeloid cell subtype as "eosinophil", a cell that stained red when put in contact with eosin, a dye primarily developed for the soda industry (Kay, 2015). Several scientists before him had described eosinophils as "granule blood cells" and "compound inflammatory globules", already putting into light the characteristic granules and lobulated nucleus of eosinophils as well as their presence in inflammatory conditions. Ehrlich further described the cell in various species and identified its origin in the bone marrow. He speculated that their granules contained secretory products and described these cells as associated with various conditions such as asthma, parasitic infections, drug reactions and cancer.

Research publications on eosinophils have tremendously increased over the last 3 decades, mainly due to research in the asthma field leading to the development of anti-IL-5 therapies, but also to the rising interest in the TME and in the role of myeloid cells within that specific compartment. While the accumulated preclinical knowledge established a role for eosinophils as agents of the innate immune system, the evidence for their role in NSCLC patients is weaker. A predictive and prognostic role for eosinophils in NSCLC patients treated with ICI for advanced disease was first reported by Tanizaki and colleagues in 2018 (Tanizaki et al., 2018). In their retrospective study the authors analysed the blood cell counts (ANC, ALC, absolute monocyte count (AMC) and AEC), clinical parameters (age, gender, ECOG PS, smoking status) and pathology criteria (histology, PD-L1 expression level and EGFR/ALK mutation status) of their patients before initiation of nivolumab in advanced or recurrent NSCLC after at least a first line of systemic treatment. The multivariate analysis demonstrated that an ALC ≥1.0/mL, an AEC ≥0.15/mL and an ANC <7.50/mL were correlated with better PFS and OS. When at least two of those factors were present, patients also showed an increased ORR. PD-L1 expression ≥50% was associated with longer PFS and OS.

OBJECTIVES

As we have just mentioned in the preceding chapter, clinical data interrogating a role for eosinophils in lung cancer are scarce. The study by Tanizaki and colleagues triggered our curiosity as it gave echo to our experience in a subset of NSCLC patients treated with ICI (Tanizaki et al., 2018). Indeed, several long-responders treated at our hospital showed (prolonged) blood eosinophilia.

The first objective of our work was to confront in our local data base blood eosinophil levels in a cohort of patients similar to the one from Tanizaki and colleagues. We therefor conducted a retrospective study on the first patients treated at the CHU de Liège for an advanced stage of NSCLC. We described the kinetics of blood eosinophils and their association with response to treatment.

We acknowledged the interest of this kind of potential blood biomarkers because they provide readily accessible material for all patients and because they are cheap. Also, response evaluation under ICI is more complex than under CT or targeted treatments and classical radiological criteria such as the RECIST do not cover all cancer evolution scenarios under checkpoint inhibition (Borcoman et al., 2019; Eisenhauer et al., 2009). A biomarker could thus aid in the assessment of a patient's tumor response to treatment and could help achieve better clinical outcomes by better selecting the patients for a specific treatment. For those reasons, we set up a second, retrospective, study where we aimed to improve the quality of data collected, including confounding factors for eosinophil levels, and a larger number of patients. In this work, we confronted the kinetics and baseline values of blood eosinophils with those of other white blood cells, looking for an association with prognosis or response to therapy.

Our literature review showed an equal lack of data in the early stage setting of NSCLC. Moreover, the existing data show contradictory results on a potential prognostic role of tissue eosinophils. For those reasons, we undertook to quantify them in a large cohort of patients whose tumor had been resected at our hospital. For this, we used an innovative tool, the QuPath® software, and a more modern immunohistochemical staining directed at the eosinophil granules. In this series, we also looked for an association with clinical outcomes such as overall survival and disease-free survival.

CHAPTER 1

Eosinophils and non-small cell lung cancer: a review for the clinician.

Adapted from:

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Study objective

The aim of this work was to review the current knowledge for the physician on the topic of eosinophils in non-small cell lung cancer. Often, clinicians' attention is attracted by the results of studies that allow the direct implementation of new therapeutic or diagnostic modalities. To understand the value of eosinophils in NSCLC patients, we first reviewed their biological properties and roles in steady-state, physiological conditions. Next, we summarized the available data from both pre-clinical and clinical research on the subject. Based on that, we suggested potential future research trajectories.

Abstract

Eosinophils are rare, multifunctional granulocytes. Their growth, survival and tissue migration mainly depend on interleukin (IL)-5 in physiological conditions and on IL-5 and IL-33 in inflammatory conditions. Preclinical evidence supports an immunological role for eosinophils as innate immune cells and as agents of the adaptive immune response. However, this role appears to be equivocal, as pro-, and anti-tumorigenic effects have been identified in tissue eosinophils. Beside this evidence, several reports show a link between outcomes of patients treated with immune checkpoint inhibitors (ICI) for advanced cancers and blood eosinophilia. In lung cancer, data regarding tissue eosinophils are scarce and, for blood eosinophils, of insufficient quality to identify them as a clear prognostic or predictive biomarker. Functional studies of tissue and blood eosinophils, more accurate techniques to highlight them in tissues and alternative materials are likely to help define more precisely the role of these myeloid cells in the particular setting of lung cancer.

Introduction

Paul Ehrlich first described eosinophils more than a century ago and already suggested that their alpha-granules contain secretary products (Kay, 2015). Eosinophils are multifunctional white blood cells (WBC) whose functions have been intensively studied in both physiological and pathological conditions. Their role in non-oncological pulmonary diseases such as asthma and chronic obstructive pulmonary disease (COPD) has been emphasized by major therapeutic developments in the field, more specifically inhaled corticosteroids (ICS) and agents targeting the interleukin (IL)-5 pathway that is essential for the expansion, recruitment, and migration of eosinophils in both physiological and pathological (inflammatory) conditions (*GINA Main Report - Global Initiative for Asthma - GINA*, n.d.; *GOLD Report - Global Initiative for Chronic Obstructive Lung Disease - GOLD*, n.d.). In oncological diseases also, the study of WBC (neutrophils, lymphocytes, and eosinophils) has gained interest, particularly since the advent of immune checkpoint inhibitors (ICI) (Delyon et al., 2013b). In this setting, WBC counts have been studied for their potential prognostic and predictive value, in various solid tumors such as non-small cell lung cancer (NSCLC) (Tanizaki et al., 2018). Paralleling this, a paradigm shift was observed in the study of solid tumors, highlighting the importance of the tumor microenvironment (TME), that consists of immune and non-immune cells, and of chemo- and cytokines interacting with each other (crosstalk) (Hanahan & Weinberg, 2011). Here, we review in the context of NSCLC the biological properties of eosinophils in humans and their roles in homeostatic and pathological conditions. We also explore possible explanations for eosinophilia during NSCLC treatment with ICI. Then, we conclude with suggestions for clinical and translational research topics on this subject.

Biology of eosinophils

Eosinophils are granulocytes that differentiate from multipotent stem cells, called common myeloid progenitors in humans and granulocyte/macrophage progenitors in mice (Iwasaki et al., 2005; Mori et al., 2009). According to recent research, the lineage of myeloid cells is set early in the development of different cell subtypes (Drissen et al., 2016). Mack EA and colleagues reviewed the major transcription factors identified in the eosinophil lineage commitment (Mack & Pear, 2020). They describe the central role of c/EBPα, GATA-1&2, FOG, PU.1, TRIB-1 and IRF8 (**Figure 1**). Not only the presence of those transcription factors seems important, but also the level and the timing of their expression. Eosinophil precursors are further matured, expanded, and activated by cytokines, among which IL-5 (in physiological and pathological conditions) and IL-33 (in pathological conditions) play a central role (Mack & Pear, 2020). The major importance of IL-5, for instance, has been demonstrated by several experiments where its deletion or overexpression in mice led to eosinophil depletion or excessive synthesis, respectively, and by clinical trials in severe asthma patients displaying a profound eosinophil depletion when treated with IL-5 antagonists, leading to a dramatic control of their symptoms and of the need for oral corticoids (Foster et al., 1996; Mishra et al., 2002; Walsh, 2020). Interestingly, it is now believed that IL-5 orchestrates the action of other cytokines, like IL-4, rather than acting as a sole direct trigger on eosinophil precursors via binding to its receptor, IL-5 Receptor unit α (IL-5Rα) (Fulkerson et al., 2014). Once triggered, eosinophils are released in a mature state in blood where they stay for a short time (half-life of 18 hours) (Steinbach et al., 1979). In physiological steady-state conditions (see below) eosinophils migrate to the gastro-intestinal tract (Mishra et al., 1999) and, to a lesser extent, to the thymus, mammalian gland and uterus (Gouon-Evans et al., 2002; Gouon-Evans & Pollard, 2001). This occurs under the action of chemokine eotaxin-1 (also called CCL11). In inflammatory conditions, the recruitment of eosinophils to alternative tissues like the lungs is triggered by CK (IL-4, IL-5, IL-13, IL-33) (Horie et al., 1997; Milovanovic et al., 2012; Moser et al., 1992; Sher et al., 1990), adhesion molecules (β-integrins) (Bochner & Schleimer, 1994), and eotaxins-1,-2 and -3 (CCL11, CCL24 and CCL26, respectively) (Zimmermann et al., 2003). Thus, expansion and survival of eosinophils depend on IL-5. Eosinophil lung infiltration depends on both IL-5 and on eotaxins. The life span of eosinophils in tissues is shorter in homeostatic conditions (2-5 days) than in inflammatory conditions (~2 weeks), at least in vitro (Kita et al., 1998; Sur et al., 1998).

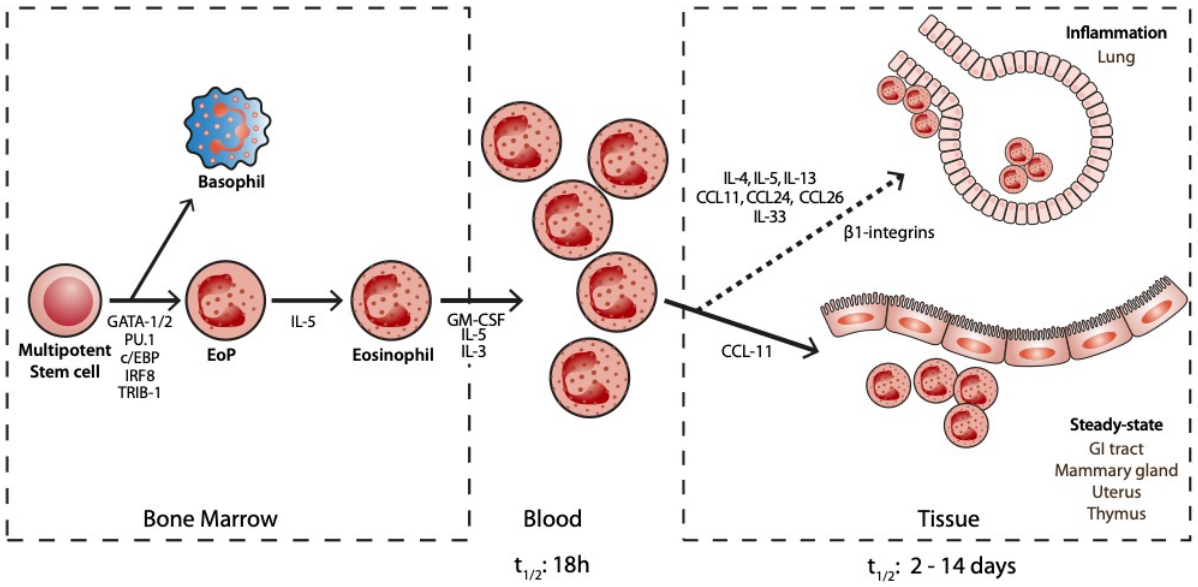

Figure 1. Biology of eosinophils.
Eosinophils derive from multipotent stem cells. They proliferate, migrate, and get activated by cytokines, mainly Interleukin-5 (IL-5). They spend a short time in blood and subsequently migrate to tissues via the interplay of several chemokines. GM-CSF: Granulocyte-Macrophage –Colony Stimulating Factor. EoP: eosinophil progrenitor. IL-5: Interleukin-5. IL-3: Interleukin-3; CCL11: CC-chemokine ligand 11(=eotaxin1); CCL24: eotaxin-2; CCL-26: eotaxin-3. $T_{1/2}$: half-life. GI tract: gastrointestinal tract.

Morphologically, eosinophils can be characterized by their intracellular content and by their surface receptors (**Figure 2**). A bilobed acidophilic nucleus and intracellular granules are common to all species (Gleich et al., 1993). The granules can be divided into primary granules (containing Charcot-Leyden crystal proteins and lipids), secondary granules and small granules. In human eosinophils, secondary granules contain four predominant, cytotoxic proteins called cationic proteins: major basic protein (MBP)-1, eosinophil peroxidase (EPX), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), the latest two also showing a ribonuclease activity. The granules also contain cytokines, chemokines and growth factors that enable eosinophils to play their role in inflammation. Cell-surface receptors of eosinophils are numerous (Hogan et al., 2008). They can be classified in adhesion molecules (selectins), chemotactic factor receptors (e.g., chemokine receptor 3 (CCR3)), cytokine receptors (e.g., IL-5Rα/β), complement receptors, immunoglobulin receptors, inhibitory receptors (e.g., sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8)) and pattern-recognition receptors (PRR, including Toll-like receptors and RAGE). The PRR recognize danger signals, also called alarmins. These can be of exogenous (infectious) origin (bacterial, fungal, or parasitic; so-called pathogen-associated molecular patterns-PAMPs) or endogenous, tumor-derived signals (so-called danger-associated molecular patterns-DAMPs). Activation of

the PRR by the alarmins leads to expansion, adhesion to blood vessels, chemotaxis, degranulation, and cell-to-cell interactions of eosinophils (Hogan et al., 2008), triggering the immune system (Kvarnhammar & Cardell, 2012). IL-33 is an epithelial- and tumor-derived cytokine belonging to the IL-1 cytokine family (Lucarini et al., 2017). It seems to be a crucial alarmin in host defense against tumors. Indeed, eosinophils recruited and activated through IL-33 were shown to be responsible for tumor growth control and for the prevention of pulmonary metastases development in melanoma-bearing mice. Mechanisms leading to these anti-tumor effects have been deciphered and are detailed further. Andreone and colleagues underline the central role of IL-33 through in vitro experiments where induction of eosinophil degranulation by IL-33 in the context of cancer is even superior to that of IL-5 (Andreone et al., 2019).

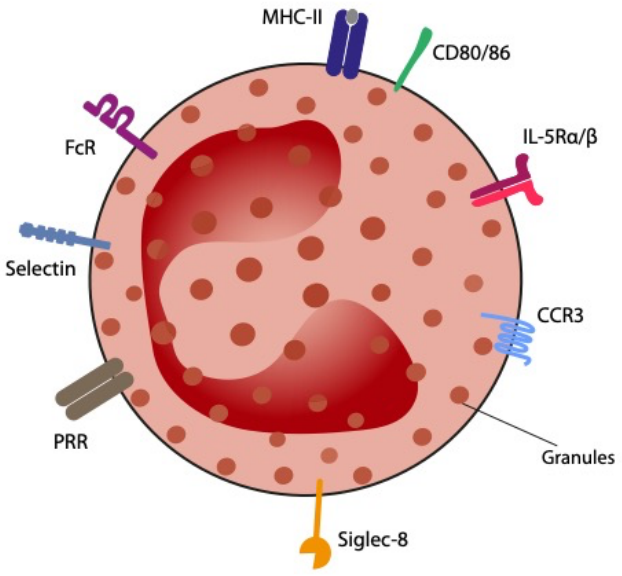

Figure 2. Structure of the human eosinophil.
Eosinophils can be characterized by their surface markers and by their intracellular content. Cell surface markers are: adhesion molecules (selectins) allowing for adhesion and endothelial transmigration; chemokine receptors (CCR) and chemotactic factors allowing for the attraction and local activation of eosinophils; cytokine and growth factor receptors (e.g. Interleukin-5 Receptor alpha subunit (IL-5Rα)); complement receptors; immunoglobulin receptors (e.g. FcR); inhibitory receptors (e.g. Sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8)) and pattern recognition receptors (PRR), e.g. Toll-like receptors whose activation is triggered by alarmins (Pathogen-associated molecular patterns (PAMPs) in case of infection and Danger-associated molecular patterns (DAMPs) in case of tumor).

Role of eosinophils in physiological steady-state conditions

Eosinophils are similarly found in various tissues of healthy humans and mice: bone marrow, blood, gastrointestinal tract, thymus, secondary lymphoid tissues, uterus, and adipose tissue. They are implicated in diverse processes, highlighted by the study of IL-5 overexpressing, eosinophil-deficient or cytokine reporter mice (Croxford & Buch, 2011; Lee, James J. & Rosenberg, 2013).

A first role of eosinophils is to contribute to tissue development as is the case in the mammary glands (Gouon-Evans et al., 2002), in the uterus (Gouon-Evans & Pollard, 2001; Timmons et al., 2009; J. Zhang et al., 2000) and in the gastrointestinal tract where they contribute to the development of the Peyer's patches (Chu et al., 2014; Mishra et al., 1999). A second role of eosinophils is tissue regeneration. As an example, the eosinophil-dependent IL-4 production has been shown to be crucial for the differentiation of fibrocyte-adipocyte progenitors into hepatocytes and myocytes in the context of liver or muscle injury (Goh et al., 2013; Heredia et al., 2013). Thirdly, eosinophils take part in metabolism. In adipose tissue, their IL-4 and IL-13 production leads to the differentiation of macrophages into the M2-phenotype that has greater insulin sensitivity (D. Wu et al., 2011) and to the increase in thermogenic, "beige" adipocytes (Qiu et al., 2014). Finally, eosinophils appear to be of great importance in immune homeostasis, playing a role as innate immune cells and as regulatory cells for the adoptive immunity. Indeed, priming of B lymphocytes as well as maintenance of plasma cells within the bone marrow or intestinal mucosa are (partly) promoted by eosinophil-linked mechanisms: production of IL-4, IL-6 and activation and proliferation-induced ligand (APRIL) cytokines (Berek, 2016; Chu et al., 2011; Jordan et al., 2004; Wang & Weller, 2008). Moreover, IgA production, microbiome composition, integrity of the mucosal barrier and the development of the Peyer's patches are, in mice at least, all eosinophil-driven through IL-6, APRIL and transforming growth factor (TGF)-β (Chu et al., 2014; Mantis et al., 2011). Lastly, eosinophils are mediators of T-cell tolerance: in the thymus, they participate to the destruction of self-reactive T cells via the secretion of indoleamine 2,3-deoxygenase (IDO) (Odemuyiwa et al., 2004).

Eosinophils and cancer: the bench side

The recruitment of eosinophils at tumor sites relies on tumor cells and on the inflammatory reaction (necrosis) they induce, as well as on peri- or intra-tumoral immune cells (lymphocytes, mast cells, dendritic cells) that can secrete eosinophil chemoattractants (Varricchi et al., 2018). Based on in vitro models of NSCLC Huang and colleagues demonstrated that eosinophils are attracted by type 2 cytokines (IL-5, IL-4, IL-10, and IL-13) that are produced by tumor cells (Huang et al., 1995). GM-CSF and CCL11 (eotaxin 1), that are present in tumor tissue, contribute to the attraction of eosinophils (Curran et al., 2011; Simson et al., 2007). Emphasizing the role of CCL11, Hollande and colleagues demonstrated that dipeptidyl peptidase DPP4 (CD26) inhibitor sitaglipitin led to enhanced tumor control through enhanced CCL11-mediated eosinophil recruitment at the tumor site (Hollande et al., 2019). Furthermore, the role of dying tumor cells in eosinophil recruitment was demonstrated in a mouse model for melanoma where eosinophil concentrations were significantly higher in the capsule (fibrotic area) and in the central (necrotic) area of the lesions (Cormier, 2006). The following alarmins promoting eosinophil infiltration of tumors were identified: high-mobility group box-1 protein (HMGB-1) and IL-33 (Lotfi et al., 2009; Lucarini et al., 2017). Recent data on colorectal cancer suggest that the gut microbiota may also influence eosinophil recruitment in such cancers (Reichman et al., 2019).

Preclinical data reveal both anti- and pro-tumorigenic activities of eosinophils, both through direct and indirect mechanisms. As a first step in exploring the hypothetical antitumor role of eosinophils, several authors manipulated eosinophil-linked cytokines (IL-4 or IL-33 injections, CCL11 and IL-5 depletion) (Lucarini et al., 2017; Simson et al., 2007; Tepper et al., 1992). They observed that tumor incidence and/or growth was indeed inversely correlated with eosinophil infiltration. Further in vitro studies showed more precisely the mechanisms by which activated eosinophils can control tumors. Beside a direct cytotoxic effect on cancer cells through degranulation (Legrand et al., 2010; Lucarini et al., 2017), activated eosinophils recruit, activate and lead to the maturation of several immune cells favoring tumor rejection (Carretero et al., 2015; Lotfi & Lotze, 2008; Lucarini et al., 2017; O'Flaherty et al., 2017) (**Figure 3**). Carretero and colleagues showed that activated eosinophils recruit cytotoxic $CD8^+$ T cells and are essential for the tumor control in their melanoma mouse model (Carretero et al., 2015). They also demonstrated that eosinophils are capable of macrophage polarization into an antitumor (M1) phenotype. A pivotal study in colorectal cancer identified that intratumoral

eosinophils exert these antitumor effects through interferon gamma (IFNγ) signaling (Reichman et al., 2019). Additionally, eosinophils tend to normalize tumor vasculature, a crucial factor for tumor maintenance and expansion. Indeed, depletion of eosinophils led to increased vascular leakiness, diminished perfusion, and diminished coverage by mature pericytes (Carretero et al., 2015).

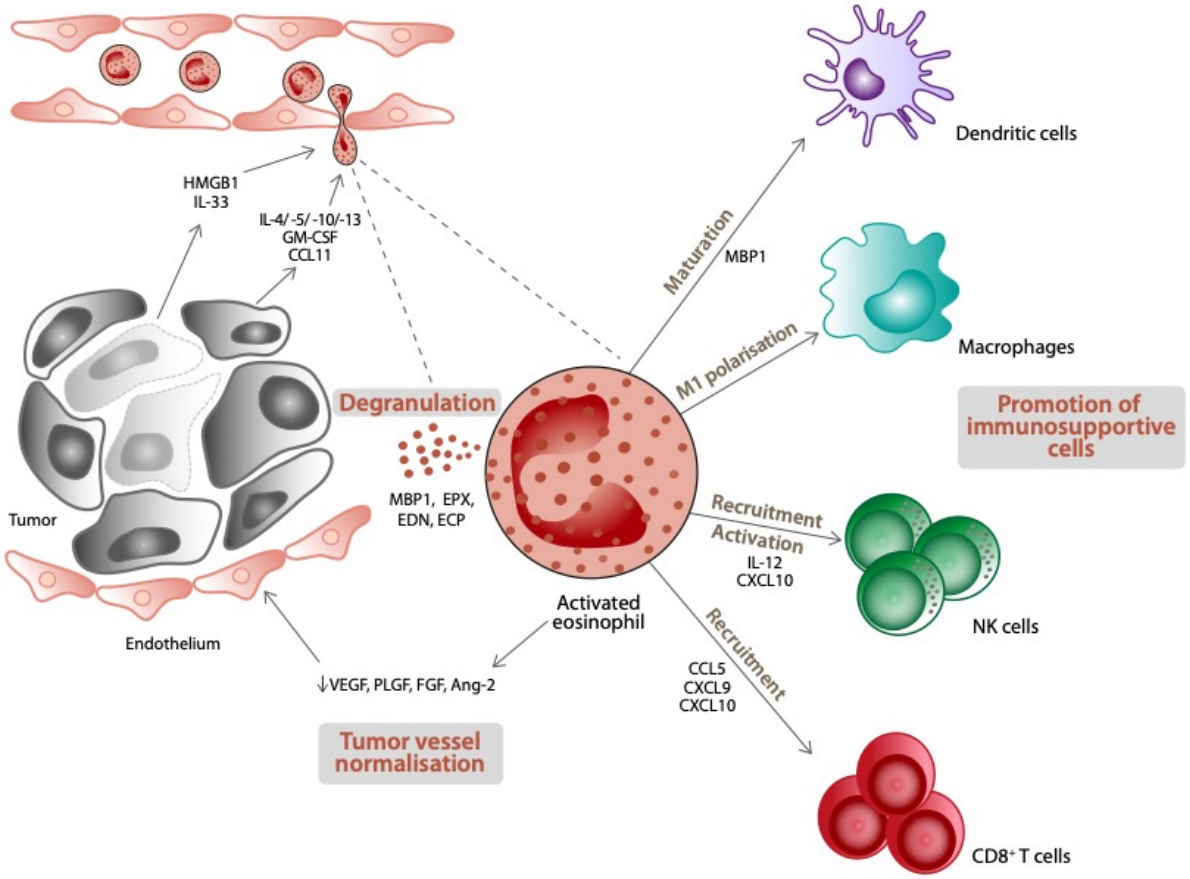

Figure 3. Eosinophil recruitment at tumor sites and anti-tumor effects of eosinophils.
In response to their recruitment and activation via different cytokines and chemokines like tumor-secreted Interleukin-5 (IL-5), or IL-33 and High Mobility Group Box-1 protein (HMGB-1), alarmins secreted by dying tumor cells, eosinophils display both direct and indirect anti-tumor effects. Degranulation of eosinophils has cytotoxic and ribonucleasic effects. Also, activated eosinophils are capable of recruiting immune cells to engage against tumors: Natural Killer (NK) cells, cytotoxic $CD8^+$ T cells and dendritic cells (DC). Additionally, they can polarise macrophages to an M1, anti-tumor phenotype. Finally, eosinophils appear to affect tumor vasculature by increasing vascular leakiness, leading to tumor necrosis. IL: Interleukin; HMGB-1: High Mobility Group Box-1 protein; PRR: Pattern Recognition Receptor; CCL11: CC-chemokine ligand 11=eotaxin1; CXCL9: CXC-chemokine ligand 9; MBP-1: major basic protein-1; EPX: eosinophil peroxidase; EDN: eosinophil-derived neurotoxin; ECP: eosinophil cationic protein; VEGF: vascular endothelial growth factor; PLGF: platelet growth factor; FGF: fibroblast growth factor; Ang-2: angiopoietin-2.

However, pro-tumorigenic effects of eosinophils have also been reported. As an example, preclinical models of oral squamous cell carcinoma showed reduced growth when eosinophil infiltration was hampered (da Silva et al., 2017; Wong et al., 1999). A model of cervix carcinoma revealed also that eosinophils, activated by tumor-generated thymic stromal lymphopoietin (TSLP), triggered tumor growth (Xie et al., 2015). Eosinophils do facilitate the recruitment of Treg (Zaynagetdinov et al., 2015), inhibit cytotoxic T cells via the production of IDO (Astigiano et al., 2005), and induce polarization of macrophages into the M2, immunosuppressive phenotype through the production of IL-13 (Kratochvill et al., 2015). Finally, eosinophils produce many growth factors, with direct effects on tumor growth, on metastatic spread, on matrix remodeling or on tumor-associated blood vessels (Grisaru-Tal et al., 2020).

Those seemingly opposing roles of eosinophils in tumors probably reflect their functional plasticity rather than underline contradictory findings. Firstly, eosinophils are, as other myeloid cells, part of the tumor microenvironment (TME), an entity where tumor cells, inflammation and immune cells interact and evolve over time (Greten & Grivennikov, 2019; Shalapour & Karin, 2019). It is reasonable to think that, as for macrophages and neutrophils, eosinophils' behavior could vary depending on the surrounding stimuli (cytokines, exosomes) (Biswas & Mantovani, 2010; Mattei et al., 2020). Indeed, while IFNγ and IL-33 trigger an anti-tumor role of eosinophils, IL-5 favors their pro-tumorigenic function (Lucarini et al., 2017; Reichman et al., 2019; Zaynagetdinov et al., 2015). Secondly, considering the data described, a differential role for eosinophils according to the histologic subtype might be suspected: immuno-supportive in melanoma, immuno-suppressive in oral squamous or cervix carcinoma. However, it may be so that different tumor types simply reflect different TME. Thirdly, phenotypic studies of eosinophils in asthma mouse models showed eosinophils with different localizations (airway lumen vs epithelium), morphology (ring-shaped vs segmented nucleus) and different gene- and cytokine expression profiles, reflecting different functions (Abdala Valencia et al., 2016; Mesnil et al., 2016; Percopo et al., 2017).This, however, remains to be demonstrated in the context of cancer.

Eosinophils and lung cancer: the bedside

Tissue eosinophils (T-Eos)

To date, these data are scarce in NSCLC. In advanced disease, we found no report on tissue eosinophils (T-Eos) for this tumor type. In early stages, two studies described eosinophils and their value in this setting. Ye and colleagues studied the expression of EPX, one of the 4 proteins contained in eosinophil granules, on 30 resection specimens of adenocarcinoma of the lung and on adjacent, normal lung tissue (Ye et al., 2019). The expression level of EPX was rated by the degree (negative/weak/medium/strong staining) and the extent (0/1-25/26-50/51-75/76-100%) of the protein expression. A score was then defined for high vs. low EPX expression. Univariate analysis revealed a higher EPX expression in the cancer areas as compared with normal tissue ($p<0.05$) and a correlation of high levels of EPX with higher pathological Tumor Node Metastases (pTNM) stage ($p=0.017$) and with lymph node involvement ($p=0.027$). T-Eos here was associated with a worse prognosis with a calculated hazard ratio (HR) for death of 3.1 ($p=0.018$) in the EPX high group. Tataroglu and colleagues published a study on the presence of mast cells, macrophages, eosinophils, their association with tumor vasculature and TNM stage of those NSCLC (Tataroğlu et al., 2004). No significant association was noted between eosinophils and tumor stage or between tumor-associated vasculature and eosinophils. It should be noted, however, that eosinophils were evaluated by light microscopy after staining with hematoxillin-eosin. Weller and Spencer described thouroughly the difficulties in detecting eosinophils in tissue and suggested that electron microscopy or the use of antibodies directed at eosinophil granule proteins are useful tools to optimize the count of these cells in tissue (Weller & Spencer, 2017). Beside technical issues, TATE could vary according to the degree of activation of the immune cascade, i.e. according to the interplay of cytokines, chemokines and immune cells shaping the tumor microenvironment.

Blood eosinophils (B-Eos)

The first data on cancer patients showing an association between anti-neoplastic treatment and eosinophilia came from a cohort of 20 patients treated with IL-2 and lymphokine-activated killer cells for advanced cancer (Rosenberg et al., 1985). van Haelst Pisani and colleagues further demonstrated that IL-2 administration was followed by IL-5 production and eosinophilia (van Haelst Pisani et al., 1991). Some 20 years later, several authors demonstrated an association between B-Eos, anti-cytotoxic T-cell lymphocyte antigen (CTLA)4 antibodies or anti-Programmed Death (PD)-1 antibodies and improved clinical outcome across various

cancer types (Bernard-Tessier et al., 2017; Chu et al., 2020; Delyon et al., 2013; Gebhardt et al., 2015; Martens et al., 2016; Moreira et al., 2017; Okauchi et al., 2021; Sibille et al., 2021; Tanizaki et al., 2018; Weide et al., 2016).

Strikingly, few data exist on the study of B-Eos in NSCLC patients treated with ICI and outcomes (**Table 1**). The studies are all retrospective in nature. Authors noted a correlation between raised blood eosinophils and a favorable clinical or radiological outcome. The princeps study by Tanizaki and colleagues suggests a prognostic and/or predictive role of B-Eos in patients treated with nivolumab for advanced NSCLC after failure of a previous systemic treatment (Tanizaki et al., 2018). Pre-nivolumab absolute eosinophil count (AEC) >0.15 cells/mL, absolute lymphocyte count (ALC) >1.0 cells/mL and absolute neutrophil count (ANC) >7.5 cells/mL were significantly associated with a better overall and progression-free survival (OS and PFS, respectively). This was confirmed in the tumors with PD-L1 expression ≥50% but was not significant for tumors with PD-L1 expression <50%. For patients with an AEC >0.15 cells/mL, the risk of death was reduced by 76% and the risk of progression by 47%. Two other studies looking at leucocytes under ICI treatment comforted those results on a slightly higher number of patients and in a similar therapeutic context (Okauchi et al., 2021; Sibille et al., 2021). Our cohort of patients will be discussed in detail in the next chapter. Okauchi and colleagues concentrated on the study of B-Eos only (Okauchi et al., 2021). They showed that pre-treatment AEC was lower in patients that would later progress under ICI (p=0.002). Under treatment, AEC and REC were lower in progressive patients (p=0.002 and <0.0001, respectively). The time to treatment failure was longer in patients with an AEC >0.15 cells/mL and a REC >3% before ICI initiation (p=0.046 and 0.003, respectively) and with an AEC >0.3 and >0.5 cells/mL (p<.001 for both) and a REC >3 and >5% under treatment (p<0.001 for both). The two latest studies further suggest, based on receiver operating characteristic (ROC) curves analysis, that a REC >5% is predictive of disease control, although with disputable sensitivity and specificity (81.9% and 32.8%, respectively (Sibille et al., 2021); 60.7% and 27.3%, respectively (Okauchi et al., 2021)). In the last study, Chu and colleagues analyzed data from 300 NSCLC patients treated with ICI for advanced disease and looked at pre-treatment peripheral blood characteristics that may predict the occurrence of immune-related pneumonitis and predict general outcomes (survival and response rates) (Chu et al., 2020). They demonstrated a link between pre-treatment AEC (cut-off value of 0.125 cells/mL) and (1) a higher objective response rate (ORR) (40.9% vs. 28.8%, p=0.029) and (2) a longer PFS (8.9 vs. 5.9 months, p=0.038).

Table 1. Studies on the association between outcomes of NSCLC patients treated with ICI and blood eosinophils.

Study	N	Stage of disease	ICI	Eosinophils	Outcome	Effects	P value
Tanizaki 2018	134	IIIB-IV	nivolumab	AEC t0; categorical; simple & composite biomarker	OS	HR=0.24 (95% CI 0.09-0.62)	0.003
					PFS	HR=0.53 (95% CI 0.31-0.91)	0.02
						if AECt0 ≥0.15 cells/mL	
Chu X 2020	300	IIIB-IV	PD-1i +/- CT +/- AAG	AEC t0; categorical; simple	ORR	40.9 % vs 28.8 %	0.029
					PFS	med.=8.93 vs 5.87mo	0.038
						HR=0.744 (95% CI 0.56–0.99)	
						if AECt0 ≥0.15 cells/mL	
Sibille 2021	191	IIIA-IV	pembrolizumab nivolumab atezolizumab durvalumab	AEC & REC t1 ; continuous	ORR	OR=0.53 (95% CI 0.32-0.88) if RECt1 >5.3%	0.014
Okauchi 2021	190	IIIA-IV	nivolumab pembrolizumab atezolizumab +/- CT	AEC & REC t0 & q2-3 wk; RECmax.*; categorical	TTF	OR=0.39 (95% CI 0.26-0.60) if RECmax. >5%	<0.001

This table illustrates the heterogeneity of study objectives and of evaluation criteria for eosinophilia : continuous/categorical variable; time of evaluation; biomarker used alone (simple) or in combinaison with others (composite). ICI: immune checkpoint inhibitor; NSCLC: non-small cell lung cancer ; PD-1i: Programmed death-1 inhibitor; AAG: anti-angiogenics; CT: chemotherapy (platinum-based doublet) ; AEC: absolute eosinophil count; REC: relative eosinophil count; categorical: studied as a categorical variable; continuous: studied as a continuous variable; t0: value before ICI treatment; t1: timing of the first RECIST evaluation under ICI treatment (at 8-12 weeks after initiation); q2-3 wk: every 2-3 weeks; *REC max.: maximal REC value noted under ICI; OS: overall survival; PFS: progression-free survival; ORR: objective response rate; TTF: time to traitement failure; CI: confidence interval; HR: hazard ratio; OR: odd ratio.

As these data come from retrospective studies, the quality of the observations is clearly weaker, with missing data regarding confounders for blood eosinophilia, except in Sibille et al. Besides, the overview given in Table 1 allows considering the heterogeneity of the studies regarding the number of patients included and the evaluation criteria for B-Eos (studied as continuous vs categorical variables; inconsistent evaluation time points; single vs composite biomarker). Yet, there is a consistent correlation between raised B-Eos under treatment with ICI and better outcome (OS, PFS, ORR).

Voorwerk and colleagues addressed the question of the specificity of ICI in inducing eosinophilia in their melanoma mouse model and demonstrated (1) that the rise in B-Eos after ICI was specific to this type of anti-neoplastic drug, as compared to chemotherapy, and that it also occurred when combining chemotherapy and ICI; (2) that the improved survival of mice treated with ICI relied upon eosinophils, as depletion of these cells by anti-Siglec8-antibodies resulted in a survival that paralleled the survival of mice not treated with ICI. The results concerning raised blood eosinophils and clinical response were confirmed for metastatic bladder and lung cancer, as well as for early-stage mismatch repair proficient colon cancer (Voorwerk et al., 2020). To the best of our knowledge, there is also no clinical report pointing at a link between B-Eos or T-Eos and efficacy of chemotherapy or tyrosine kinase inhibitors.
Other pre-clinical research models brought some light on this topic. In a mouse model for breast cancer, Zheng and colleagues suggested that the accumulation of tissue eosinophils under CTLA-4 blockade was correlated with upregulation of CCL11, CCL5 and IL-5 expression on $CD4^+$ and $CD8^+$ T lymphocytes (Zheng et al., 2020). However, these data were based on blood analysis by flow cytometry and not correlated with tissue by immunohistochemistry. Furthermore, in mice bearing melanoma tumors and treated with a PD-1 inhibitor, tumor regression correlated with eosinophil infiltration at the tumor site, mediated by GM-CSF produced by type 2 innate lymphoid cells (ILC2) that are present in the TME of such tumors (Jacquelot et al., 2021). ILC2 expressed PD-1 and the concomitant injection of IL-33, the ligand of the constitutively present ST2 receptor on ILC2, and of a PD-1 inhibitor to the mice led to a clear tumor shrinkage.

Blood eosinophilia has also been reported in cancer patients who display toxicity to ICI. So-called immune-related adverse events (irAE) are specific to the use of these drugs and reflect excessive immune activation (Postow & Hellmann, 2018). There are case reports as well as (mostly retrospective) studies showing an association between the occurrence of irAE and eosinophilia. In the context of NSCLC, the series of Chu et al revealed a correlation between baseline AEC and the occurrence of pneumonitis (27.7% if AEC ≥0.125 cells/mL vs. 9.8% if AEC <0.125 cells/mL, p<0.0001) (Chu et al., 2020).

Some authors advocate for the existence of a drug-driven, irAE-independent eosinophilic syndrome in the context of ICI (Bernard-Tessier et al., 2017; Scanvion et al., 2020). Both groups demonstrated the existence of B-Eos (>0.5cells/mL in Bernard-Tessier, >1.0cells/mL in Scanvion) in the absence of irAE, although the retrospective nature of the study may not allow for a complete recording of toxicity events. However, the correlation between various drugs and eosinophilia is already known for long and, as such, there is no reason that ICI could not lead to a similar phenomenon. In that case, the rise in eosinophils can be the consequence of an increased production of these cells, e.g. IL-2 triggering IL-5 production, leading to increased eosinophilopoïesis, as observed in mouse models (Van Gool et al., 2014; Yamaguchi et al., 2021). It can also be the result of a type IVb allergic reaction characterized by the occurrence of a Th2-mediated immune response, as seen in some patients taking various types of medication (Werner J., 2003). Given the wide clinical spectrum of medication-induced eosinophilia and possible overlap of clinical signs with irAE (like rash), this drug-induced eosinophilia may in fact well be underestimated.

Conclusion

Preclinical models have established a role, although not unique, for tissue eosinophils in cancer. Despite their questionable quality, clinical data suggest that raised B-Eos may reflect a favorable outcome in patients treated with immune checkpoint inhibitors for advanced NSCLC. Data on T-Eos in NSCLC are too scarce at present to draw any firm conclusion. This relative lack of data calls for more stringent clinical research and for functional studies to further elucidate the role of eosinophils in lung cancer and their potential value as a biomarker.

CHAPTER 2

Clinical benefit to programmed death-1 inhibition for non-small cell lung cancer is associated with higher blood eosinophil levels.

Sibille, A., Henket, M., Corhay, J. L., Louis, R., & Duysinx, B. (2020). Clinical benefit to programmed death-1 inhibition for non-small-cell lung cancer is associated with higher blood eosinophil levels. *Acta Oncol*, *59*(3), 257–259. https://doi.org/10.1080/0284186X.2019.1695063

Study objectives

Earlier in this manuscript, we described the first reported study on white blood cells pointing at a prognostic and predictive role of eosinophils in NSCLC patients treated with the PD-1 inhibitor nivolumab for advanced-stage disease (Tanizaki et al., 2018). Facing new challenges in the evaluation of patients' response to ICI, the search for predictive biomarkers was open.

In this study, we wanted to confirm the results obtained by Tanizaki and colleagues regarding the potential predictive role of blood eosinophils under ICI in a group of patients treated for advanced NSCLC. Patients were recruited at the Centre Hospitalier Universitaire de Liège: a computer-based search retrieved the files from patients who received a PD-(L)1 inhibitor for relapsed stage III or progressing stage IV NSCLC after at least one line of systemic therapy. We analysed the kinetics and the value of blood eosinophils in predicting a patient's response at the first radiological evaluation, i.e., two to three months after treatment initiation, and at the second evaluation, i.e., four to six months after treatment initiation.

Introduction

The use of immune checkpoint inhibitors (ICI) for non-small-cell lung cancer (NSCLC) is increasing. Currently validated indications include advanced and locally advanced disease (Planchard et al., 2019). Classically, response evaluation relies on radiological assessment with the REsponse Criteria In Solid Tumors (RECIST) version 1.1 (Eisenhauer et al., 2009). However, in the setting of ICI these criteria seem imperfect. Indeed, atypical response patterns have been observed that make radiological evaluation less clear than it is with chemotherapy (Borcoman et al., 2019). Pseudoprogression, one of these atypical radiological responses, is defined as radiological progression in the absence of clinical deterioration. It correlates with immune cell infiltration and/or transient tumor growth before response to ICI (Borcoman et al., 2019). Discontinuing ICI in this case would mean stopping an efficient therapy. Alternative or additional tools for the evaluation of response could help to evaluate the efficacy of ICI effectively and more accurately. Blood eosinophil counts are routinely available at a negligible cost. Most of the objective responses to PD-(L)1 inhibitors occur within the first two months of PD-1 inhibitor use (Borghaei et al., 2015; Brahmer et al., 2015). Here, we report on blood eosinophil evolution during the first months of treatment with anti-Programmed Death (PD)-(L)1 antibodies and on their value as early indicators of response in patients treated for advanced stage NSCLC.

Materials and methods

Medical records from patients consecutively treated at our institution with any anti-PD-1/anti-PD-L1 in monotherapy for advanced stage NSCLC between 1/8/2015 and 30/4/2018 were investigated. In this time frame only two agents were used: pembrolizumab, given at the dose of 2mg/kg every 3 weeks during the early access program then at the fixed dose of 200mg every 3 weeks, and nivolumab, given at 3mg/kg every 2 weeks. We collected the following data : (i) patients characteristics (age at the start of immunotherapy, gender, smoking status, concurrent airway disease), (ii) lung cancer characteristics (histological subtype, stage, line of treatment of the anti-PD-1, PD-L1 status), (iii) treatment characteristics (agent, response at t1 (time of first evaluation, i.e. after three (for pembrolizumab) to four (for nivolumab) cycles of immunotherapy) and t2 (time of second evaluation, i.e. after four (for pembrolizumab) to six (for nivolumab) additional cycles of immunotherapy) using REsponse Criteria for Solid Tumors (RECIST) v1.1), (iv) eosinophil counts (absolute and relative) at t0 (before treatment), t1 and t2 . Of the 191 patients identified the following patients were excluded: loss of follow-up (n=8), treatment discontinuation before t2 due to toxicity (n=2), progressive disease (n=4), patient's will (n=3) or death (n=57). Response was assessed according to the RECIST criteria version 1.1 (Eisenhauer et al., 2009). We further describe patients as responders (complete (CR) or partial (PR) response), stable or progressive. We focused on the first two radiological evaluations as the majority of objective responses (i.e., CR and PR) occur in the first two months of treatment with anti-PD-1/-PD-L1 in monotherapy for NSCLC, corresponding to the first time point (t1) in our study (Borghaei et al., 2015; Brahmer et al., 2015). We extended the evaluation period to the second radiological evaluation (t2) in order to include the patients showing a non-significant response at t1 further evolving towards PD or PR. Blood eosinophils were expressed as median number of cells/mL for the absolute eosinophil count (AEC) and in percentage of the total white blood cell count for the relative eosinophil count (REC) with interquartile range (IQR).

Regarding the statistical analyses paired comparisons of eosinophil values between the three visits of patients were performed with a non-parametric test: Wilcoxon's signed rank test. The comparison of eosinophil levels of the 3 groups of patients ranked according to the response to the treatments were performed by an unpaired test for non-parametric continuous variables: Kruskal-Wallis test followed by the Dunn's post-hoc testing if Kruskal-Wallis tests were positive. A p value <0.05 was considered statistically significant. Analyses were conducted by the statisticians of the Pneumology laboratory Unit of the CHU de Liège using GraphPad Prism V.7.03 (GraphPad Software, La Jolla, California, USA) for the statistical analyses and for the figures.

Results

In the 117 patients analyzed baseline blood eosinophils were not statistically different in responders, stable or progressive patients. For the whole study population, the AEC and REC were significantly raised at t1 compared to t0 ($p<0.01$ for both AEC and REC) (**Figure 1**). Responders and stable patients had significantly higher eosinophils than progressive patients at t2 ($p<0.05$ for AEC and $p<0.01$ for REC for responders and $p<0.05$ for both AEC and REC for stable patients). Stable patients showed an early (t1) and persistent (t2) significant rise in eosinophils ($p<0.01$ for AEC and REC at t1 and $p<0.05$ for AEC and REC at t2) (**Table 1**). Performing univariate analysis (two-way factorial ANOVA) we did not find any impact of histology, type of anti-PD-1 agent, smoking status (current versus former smoker) or PD-L1 status on those results.

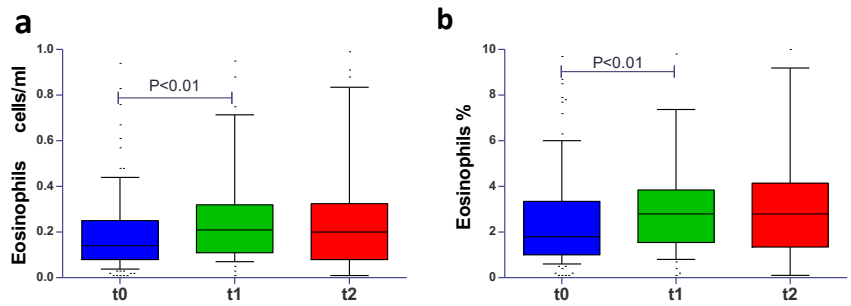

Fig. 1: Blood eosinophil levels of the entire study cohort. Results expressed as median ± IQR, 10%-90% quantiles.

Table 1: Blood eosinophil levels according to the type of response.

Eosinophils	RESPONDERS		STABLE		PROGRESSIVE	
	AEC	REC	AEC	REC	AEC	REC
t0	0,16 (0,12-0,29)	2 (1,4-3,4)	0,14 (0,08-0,27)	2 (0,95-3,6)	0,11 (0,06-0,2)	1,4 (0,75-2,4)
t1	0,2 (0,1-0,29)	2,7 (1,6-3,8)	0,23** (0,17-0,35)	3,3** (2,05-4,3)	0,16 (0,1-0,32)	1,8 (1,3-3,15)
t2	0,23*$^{\#}$ (0,14-0,33)	3,6*$^{\#\#}$ (2,1-5,2)	0,21*$^{\#}$ (0,12-0,35)	2,8*$^{\#}$ (1,75-4,05)	0,08 (0,04-0,21)	1,4 (0,4-3,4)

AEC: absolute eosinophil count; expressed as number of cells/mL. REC: relative eosinophil count; expressed as percentage of the total white blood cell count. Responders (n=27), stable (n=61) and progressive (n=29) patients: according to the RECIST criteria (see materials and methods). Inter-group analysis: Kruskal-Wallis test followed, if positive, by Dunn's test; p-value versus progressive: $^{\#\#}p<0.01$; $^{\#}p<0.05$. Intra-group analysis: Wilcoxon's paired test; p-value: **p<0.01, *p<0.05.

Discussion

Response evaluation of patients treated with PD-1 inhibitors remains a challenge. In routine clinical practice RECIST criteria remain the core element for the evaluation of response. However, atypical response patterns have been described following anti-PD-1 use showing the limitations of these criteria. We hypothesized that blood eosinophil kinetics might be an early indicator of response to ICI.

Considering the whole study population, we found a significant and early rise in blood eosinophils, i.e., after two to three months of PD-1 inhibition, compared to baseline values. In a large series (n=909) of patients treated with anti-PD-1 antibodies for various types of cancers the rise in AEC was seen from 3 months after the start of the treatment and peaked at a median of 6.4 months (Bernard-Tessier et al., 2017). The significant increase at t1 in our cohort is in keeping with this previous study although we found no further significant rise between t1 and t2, possibly due to the lower number of patients.

Baseline eosinophil counts did not differ between responders, stable or progressive patients. This contrasts with retrospective data from one melanoma series treated with anti-PD-1 agent pembrolizumab where baseline REC >1.5% was associated with an improved overall survival and more objective responses according to the RECIST criteria, although this positive prognostic and predictive value of eosinophils was only noted for the REC, not for the AEC, and in combination with the relative lymphocyte count (Weide et al., 2016). The predictive role of a composite blood biomarker was retrospectively investigated in one series of NSCLC patients and showed a longer progression-free survival, defined as the time between the start of a PD-1 inhibitor and radiological progression, in patients showing the following baseline characteristics: an absolute lymphocyte count >1 cells/mL, an absolute eosinophil count ≥0.15 cells/mL and an absolute neutrophil count <7.5 cells/mL) (Tanizaki et al., 2018).

Our main results indicate a clear association between blood eosinophils kinetics and the type of response. Indeed, eosinophils were significantly higher in responders and in stable patients than in patients with progressive disease at the time of second evaluation. Also, stable patients showed an early and persistent significant increase in eosinophils. This was not the case for responders, possibly due to a lower number of patients (27 responders vs 61 stable patients).

To the best of our knowledge no data exist to date regarding the evolution of blood eosinophil levels and the type of response to ICI in NSCLC.

The exact role of eosinophils in (lung) cancer remains uncertain at present (Simon et al., 2019a). Indeed, some preclinical studies have shown a lower incidence of squamous cell carcinoma in eosinophil-deficient mice. Most studies, however, highlight their multiple anti-tumor effects: maturation of dendritic cells, polarization of macrophages to an M1 phenotype, inhibition and normalization of tumor vasculature, recruitment and activation of T lymphocytes and NK-cells, direct cytotoxic effects on tumor cells (Carretero et al., 2015; Simon et al., 2019). Even though we cannot state whether raised blood eosinophils are a consequence or a driver of enhanced activity of PD-1 antibodies our results indicate that they might be indicators of response to anti-PD-1 drugs for NSCLC. Although we acknowledge the need for a validation study with a greater and ideally prospective cohort, we believe that the highly significant differences between eosinophils of responders and stable patients versus non-responders in our study warrant reporting.

In conclusion, our retrospective cohort suggests a role of blood eosinophils in the early response to PD-1 inhibitors in NSCLC patients.

CHAPTER 3

White blood cells in patients treated with programmed cell death-1 inhibitors for advanced non-small cell lung cancer.

Sibille A., Henket M., Corhay J-L., Louis R., Alfieri R., Duysinx B. (2021) White blood cells in patients treated with programmed cell death-1 inhibitors for advanced non-small cell lung cancer. *Lung, Oct;199*(5):549-557
https://doi.org/10.1007/s00408-021-00474-2

Study objective

In patients with advanced-stage NSCLC treated with an anti-PD-1 antibody, our first study suggested a predictive role for eosinophils at the time of the second radiological evaluation, i.e., four to six months after the start of treatment (Sibille et al., 2020).

In the present study, we investigated the association of more myeloid cells (eosinophils, total white blood cells, neutrophils, lymphocytes) with clinical outcomes such as response to PD-(L)1 inhibition, overall survival, and treatment duration. Several reports had, by the time we initiated this study, pointed at the prognostic role of neutrophils, lymphocytes, and their ratio but only one concluded to a potential prognostic role for eosinophils (Mezquita et al., 2018; Park et al., 2018; Tanizaki et al., 2018). Next, in this larger patient cohort, we aimed at gathering information on potential confounding factors for blood eosinophilia, such as the presence of a concomitant asthmatic disease, an information that was lacking in previous reports.

Abstract

Purpose: To investigate whether eosinophils and other white blood cell (WBC) subtypes could be used as response and prognostic markers to anti-Programmed cell Death-1 (PD-1) or anti-PD-Ligand-1 (PD-L1) treatments in non-small cell lung cancer (NSCLC) patients.

Methods: We retrospectively analyzed data from the NSCLC patients consecutively treated at our hospital with a PD-1/PD-L1 inhibitor in monotherapy for advanced disease. A total of 191 patients were evaluated at three time-points to investigate any relation between tumor response and WBC counts.

Results: Baseline WBC and subtypes did not differ according to the type of response seen under treatment. A higher relative eosinophil count (REC) associated with more objective responses ($p=0.019$ at t1 and $p=0.014$ at t2; OR for progression=0.54 and 0.53, respectively) independently of the smoking status, PD-L1 status and immune-related toxicity (IRT). Higher REC was also associated with a longer duration of treatment ($p=0.0096$). Baseline absolute neutrophil count was prognostic ($p=0.049$). At t1 relative lymphocytes, absolute and relative neutrophils and neutrophil-to-lymphocyte ratio were prognostic ($p=0.044$, $p=0.014$, $p=0.0033$ and $p=0.029$, respectively).

Conclusion: Our results show that in NSCLC patients anti-PD-1/PD-L1 therapy induces an early increase only in blood eosinophils, more prominent in responding patients and independent of the smoking status, PD-L1 status and IRT. Eosinophils are also associated with a longer duration of treatment. Furthermore, our data support a prognostic role of neutrophils, lymphocytes and their ratio for NSCLC patients with advanced disease treated with PD(L)-1 blockade.

Introduction

The use of immune checkpoint inhibitors (ICI) for non-small-cell lung cancer (NSCLC) is increasing. Currently validated indications include advanced and locally advanced disease (Planchard et al., 2019). One of the challenges regarding ICI lies in the evaluation of objective response to these drugs. Classically, response evaluation relies on radiological criteria based on the REsponse Criteria In Solid Tumors (RECIST) (Eisenhauer et al., 2009).However, in the setting of ICI, these criteria seem imperfect. Indeed, several atypical response patterns like pseudoprogression have been observed that make radiological evaluation less clear than it is with chemotherapy (Borcoman et al., 2019). In the search for additional evaluation tools, white blood cell (WBC) count has been investigated, among others, in melanoma and in NSCLC patients treated with Programmed cell Death (PD) Ligand (L)-1 inhibitors (Bagley et al., 2017; Diem et al., 2017; Tanizaki et al., 2018; Weide et al., 2016). Some reports also mention a potential prognostic role of WBC subtypes and/or their ratio for various malignancies among which NSCLC (Bagley et al., 2017; Park et al., 2018, 2020; Tanizaki et al., 2018). We previously reported a retrospective study investigating peripheral blood eosinophil counts as a parameter in the evaluation of response in NSCLC patients receiving PD-1 blockers (Sibille et al., 2020). In the present study, we first aimed to investigate whether the results obtained in our former cohort could be confirmed. Then, we compared the association of different subtypes of WBC with response to PD-(L)1 inhibitors and investigated the prognostic value of baseline WBC subtypes.

Material and methods

Patients

All consecutive cases of advanced stage NSCLC were collected from our internal cancer registry from August 1st 2015 until September 30th 2019. A computer-based search was performed with the following inclusion criteria: (1) use of an anti-PD-1 or anti-PD-L1 agent (pembrolizumab at 2mg/kg/3 weeks during the early access program (EAP) then at 200mg/3 weeks; nivolumab at 3mg/kg/2 weeks during the EAP then at 240mg/2 weeks; atezolizumab at 1200mg/3 weeks; durvalumab at 10mg/kg/2 weeks) or (2) a pathological diagnosis of NSCLC for which the patient was registered in the electronic treatment prescription system. For the 388 patients identified the following exclusion criteria were applied: histology other than NSCLC (n=27), missing laboratory values (n=15), loss of follow-up (n=22); early treatment discontinuation, i.e. before the second evaluation (n=106) due to death, toxicity, progressive disease without death or patient's will; ongoing treatment (n=7) or chemotherapy combined with anti-PD-1 (n=20). Based on this, 191 patients were included in the present analysis.

Data collection

We collected the following data: (i) patient characteristics: age at the start of immunotherapy, gender, smoking status, concomitant obstructive airway disease, use of inhaled or oral corticoids and the reason for it (underlying respiratory condition, immune-related toxicity (IRT), other), date of death, baseline Eastern Cooperative Oncology Group (ECOG)-Performance Status (PS); (ii) lung cancer characteristics: histology, stage of disease, line of treatment of the anti-PD (L)-1, PD-L1 expression level, based on immunohistochemistry (monoclonal antibody clone 22C3 with Automated Stainer, Dako), presence or absence of a mutation based on next generation sequencing analysis and ALK immunohistochemistry; (iii) treatment characteristics: dates of the start of treatment (t0), first evaluation (t1) and second evaluation (t2), immunotherapeutic agent, response at t1 and t2 using the RECIST criteria v1.1, immune-related toxicity (IRT), duration of treatment; (iv) biological variables: total WBC counts and differential WBC counts (neutrophils, lymphocytes, eosinophils; absolute and relative) at t0, t1 and t2 .

Response evaluation

A total of 191 patients were assessed for tumor response based on the RECIST criteria v 1.1 at two time points (t1, t2; 8 to 12 weeks interval in between) and compared with baseline data. We describe patients as responders (R; for complete or partial response), stable (S) or progressive (P). We focused on the first two radiological evaluations as the majority of objective responses occur in the first two months of treatment (t1) with PD-1 blockers in monotherapy for NSCLC (Borghaei et al., 2015; Brahmer et al., 2015). We extended the evaluation period to the second radiological evaluation (t2) in order to include the patients showing a non-significant response at t1 further evolving towards progression or response.

Duration of treatment

Duration of treatment with anti-PD (L)-1 drugs was calculated from the time of first administration until the last recorded dose administration (data cut-off December 5th 2019) and expressed in weeks.

Overall survival

Overall survival (OS) was defined as the time between the first dose of PD (L)-1 blocker and the date of death from any cause and expressed in months. If still alive at data cut-off (December 5th 2019) the patient was censored.

Statistical analyses

Biological variables were studied as continuous variables and are described as medians and interquartile range. Qualitative data are described using frequencies and percentages. For the analyses on biological variables logarithmic analyses were performed (translated logarithm log (.+1) for the relative eosinophil count (REC) in percentage and log (.+0.01) for the absolute eosinophil count (AEC) in 10^3cells/mm^3). Univariate logistic regression analyses were performed with determination of the Odds ratio (OR), with confidence interval (CI) at 95% and p-values. Survival was calculated, expressed in months and reported with Kaplan-Meier curves and Cox regression models were used to analyze the impact of the different variables on the survival and reported as Hazard Ratio (HR), with CI at 95% and p-values. Results were considered significant with an uncertainty level of 5% ($p<0.05$). Calculations were made with the help of SAS software (version 9.4) and graphs with R software (version 3.6.2).

Results

Patient characteristics

A total of 191 patients were included in the study (**Table 1**). Approximately two thirds of the patients were male with a large majority (94.8%) of (former) smokers and in good performance status (PS; 92.7% PS 0-1). Slightly more than half of the patients presented with a chronic obstructive airway disease at the time of PD(L)-1 blocker initiation but only 10.5% used inhaled corticoids and none used oral corticoids during the study period. The predominant histology was adenocarcinoma (55.5%). The majority of patients (69.7%) had stage IV disease at the time of treatment with PD(L)-1 blockade. Most of the patients (67.7%) received an anti-PD(L)-1 antibody in second or later line of treatment.

Table 1: Patients characteristics.

Characteristic		Total (n=191)	Number (Percent)
Age-years			
	Median		66
	Range		42-85
Gender		191	
	Male		122 (63.9)
	Female		69 (36.1)
Smoking status		191	
	Non smoker		10 (5.2)
	Former smoker		117 (61.3)
	Current smoker		64 (33.5)
Obstructive airway disease		191	
	None		83 (43.5)
	COPD		88 (46.1)
	Asthma		20 (10.4)
Inhaled corticosteroids		191	
	No		171 (89.5)
	Yes		20 (10.5)
ECOG-PS		191	
	0		26 (13.6)
	1		151 (79.1)
	2+		14 (7.3)
Histology		191	
	Adenocarcinoma		106 (55.5)
	NOS		7 (3.7)
	Squamous cell carcinoma		72 (37.7)
	LCNE carcinoma		6 (3.1)
Oncogenic driver		119	
	None		77 (64.7)
	EGFR		3 (2.5)
	ALK		0 (0)
	Other		19 (16)
	Unknown		20 (16.8)
Disease stage		191	
	II		2 (1.0)
	III		56 (29.3)
	IV		133 (69.7)
PDL-1 category		191	
	1		64 (33.5)
	2		26 (13.6)
	3		29 (15.2)
	4		72 (37.7)

IT line stage IV		133	
	1L		43 (32.3)
	2L^{+}		90 (67.7)
IT Agent		191	
	Nivolumab		100 (52.3)
	Pembrolizumab		58 (30.4)
	Durvalumab		22 (11.5)
	Atezolizumab		11 (5.8)

Smoking status: as registered at the start of PD(L)-1 blockade. Obstructive airway disease: COPD=chronic obstructive pulmonary disease. ECOG-PS: Eastern Cooperative Oncology Group-Performance Status. Histology: NOS=not otherwise specified. Oncogenic driver: EGFR=epidermal growth factor receptor (Tumor Hotspot Mastr kit, Illumina MiSeq); ALK=anaplastic lymphoma kinase (monoclonal antibody with Automated Stainer Benchmark, Roche); other= BRAF, KRAS, PIK3CA mutations (Tumor Hotspot Mastr kit, Illumina MiSeq); unknown= no NGS or EGFR/ALK testing done; no= at least no EGFR mutation/ALK rearrangement identified. Disease stage: according to the TNM 7th classification. PD-L1 category: 1= ≥50%; 2=1-49%; 3=<1%; 4=unknown. IT line stage IV: line of treatment for the PD(L)-1 blockade: 1L=first line; 2L+= second or later line.

White blood cell counts over time under PD(L)-1 blockade

Among the studied biological variables only eosinophils rose under PD(L)-1 inhibition between the start of treatment and the time of first or second evaluation (p<0.0001) (**Table 2**).

Table 2: Kinetics of white blood cell counts over time.

	t0	t1	t2	p -value
WBC 10^3cell/mm³	8.47 ±3.70	8.09 ± 3.16	8.56 ± 4.94	0.70
Eosinophils %	2.34 ± 2.00 [ab]	3.38 ± 2.79 [a]	3.29 ± 2.83 [b]	<0.0001
10^3cell/mm³	0.19 ± 0.20 [ab]	0.27 ± 0.27 [a]	0.29 ± 0.40 [b]	<0.0001
Lymphocytes %	20.16 ± 9.67	20.66 ± 8.54	20.41 ± 9.50	0.43
10^3cell/mm³	1.56 ± 0.75	1.55 ± 0.62	1.58 ± 0.70	0.43
Neutrophils %	67.13 ± 11.93	65.68 ± 9.81	65.65 ± 13.32	0.20
10^3cell/mm³	5.90 ± 3.28	5.47 ± 2.85	5.99 ± 4.72	0.15
NLR	4.64 ± 3.83	4.38 ± 5.26		0.20

t0:pre-treatment, t1: first evaluation, t2: second evaluation. Comparisons made with Scheffé's test between t0 – t1 (p^a <0.0001) and t0-t2 (p^b <0.0001). WBC: white blood cells. NLR: neutrophils-to-lymphocytes ratio.

Baseline WBC and subtypes did not differ between responding, stable and progressive patients (**Table 3**).

Response

At the time of first evaluation 51 (26.7%) of the 191 patients were responders (R), 103 (53.9%) stable (S) and 37 (19.4%) progressive patients (P). At t2, we found 64 R (33.5%), 67 S (35.1%) and 60 P (31.4%). We found 3 patients (4.7%) showing progression at t1 but response at t2, so-called pseudo-progression. Five R (8.3%) became P at t2.

Higher response rates were noted for high (≥50%) PD-L1 expression (unknown vs high PD-L1 status at t1 p=0.0001, odd ratio (OR) for response =0.30 (95% confidence interval (CI) 0.20-0.39; and at t2 p=0.0031, OR=0.30 (95% CI 0.16-0.56)), for pembrolizumab (P) and durvalumab (D) use vs atezolizumab (A) or nivolumab (N) (at t1: p<0.0001, OR for response with A vs P/D=0.23 (95% CI 0.06-0.98) and OR with N vs D=0.21 (0.08-0.53); at t2: p=0.0096, OR P vs N=2.59 (95% CI 1.40-4.77) and OR D vs N=2.40 (95% CI 1.01-5.71)) and in former (FS)/active (AS) smokers vs non-smokers (NS) (at t2, p=0.024; OR FS vs NS=2.88, 95% confidence interval (CI) 0.85-9.76) and OR FS vs NS=1.43, 95% confidence interval (CI) 0.41-5.02)).We also noted higher odds of clinical benefit (objective response and stable disease) in stage II&III vs stage IV (at t2, p=0.0066, OR=2.89 (95% CI 1.34-6.22)).

Regarding biological variables, none of the baseline values predicted the response at t1 or at t2. Responders had a significantly higher REC than progressive patients at t1 (p=0.019 with OR=0.54) and at t2 (p=0.014 with OR=0.53). By univariate analysis (two-way factorial ANOVA) PD-L1 status (p=0.18 for REC and p=0.067 for AEC), smoking status (p=0.43 for REC and p=0.13 for AEC) and immune-related toxicity (IRT) (p=0.87 for REC and p=0.93 for AEC) had no influence on eosinophil levels. No biological variable other than eosinophils was predictive of the response at t1 or at t2 (**Table 3**).

Table 3. Biological variables according to the type of response at t2.

	Responders		**Stable**		**Progressive**	
WBC						
t0	8.53 (5.92-10.61)		7.76 (6.22-18.49)		7.68 (6.04-10.36)	
t1	6.82 (5.74-8.98)		7.79 (5.94-8.60)		7.66 (6.25-9.42)	
t2	6.63 (5.66-8.89)		7.58 (6.54-9.13)		8.52 (6.55-10.61)	
Eosinophils	**AEC**	**REC**	**AEC**	**REC**	**AEC**	**REC**
t0	0.14 (0.08-0.28)	1.85 (0.90-3.40)	0.13 (0.09-0.23)	1.90 (1.00-3.10)	0.12 (0.06-0.21)	1.65 (0.80-8.70)
t1	0.22 (0.14-0.35)	3.1* (2.05-4.75)	0.2 (0.11-0.30)	2.9* (1.50-4.00)	0.19 (0.10-0.34)	2.6 (1.30-3.60)
t2	0.24 (0.15-0.39)	3.55 (1.85-5.50)	0.22 (0.13-0.30)	2.50 (1.80-3.70)	0.13 (0.06-0.31)	1.90 (0.80-3.80)
Neutrophils	**ANC**	**RNC**	**ANC**	**RNC**	**ANC**	**RNC**
t0	5.51 (3.75-7.70)	68.75 (60.40-74.55)	5.06 (3.96-6.87)	66.60 (60.20-74.20)	5.31 (3.88-7.59)	68.95 (62.65-74.00)
t1	4.54 (3.67-5.77)	64.45 (56.75-69.70)	5.27 (3.76-5.98)	66.90 (62.00-72.10)	5.30 (4.03-6.63)	67.15 (60.85-74.65)
t2	4.14 (3.19-5.77)	63.90 (52.70-68.50)	5.07 (4.23-6.31)	67.60 (59.50-74.00)	5.95 (4.26-7.77)	69.35 (62.90-80.15)
Lymphocytes	**ALC**	**RLC**	**ALC**	**RLC**	**ALC**	**RLC**
t0	1.62 (1.13-2.02)	19.80 (14.25-25.15)	1.33 (1.04-1.85)	18.20 (13.60-24.40)	1.51 (1.09-1.89)	17.55 (14.40-25.10)
t1	1.55 (1.10-1.86)	21.25 (15.95-26.40)	1.45 (1.10-1.82)	19.90 (15.00-24.40)	1.48 (1.01-1.85)	19.95 (12.85-24.65)
t2	1.67 (1.17-2.06)	22.20 (16.75-29.30)	1.46 (1.15-1.84)	18.50 (13.80-24.90)	1.29 (1.03-1.89)	16.80 (10.75-24.95)

WBC: white blood cells (.10^3cells/mm^3); AEC: absolute eosinophil count (.10^3cells/mm^3); REC: relative eosinophil count (%); ANC: absolute neutrophil count (.10^3cells/mm^3); RNC: relative neutrophil count (%); ALC: absolute lymphocyte count (.10^3cells/mm^3); RLC: relative lymphocyte count (%). Responders (n=64), stable (n=67), progressive (n=60) patients: according to the RECIST criteria (see materials and methods). Results expressed as medians and interquartile range. Logistic regression analysis; p-value vs progressive *p<0.05

Toxicity

The overall rate for IRT was 24.1%. Most IRT was of low intensity, requiring no immunosuppressive therapy. Indeed, only 12 out of 191 patients (6.3%) required oral corticoids (OCS) for the control of their IRT. Skin (22/191 patients, 11.6%), thyroid (9 patients, 4.7%), joints (5 patients, 2.6%) and lungs (5 patients, 2.6%) were the most frequently involved. For the durvalumab subgroup we identified significantly more IRT (40.9% reported at t1 or t2 vs. 21.9% for non-durvalumab drugs, p=0.017), a higher use of OCS (18.1% vs. 4.7%, p=0.0057) and higher pulmonary and thyroid toxicity (13.6% for both in the durvalumab group, compared to 1.2% and 3.6% for patients receiving non-durvalumab drugs, respectively; p=0.0037). There was no correlation between baseline WBC subtypes and toxicity and no correlation between toxicity and response.

Duration of treatment

At the time of first evaluation a higher REC and a lower ANC were associated with a longer duration of treatment (p=0.0096 and p=0.021, respectively) (**Figure 1**). At t2 all biological variables were predictive for the duration of treatment (data not shown).

Overall survival

The median OS was 18.8 months with 98 patients (51.3%) alive at data cut-off. No clinico-pathological feature was prognostic in this cohort. The OS was longer in patients responding at t1 (p<0.0001) with medians of OS of 30.4 months for responders, 19.9 months for stable and 12.8 months for progressive patients. A lower baseline absolute neutrophil count (ANC) correlated with longer OS (p=0.049) while at t1, the relative lymphocyte count (RLC), relative neutrophil count (RNC), ANC and neutrophil-to-lymphocyte ratio (NLR) were correlated with OS (p=0.044, p=0.014, p=0.0033 and p=0.029, respectively) (**Table 4**).

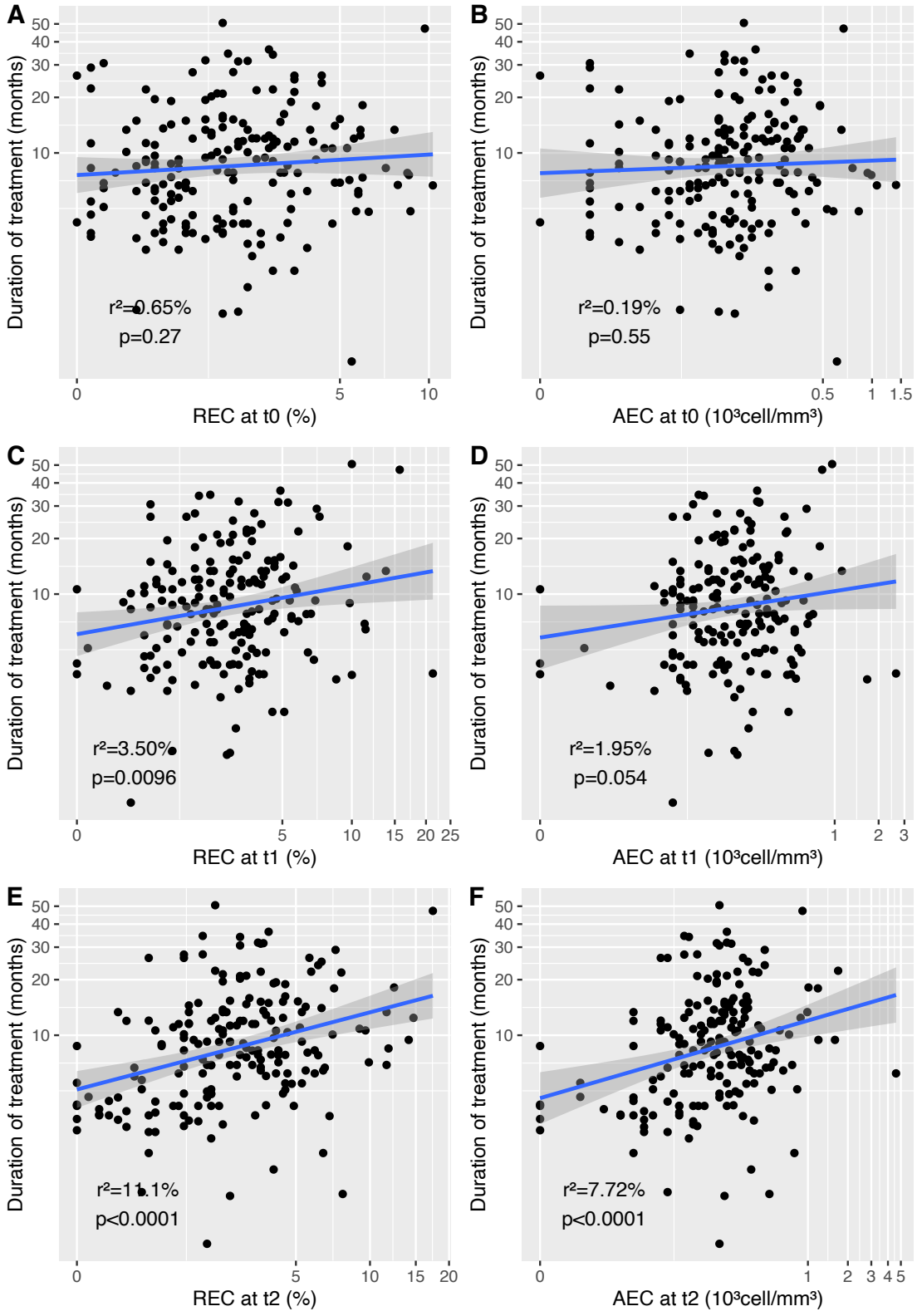

Fig. 1: Eosinophils and duration of treatment.
Logarithmic scale representation for relative eosinophil counts (REC) and absolute eosinophil counts (AEC).t0: before treatment; t1: at time of first evaluation; t2: at time of second evaluation.

Table 4: Risk of death in the 191 patients according to biological variables at t1.

Variable	t1 values	p-values	HR	95% CI
White blood cells (10^3cells/mm^3)				
Alive	7.28 (5.74-8.90)	0.094	1.78	0.91-3.51
Dead	7.80 (6.32-8.97)			
Eosinophils (%)				
Alive	2.90 (1.90-4.00)	0.081	0.70	0.47-1.04
Dead	2.80 (1.30-4.10)			
Eosinophils (10^3cells/mm^3)				
Alive	0.21 (0.12-0.33)	0.22	0.84	0.64-1.10
Dead	0.20 (0.11-0.33)			
Lymphocytes (%)				
Alive	20.85 (15.90-25.50)	0.044*	0.64	0.42-0.99
Dead	19.10 (13.80-23.00)			
Lymphocytes (10^3cells/mm^3)				
Alive	1.56 (1.10-1.88)	0.39	0.81	0.50-1.31
Dead	1.47 (1.09-1.81)			
Neutrophils (%)				
Alive	64.10 (59.40-69.20)	0.014*	1.03	1.00-1.05
Dead	68.90 (60.80-73.60			
Neutrophils (10^3cells/mm^3)				
Alive	4.62 (3.54-5.87)	0.0033*	1.11	1.04-1.20
Dead	5.21 (4.11-6.32)			
NLR				
Alive	2.96 (2.38-4.27)	0.029*	1.46	1.04-2.06
Dead	3.56 (2.67-5.35)			

Results expressed as medians and interquartile ranges. Alive (n=98)/dead (n=93): as recorded at data cut-off (see Materials and methods). HR: hazard ratio for death. CI: confidence interval. NLR: neutrophils-to-lymphocytes ratio. *: significant p-value (<0.05).

Discussion

In this cohort of advanced stage NSCLC patients treated with PD(L)-1 blockade, PD-L1 expression levels and smoking history were associated with response, confirming earlier data (Herbst et al., 2016; Mazieres et al., 2019). Pembrolizumab was associated with more responses, as it was the only drug used in patients with high PD-L1 expression levels.. Durvalumab was also associated with more responses, owing to its use in early stage of disease and after chemoradiation. We further noticed a higher probability of clinical response in earlier stages of disease. Regarding biological data we noted an early rise only in eosinophils. Moreover, a higher proportion of eosinophils was associated with an early response and with a longer duration of treatment. Neutrophils, lymphocytes and their ratio, either at baseline or early in the course of treatment, appeared to be prognostic.

The role of eosinophils in tumors is still a matter of debate. In various tumor types in vitro data and preclinical models show direct and indirect anti-tumor effects (Carretero et al., 2015; Simon et al., 2019) but also pro-tumorigenic effects (Astigiano et al., 2005; Kratochvill et al., 2015; Puxeddu et al., 2009; Zaynagetdinov et al., 2015). Neutrophils can, like eosinophils, have both anti- and pro-tumor functions (Coffelt et al., 2016; Fridlender et al., 2009).

The prognostic and predictive value of blood biomarkers and more specifically WBC and their subtypes in patients treated with ICI has been reported in several tumor types e.g. colorectal cancer (Wei et al., 2018), breast cancer (Gündüz et al., 2015; Ownby et al., 1983), prostate cancer (McNeel et al., 2014), melanoma (Gebhardt et al., 2015; Heppt et al., 2017; Moreira et al., 2017; Weide et al., 2016) and NSCLC (Tanizaki et al., 2018). However, these studies lack homogeneity: absolute vs. relative WBC counts, single vs. composite markers, continuous vs. categorized variables. Weide and colleagues proposed a prognostic model based on categorized serum lactate dehydrogenase (LDH), WBC count and clinical characteristics (Weide et al., 2016). The risk of death was 2.4-fold ($p=0.003$) and 2.2-fold ($p<0.001$) for patients with pre-treatment RLC <17.5% and REC <1.5%, respectively. In part based on these results Tanizaki and colleagues studied the prognostic and predictive value of peripheral blood biomarkers in a population of NSCLC patients treated with nivolumab for advanced disease (n=137) (Tanizaki et al., 2018). They found a strong association between baseline low (<7.5 cells/mL) ANC, high (>1.0 cells/mL) absolute lymphocyte count (ALC) and high (>0.15 cells/mL) AEC and higher response rates, progression-free survival (PFS) and OS. In those two studies, authors used

categorized variables, i.e., AEC > or <0.15 cells/mL and REC > or <1.5%. We, however, considered the variables as continuous. Keeping this in mind, in our cohort a higher proportion in eosinophils at the time of the first evaluation (t1) was associated with a higher chance of objective response to treatment at t1 and t2. In our series, this was independent of the smoking history, PD-L1 status and immune-related toxicity (IRT). We could, however, not identify a cut-off value for REC at t1 with satisfying sensitivity for discriminating responders from stable and from progressive patients at t2 (32.8% sensitivity and 81.9% specificity for a cut-off of 5.3% REC, p-value=0.0137) (**Figure 2**). Our study also emphasizes the association between blood eosinophils and the durability of clinical benefit for NSCLC patients, as expressed by the duration of treatment. A series of melanoma patients comforts these findings with response to ipilimumab correlated with an early rise in eosinophils (Gebhardt et al., 2015).

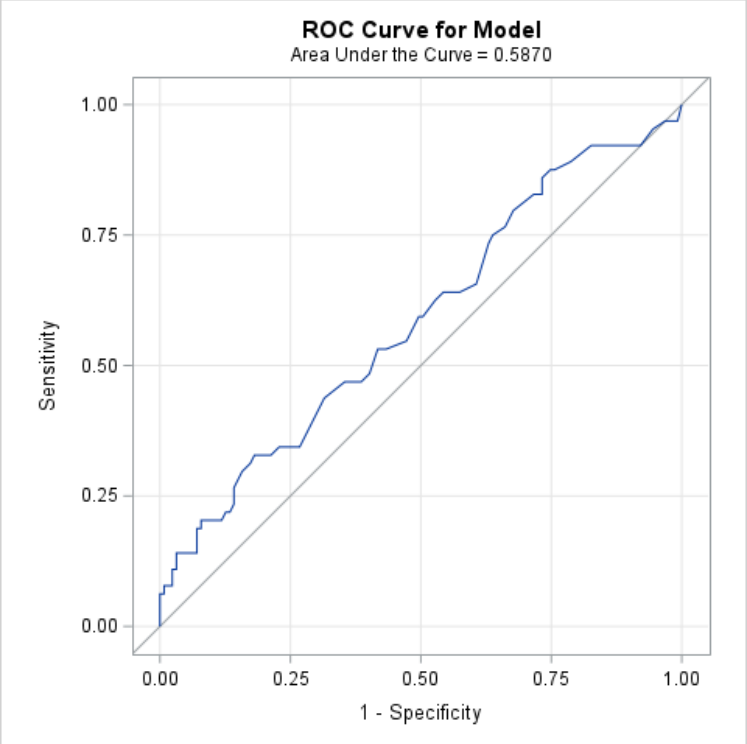

Figure 2. Receiver operating characteristic (ROC) curve based on the logistic regression analysis from the 191 patients cohorts, depicting the sensitivity and specificity of the relative eosinophil count to predict response.

In contrast to other series, toxicity in our cohort was not correlated with a higher probability of response and also not with raised eosinophils. Several retrospective studies (Haratani et al., 2018; Hasan Ali et al., 2016; Osorio et al., 2017) and one prospective report (Teraoka et al., 2017) showed an association between early IRT for advanced NSCLC and outcome. Although these studies are small-sized and mostly lack pathological correlation, there is some rationale

to explain this link: similarity between tumor antigens and self-antigens leading to cross-reactivity of T-cells that are reactivated by the ICI (Cui & Bystryn, 1995), pre-existing auto-immunity with reactivation of T-cells primarily directed at self-antigens (Yoest, 2017) or B-cell reactivation through PD-1 blockade (Zhang et al., 2019). The fact that we did not find a correlation with response may be due to the retrospective nature of the study with incomplete data collection during patients' follow-up. On the other hand, a correlation between eosinophilia (i.e. AEC >0.5 cells/mL) and immune-related toxicity (p=0.0042) has been demonstrated in a retrospective series including 146 patients with various solid tumor types treated with anti-PD(L)-1 (Krishnan et al., 2020). As a correlation between eosinophils and response to ICI and between eosinophils and toxicity under ICI were shown, it is tempting to think that both clinical results (response and toxicity) are two sides of one phenomenon: immune (re-) activation. This, however, remains to be formally proven.

Some authors found a prognostic value of baseline eosinophils (Tanizaki et al., 2018; Weide et al., 2016). This was not the case in our series. However, we found a clear association between eosinophils and response to treatment and between response and OS. The lack of prognostic value of REC at t0 may be due to small sample size when compared to the work of Weide and colleagues. Moreover, the prognostic value of baseline neutrophils, lymphocytes and neutrophils/lymphocytes ratio (NLR) demonstrated in our work support the findings of several authors (Bagley et al., 2017; Park et al., 2018; Wang et al., 2019). Illustratively, the prognostic value of the iSEND model (immunotherapy, Sex, ECOG-PS, NLR, and Delta NLR) is being investigated in a prospective manner after it showed its value as a predictive tool for patients with advanced NSCLC treated with nivolumab (Park et al., 2018). In earlier stages of disease a study on operated NSCLC specimen revealed an inverse correlation between neutrophils and CD8+ cytotoxic T cells (Kargl et al., 2017).

An additional interesting finding of the present study is that blood eosinophils is the only WBC subtype displaying a rise during the first six months of anti-PD (L)-1 therapy for NSCLC, data that are in keeping with results from a large French cohort and from our previously published data (Bernard-Tessier et al., 2017; Sibille et al., 2020). Further studies will have to explore why this rise is transient and whether raised eosinophils in responders are a consequence of or a trigger for immune anti-tumor activation.

Conclusion

In this study patients receiving PD-(L)1 blockade for advanced NSCLC and showing a raised proportion of eosinophils at the time of first evaluation were more likely to show an objective response according to the RECIST criteria at the time of second evaluation, regardless of smoking history, PD-L1 status and IRT. A higher REC also correlated with a longer duration of treatment. We could, however, not identify a clear cut-off value to propose eosinophils as a predictive biomarker. It seems necessary to identify the underlying mechanism(s) leading to a rise in blood eosinophils in patients deriving clinical benefit from anti-PD-(L)1 drugs. Further results of this cohort support the prognostic role of neutrophils, lymphocytes and their ratio, either at baseline or early in the course of treatment.

CHAPTER 4

Tissue eosinophils in non-small cell lung cancer: the TEN study.

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Study objective

While the first part of our work focused on blood (B-)eosinophils (Eos), our literature review showed that information on tissue (T-)Eos in NSCLC was scarce in early-stage and (almost) inexistent in later-stage disease (Tataroğlu et al., 2004; Ye et al., 2019). Reviewed in the same work, pre-clinical evidence showed a-at first glance- conflicting evidence on the role of eosinophils based on tissue data (Sibille et al., 2022).

On the clinical side, Hu and colleagues conducted a meta-analysis on the prognostic role of tumor-associated tissue eosinophils (TATE) in various solid tumors, totalizing more than 6000 cases (Hu et al., 2020). At a global level, the pooled studies showed a favorable prognostic role for TATE in terms of OS (HR for death=0.82, p=0.041) but not in terms of disease-free survival (DFS) (HR for progression or death=1.13, p=0.598). Looking deeper at the data by histological subgroup, TATE had a higher prognostic value in colorectal (HR for death=0.70, p<0.001) and in esophageal cancer (HR for death=0.35, p=0.026). Of note, this meta-analysis did not include lung cancer cases.

For lung cancer, two authors have reported on T-Eos so far. In a series of 63 NSCLC resection specimen, Tataroglu and colleagues found no correlation between TATE and disease stage, a surrogate for DFS and OS (Tataroğlu et al., 2004). Eosinophils were identified with the use of a classical hematoxylin-eosin (HE) stain and manually counted under a light microscope. In another series of 30 adenocarcinoma samples from the lung, Ye et al described a correlation between TATE, as assessed by manual counting but with a staining using an antibody directed at the eosinophil peroxidase (EPO), and pTNM stage (Ye et al., 2019). The two main findings were that the EPO concentration was significantly higher in tumor than in paired, non-tumoral tissue and that EPO intensity level was an independent prognostic marker in lung adenocarcinoma.

Considering these results, we undertook to quantify TATE in a large cohort of patients who underwent anatomical lung resection for early-stage to locally advanced NSCLC at our academic hospital. HE stains yielding frequently poor tissue eosinophils quantification results, we decided to use a stain directed at the eosinophils' granules, the major basic protein (MBP) (Weller & Spencer, 2017). Because of the large number of samples, we chose a semi-automated system for eosinophil quantification, i.e., the QuPath® software. QuPath® is a well-recognized

and widely used image analyzer used in pathology, among others in cancer research (Berben et al., 2020). Next, we were interested in their localization because a difference had been noted in preclinical models of melanoma, a finding that we now can explain by the production of IL-33 and HMGB-1 by dying tumor cells (Cormier et al., 2006). Then, TATE in our patients was correlated with survival outcomes. Finally, we gathered information on B-Eos given the lack of data on this topic in early-stage NSCLC.

Abstract:

Background: Prognostic biomarkers in early stage (e), resected non-small cell lung cancer (NSCLC) are lacking. Eosinophils (Eos) are leucocytes with equivocal roles in cancer immunity. Tissue (T-) Eos in eNSCLC are poorly described.

Research question: We investigated the prognostic value of T- and blood (B)-Eos in eNSCLC.

Study design and methods: 316 consecutive eNSCLC (307 paired, i.e., tumor (T) and healthy (H) tissues) resected at our hospital with adequate tissue were retrospectively included. T-Eos were stained with an antibody against the major basic protein (MBP). QuPath®, an image analysis software was used to detect and quantify T-Eos in T and H slides. We defined 6 zones and 3 staining intensity levels. We compared the QuPath® and manual counts in a test series (N=60). Preoperative white blood cell (WBC) data were collected.

Results: T-Eos were detected in 80.8% of the samples. T-Eos numbers varied significantly across staining batches ($p<0.001$). More T-Eos were detected in the tumor compared to the adjacent parenchyma ($p=0.0059$) and compared to H tissue ($p<0.0001$), even after adjustment for the batch effect. Total WBCs were associated with overall survival (OS) and disease-free survival (DFS) and the absolute neutrophil count only with OS. T-Eos were not associated with OS (HR=1.03, p=0.49; median follow-up not reached). DFS was longer when T-Eos were detected on the T slide, at a distance from the tumor (HR=0.62, p=0.023). The intraclass correlation coefficient between QuPath® and manual counting was 0.552 (95% confidence interval 0.349; 0.705) for intermediate to high MBP staining intensity.

Interpretation: In this QuPath® quantified, large series of resected eNSCLC, T-Eos were frequently identified. They concentrated in the tumor zone. No association was found between T-Eos and OS; higher T-Eos at a distance from the tumor were associated with a longer DFS. The inter-rater reliability of QuPath® vs manual counting was good.

Trial registration: ClinicalTrials.gov ID: NCT05537701

Lung cancer is the second most frequent cancer type worldwide, the first in men and the third in women (Sung et al., 2021). With an aging population and lung cancer screening programs, those figures are expected to increase further. The most frequent type of lung cancer is non-small cell lung cancer (NSCLC), accounting for an approximate 85% of the cases. Despite adequate treatment, 30 to 55% of early stage (e) NSCLC will recur (Taylor et al., 2012; Uramoto & Tanaka, 2014). The 5-year overall survival (OS) rates are low, ranging from 90% for stage IA1 to 24% for stage IIIB Tumor Node Metastasis (TNM) classification 8th edition (Goldstraw et al., 2016). Predicting recurrence might help develop patient-tailored strategies to optimize their management and, ultimately, their survival.

Over the last decade, the study of the tumor microenvironment (TME) has gained a lot of interest (Hanahan & Weinberg, 2011). Among the cells that shape the TME, myeloid cells are the focus of intensive research efforts, particularly since the advent of immune checkpoint inhibitors (ICI) (Delyon et al., 2013). Eosinophils (Eos) are rare (normal range <5% relative eosinophil count (REC), <0.5 cells/mL absolute eosinophil count (AEC)), multipotent leucocytes. Their role as agents of the innate and adaptive immune system in the context of cancer has been ascertained in preclinical models (Grisaru-Tal et al., 2020; Sibille et al., 2022). In humans, tissue (T-)Eos have been reported in several tumor types and correlate with favourable outcomes in terms of overall survival (OS), particularly in colorectal and oesophageal cancer (Hu et al., 2020). In resected NSCLC, two publications reported on T-Eos with variable methodology and contrasted results, both on a limited number of samples (Tataroğlu et al., 2004; Ye et al., 2019). Finally, only one study reported on the preoperative B-Eos values (Dai et al., 2023). In that cohort, Dai and colleagues found that a preoperative low and postoperative normal B-Eos was associated with a favourable prognosis.

In view of the paucity and heterogeneity of data on tissue eosinophils (T-Eos) in NSCLC, we decided to quantify them in a large series of eNSCLC resected at the University Hospital of Liège. To be able to handle a large number of samples we chose to use the QuPath® software, an open-source, digital image analysis software frequently used in digital pathology (Bankhead et al., 2017; Viswanathan et al., 2022). We hypothesised that T-Eos would be correlated with OS and/or disease-free survival (DFS). Additionally, we wanted to describe the T-Eos localization on the tumor slide and aimed at comparing, paired-wise, their concentrations with those in the adjacent, non-tumoral lung parenchyma. Indeed, in a melanoma mouse model, Cormier and colleagues reported that Eos concentrated in the necrotic and fibrotic zones of and

around tumors (Cormier, 2006). We postulated that, if prognostic, T-Eos numbers would be differentially located in tumoral vs non-tumoral tissue, and, perhaps, even show differences within the tumoral tissue. Lastly, we gathered data on B-Eos before surgery in this eNSCLC patient population.

Study design and methods:

Clinical and pathological data collection:

A computer-based search was conducted for all consecutively resected NSCLC at the University Hospital of Liège, Belgium, between 01-01-2015 and 02-28-2022. We excluded 51 patients, based on: histology other than NSCLC (n=11), incomplete pathological stage of disease (n=9), incomplete clinical data (n=9), surgery date (n=1), double extraction (n=1), loss of follow up (n=20) (**Figure S1**). Archived tissue blocks were retrieved for analysis of both tumoral and non-tumoral lung parenchyma. Here, further exclusion of 27 patients occurred, due to unmet pathology criteria (**Figure S1**). A total of 316 patients were included in the analysis. The clinical data collected from patients' charts are: (1) patient characteristics (**Table 1a**); (2) tumor characteristics (**Table 1b**); (3) survival data: overall survival (OS), defined as the time elapsed between surgery and death or last survival follow-up, and disease-free survival (DFS), defined as the time elapsed between surgery and routine clinical follow-up pointing at relapse or death, whichever occurred first. The censoring date for the survival data was September 1, 2023. The median duration of follow-up was 44.9 months (mo.). The study protocol was approved by the Ethics committee of the Centre Hospitalier Universitaire de Liège with reference 2022/258 and registered at clinicaltrials.gov with reference NCT05537701.

Table 1A. Patient characteristics.

Variable	Categories	N	N (%)	Mean ± SD	Med (Q1 ; Q3)	Extremes
Gender	Female	316	127 (40.2)			
Age (years)		316		64.8 ± 8.6	66 (59.5; 71)	29; 89
Smoking		316	278 (88.0)			
Atopy		316	34 (10.8)			
Airway disease		316	160 (50.6)			
	Asthma		*32 (10.1)*			
	COPD		*122 (38.6)*			
	Overlap		*6 (1.9)*			
Parasitic disease		316	0 (0.0)			
Corticoids		316	61 (19.3)			
	Inhaled		*53 (16.8)*			
	Systemic		*8 (2.5)*			
Performance status: 1 vs 0		316	118 (37.3)			
History of cancer		316	65 (20.6)			
Relapse		316	121 (38.3)			
Death		316	81 (25.6)			

Smoking : current or past active smoking history ; atopy : any history of allergy (air-borne, food, drug, contact); COPD : chronic obstructive pulmonary disease ; overlap : as mentioned in patient's file, obstructive airway disease either will significant reversibility after beta-agonist inhalation or fixed obstructive defect in a non-smoker ; corticoids : as registred at the time of surgery ; performance status : according to the Eastern Cooperative Oncology Group scale ; history of cancer: prior cancer.

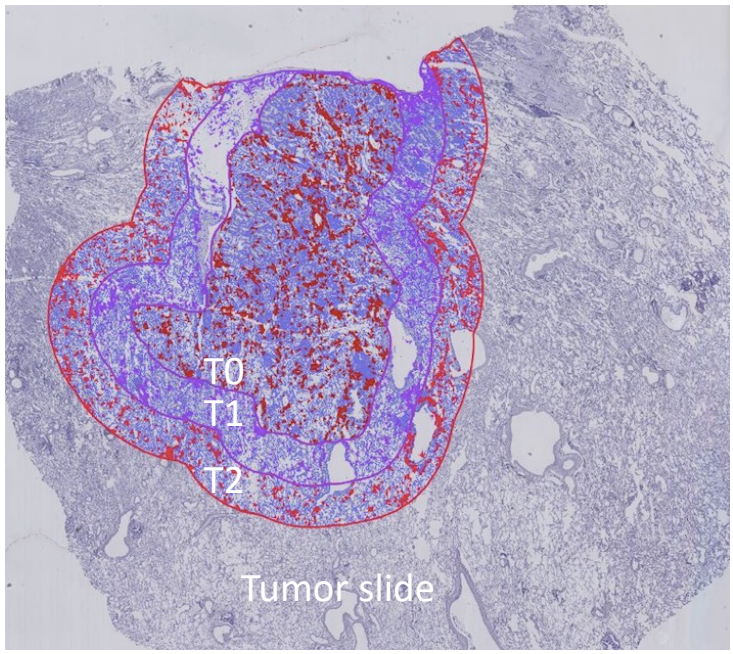
Table 1B. Tumor characteristics.

Variable	Categories	N	N (%)
Resection type		316	
	Lobectomy		297 (94.0)
	Bilobectomy		11 (3.5)
	Pneumonectomy		7 (2.2)
	Wedge or segmentectomy		1 (0.3)
Histology		316	
	Adenocarcinoma		220 (69.6)
	Squamous cell carcinoma		71 (22.5)
	Neuroendocrine NSCLC		16 (5.1)
	Mixed type		3 (1.0)
	NOS		6 (1.9)
Neoadjuvant treatment		316	
	No		266 (84.2)
	Yes		50 (15.8)
pTNM		316	
	IA		179 (56.7)
	IB		33 (10.4)
	IIA		50 (15.8)
	IIB		23 (7.3)
	IIIA		30 (9.5)
	IIIB		1 (0.3)
PD-L1		316	
	≥50%		85 (26.9)
	1-49%		67 (21.2)
	<1%		67 (21.2)
	Unknown		97 (30.7)
Oncogenic driver		316	
	Yes		102 (32.3)
	KRAS		39 (12.3)
	EGFR		22 (7.0)
	ALK		4 (1.3)
	BRAF		1 (0.3)
	PIK3CA		2 (0.6)
	METex14 skipping		3 (1.0)
	STK11		9 (2.9)
	other		22 (7.0)
	No		101 (32.0)
	Unknown		26 (8.2)
	NA		87 (27.5)

Neuroendocrine NSCLC : carcinoid tumors and large cell neuroendocrine tumors; NOS : not otherwise specified ; pTNM : pathological Tumor, Node and Metastasis according to the TNM^{7th} classification ; PD-L1 : programmed death-ligand1 ; oncogenic driver : as assessed by the NGS kit Tumor Hotspot MASTR plus, Multiplicom; KRAS : kirsten rat sarcoma ; EGFR : epidermal growth factor receptor ; ALK : anaplastic lymphoma kinase ; NA : not applicable (carcinoids+squamous carcinomas).

Tissue eosinophils (T-Eos):

Tissue blocks were retrieved from archived resected NSCLC specimen: tumor (T) and paired, non-tumoral («healthy», H) blocks, i.e., remote from the tumor. Blocks were sliced at a 4µm thickness per slide. All slides were stained with hematoxilin-eosin and with an antibody (AB) against the Major Basic Protein (MBP) in eosinophil granules (**Tables S1-S3** for the detailed staining protocol). We defined 3 levels of Eos granule staining intensity: low (some MBP deposit, MBP1), intermediate (moderate MBP deposit, MBP2), and high (cell full of MBP deposit, MBP3). We used nasal polyps, that are Eos-rich, to check the good performance of the MBP stain. Stained slides were scanned with a Hamamatsu NanoZoomer scanner. For a more precise localization of Eos, we created six zones: T0, corresponding to the tumor; H0, corresponding to a central zone on the H slide, with a surface equalling the average surface of T0; T1/H1, an expand of 1200µm around the T0/H0 margin; T2/H2, an expand of 1200µm around the T1/H1 margin (**Figure 1a**).



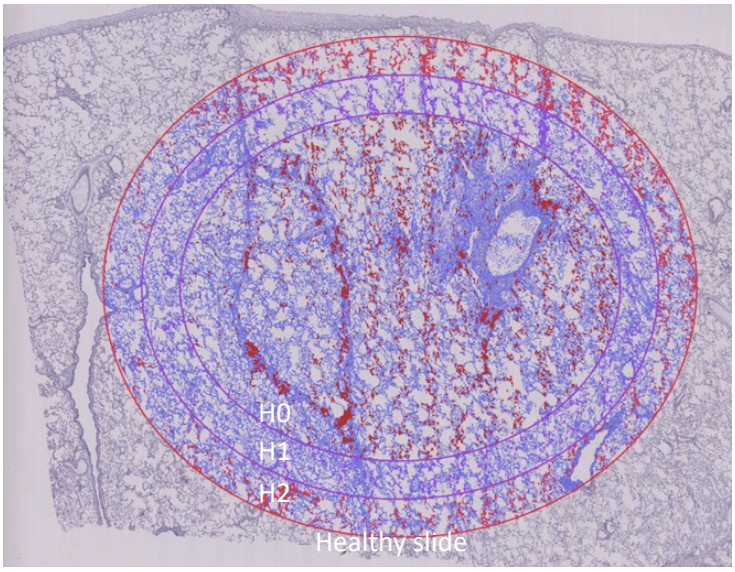


Figure 1A. Eosinophil quantification process by QuPath®. Regional cell detection in the 3 delineated areas: T0 or H0=tumor or healthy core tissue; T1 or H1=inner margin, i.e., the zone between tumor (on T slides) or healthy core zone (on H slides) and outer margin; T2 or H2= outer margin; areas 1 and 2 are at 1200 µm distance from each other. The limit of the tumor was manually annotated by a certified pathologist. Total H area=mean of the total area on T slides. T: tumor; H: healthy; MBP: major basic protein.

We used the QuPath® version 0.4.1 software for T-Eos quantification (see **Supplemental file** for the detailed methodology). QuPath® is an open-source image analysis software frequently used in digital (lung) pathology (Bankhead et al., 2017; Viswanathan et al., 2022). Scripts are available at https://github.com/AlexHego/Eosino_Tumor. The software detects and quantify the number of Eos according to the staining colour, in the 6 pre-defined zones (**Figure 1b**). Empty spaces and anthracosis were removed by QuPath®, and manually thereafter if needed. Then, the numbers and concentrations of T-Eos in the different zones were exported for further analysis. To ensure a reasonable correlation between the QuPath® and manual quantification, both methods were used on 60 zones and the results compared.

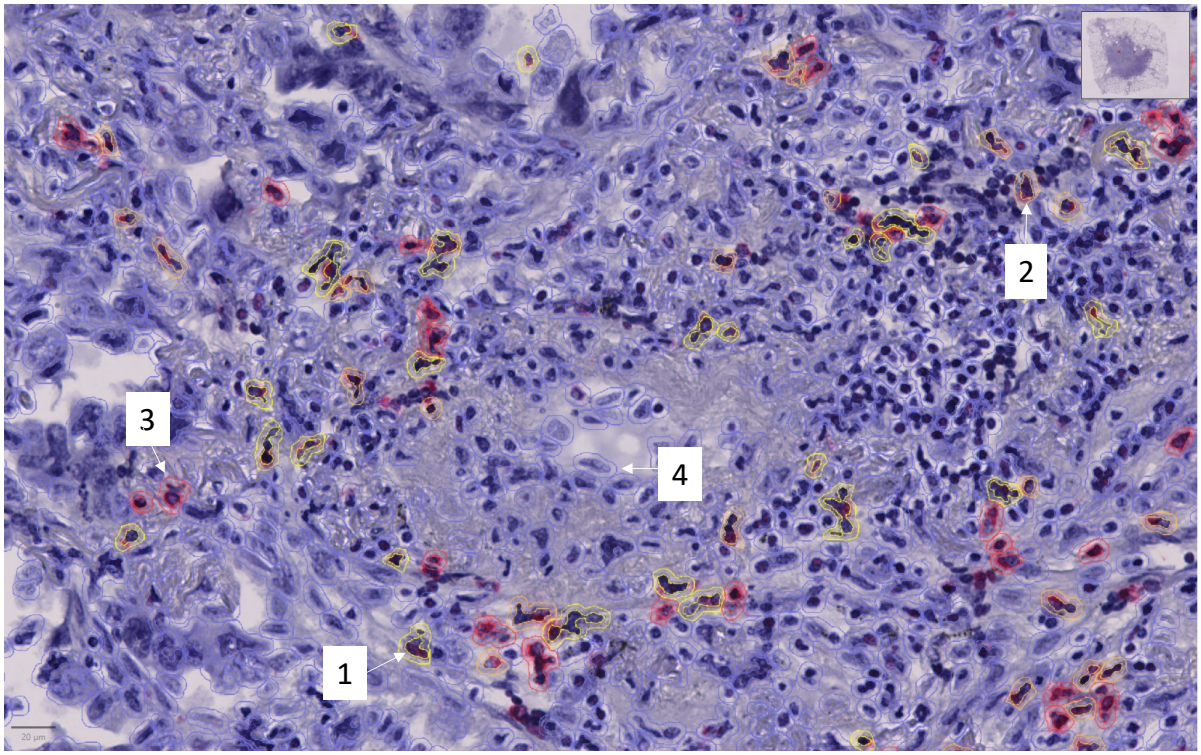


Figure 1B. Annotation of eosinophils on a tumor slide. 1: eosinophil weakly staining for MBP (MBP1); 2: eosinophil moderately staining for MBP (MBP2); 3: eosinophil strongly staining for MBP (MBP3). 4: negative (i.e., non-eosinophilic) cell. MBP: major basic protein. Scale: 20µm.

Of the 316 patients included, 9 had unpaired slides. For 60 patients we had data doublets/triplets (2/3 T or 2/3 H slides per patient). The quantification expressed was then the mean of the quantification of each slide. Five outliers (anthracosis not detected by QuPath®) were detected late in the process, manually checked, and excluded. Eleven slides did not have H2 and/or T2 zones, totalizing 1853 zones analysed.

Blood eosinophils (B-Eos):

White blood cell (WBC) data including B-Eos were retrieved from the patients' charts (range day-30 before surgery to day 0, i.e., the day of the surgery). Blood samples were analysed by laser flow cytometry as per laboratory protocol (on the XN-9000 platform from Sysmex). We studied B-Eos in a continuous and in a categorical manner (REC cutoff=1.5%; AEC cutoff=0.15 cells/mL), as both methods have been used (Chu et al., 2020; Okauchi et al., 2021; Sibille et al., 2021; Tanizaki et al., 2018).

Statistics:

Qualitative data are described with frequencies (numbers and percentages). Quantitative data are described as means ± standard deviation (SD), medians and quartiles, minimum and maximum values. A normalization of the following data was needed through logarithmic transformations: a classical logarithm for WBC (cells/mL), absolute neutrophil and lymphocyte counts (ANC and ALC, respectively; cell/mL) and their ratio (ANC/ALC, neutrophil-to-lymphocyte ratio, NLR); a translated logarithm log (. +1) for the REC (%); a translated logarithm log (. +0.01) for AEC (cell/mL); and a logarithmic translation log (·+0.0001) for the graphical representation of percentages and concentrations of Eos. To determine the impact of individual variables on Eos quantification, generalized logistic regression models (genmod) were performed to deal with paired data and an adjustment for the batch was considered as a batch effect was detected (**Figure S2**). For each model, we provide the adjusted odd ratios (OR), with confidence interval (CI) at 95% and associated p-values. Survival was expressed in months, and reported with Kaplan–Meier curves, and Cox regression models were used to analyze the impact of the different variables on survival, which were reported as Hazard Ratio (HR), with CI at 95% and associated p-values. Results were considered significant with an uncertainty level of 5% ($p<0.05$). An intraclass correlation test was carried out for the comparison of the manual and the QuPath® T-Eos counting. The concordance was rated as poor/moderate/good/excellent if the CI was <0.5/0.5-0.75/>0.75 (Koo & Li, 2016). Calculations were performed using SAS software (version 9.4) and graphs with R software (version 4.2.2).

Results:

Study population:

Of the 316 patients in our study, a majority were male patients (59.8%) with a median age of 66 years (**Table 1a**). A vast majority were (ex-)smokers (88%) and 10.8% had an atopic condition. Obstructive airway disease was reported in half of the patients. 53 patients (16.8%) used inhaled corticoids and 8 patients (2.5%) took oral corticoids at the time of surgery. Approximately two thirds of the patients had an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0. Lobectomy was the most frequently used surgical procedure (94%). 50 patients (15.8%) underwent neoadjuvant chemotherapy. The most frequent histology was adenocarcinoma (69.6%). 90.2% of the tumors were classified as pathological stages I and II. In 30.7% of the cases, no PD-L1 expression was determined and for 102 patients (32.3%) an oncogenic driver was found (**Table 1b**).

T-Eos quantification:

QuPath® quantification and manual quantifications were compared on 60 zones, using the Koo & Li methodology. Including all MBP levels, the inter-rater reliability was initially poor (intraclass correlation coefficient (ICC)=0.0927, 95% CI 0.000-0.336). For MBP2 & 3, the reliability was moderate to good (ICC=0.552, 95% CI 0.349-0.705).

In the whole cohort, T-Eos were detected in 1498 zones (80.8%) (**Table 2**). The mean T-Eos rate and concentration were 0.35 (± 1.18 SD) and 6.43 (± 21.94 SD), respectively. We observed significant differences in the detection of T-Eos, depending on the staining batch (**Figure S2**). This parameter was thus added in the statistical models to correct for this bias. We noted higher T-Eos concentrations in T than in H slides, and higher in the T0 than in the T2 zones (**Table 3**). This difference was also significant between T0 and T1 zones but at a lower significance level. The adjusted odd ratios for Eos detection in H0/H1/H2/T1/T2 vs in T0 were 0.39/0.27/0.31/0.54/0.41, respectively ($p<0.0001$ for all except $p=0.0059$ for T1) (**Figure 2** and **Table S4**). Squamous cell carcinomas showed more T-Eos (median 1.76 (0.14-11.30)) than other histologies (median 0.43 (0.06-2.70)), $p=0.023$. No statistical difference was noted in T-Eos quantification according to corticoid intake ($p=0.50$ for the AEC and $p=0.38$ for the REC; **Table S5**). Rates and concentrations of T-Eos were lower in patients with neoadjuvant compared to without chemotherapy but without statistical significance (**Table S6**). The Eos rate and concentration differed according to the stage of disease, although these differences

lacked significance (stage II vs I, p=0.71 (rate) and p=0.37 (concentration); stage III vs I, p=0.20 (rate) and p=0.92 (concentration)).

Table 2. Eosinophil quantification for the entire cohort.

Variable	N	Mean ± SD	Med (Q1 ; Q3)	Extremes	Detected N (%)
Positive rate (%)	1853	0.35 ± 1.18	0.01 (0.00; 0.12)	0.00; 17.42	1498 (80.8)
Concentration of total MBP (n/mm²)	1853	6.43 ± 21.94	0.21 (0.02; 2.26)	0.00; 304.69	1498 (80.8)
Concentration of negative cells (n/mm²)	1853	1987.3 ± 1151.3	1708.5 (1053.3; 2725.1)	0.0; 7214.5	1849 (99.8)
Concentration of detected cells (n/mm²)	1853	1993.7 ± 1154.6	1709.9 (1057.8; 2736.9)	0.0; 7282.9	1849 (99.8)
Concentration of MBP1 (n/mm²)	1853	4.34 ± 14.21	0.16 (0.02; 1.36)	0.00 ; 196.86	1474 (79.6)
Concentration of MBP2 (n/mm²)	1853	1.16 ± 4.59	0.02 (0.00; 0.36)	0.00; 65.73	1014 (54.7)
Concentration of MBP3 (n/mm²)	1853	0.93 ± 5.50	0.00 (0.00; 0.12)	0.00 ; 131.45	813 (43.9)
Positive rate of MBP1 (%)	1853	0.24 ± 0.81	0.01 (0.00; 0.07)	0.00; 11.02	1474 (79.6)
Positive rate of MBP2 (%)	1853	0.06 ± 0.25	0.00 (0.00; 0.02)	0.00; 4.95	1014 (54.7)
Positive rate of MBP3 (%)	1853	0.04 ± 0.26	0.00 (0.00; 0.01)	0.00; 6.28	813 (43.9)

Positive rate: number of eosinophils/total number of cells detected, in %; MBP: major basic protein. Total MBP: eosinophils detected with any MBP intensity level. Negative cells: non-eosinophilic cells. Detected cells: eosinophils + negative cells. MBP1/2/3: eosinophils with weak/intermediate/strong staining for MBP. N: number.

Table 3. Eosinophil quantification according to the zone.

Variable	Zone	N	Mean ± SD	Med (Q1 ; Q3)	Extremes	Detected N (%)
Positive rate (%)						
	H0	309	0.36 ± 1.13	0.01 (0.00; 0.08)	0.00; 9.89	246 (79.6)
	H1	310	0.39 ± 1.29	0.01 (0.00; 0.11)	0.00; 10.73	230 (74.2)
	H2	299	0.37 ± 1.36	0.01 (0.00; 0.10)	0.00; 17.42	228 (76.2)
	T0	311	0.23 ± 0.73	0.01 (0.00; 0.12)	0.00; 7.39	282 (90.7)
	T1	312	0.35 ± 1.20	0.01 (0.00; 0.17)	0.00; 13.62	261 (83.6)
	T2	312	0.37 ± 1.28	0.02 (0.00; 0.15)	0.00; 15.30	251 (80.4)
Concentration of MBP1 (n/mm²)						
	H0	309	3.28 ± 12.16	0.10 (0.01; 0.69)	0.00; 158.00	243 (78.6)
	H1	310	3.75 ± 15.15	0.09 (0.00; 0.82)	0.00; 196.86	225 (72.6)
	H2	299	3.22 ± 10.33	0.11 (0.02; 0.87)	0.00; 108.14	226 (75.6)
	T0	311	5.60 ± 16.67	0.32 (0.04; 2.63)	0.00; 158.05	278 (89.4)
	T1	312	5.39 ± 15.74	0.25 (0.03; 2.12)	0.00; 137.87	256 (82.0)
	T2	312	4.76 ± 13.97	0.21 (0.02; 1.73)	0.00; 122.94	246 (78.8)
Concentration of MBP2 (n/mm²)						
	H0	309	0.80 ± 3.45	0.01 (0.00; 0.16)	0.00; 38.90	169 (54.7)
	H1	310	0.86 ± 4.33	0.00 (0.00; 0.16)	0.00; 65.73	146 (47.1)
	H2	299	0.69 ± 2.89	0.00 (0.00; 0.15)	0.00; 35.68	135 (45.1)
	T0	311	1.81 ± 6.54	0.08 (0.00; 0.71)	0.00; 59.34	210 (67.5)
	T1	312	1.54 ± 5.10	0.03 (0.00; 0.76)	0.00; 37.83	183 (58.6)
	T2	312	1.25 ± 4.21	0.02 (0.00; 0.51)	0.00; 37.69	171 (54.8)
Concentration of MBP3 (n/mm²)						
	H0	309	0.51 ± 3.34	0.00 (0.00; 0.04)	0.00; 46.07	121 (39.2)
	H1	310	0.40 ± 2.24	0.00 (0.00; 0.04)	0.00; 26.63	106 (34.2)
	H2	299	0.37 ± 2.76	0.00 (0.00; 0.05)	0.00; 45.26	102 (34.1)
	T0	311	1.92 ± 8.44	0.02 (0.00; 0.41)	0.00; 89.30	182 (58.5)
	T1	312	1.37 ± 8.09	0.01 (0.00; 0.31)	0.00; 131.45	157 (50.3)
	T2	312	0.97 ± 4.33	0.00 (0.00; 0.15)	0.00; 48.85	145 (46.5)
Concentration of total MBP (n/mm²)						
	H0	309	4.6 ± 16.6	0.1 (0.0; 1.0)	0.0; 192.8	246 (79.6)
	H1	310	5.0 ± 20.7	0.1 (0.0; 1.2)	0.0; 284.6	230 (74.2)
	H2	299	4.3 ± 14.3	0.1 (0.0; 1.1)	0.0; 131.7	228 (76.2)
	T0	311	9.3 ± 29.2	0.5 (0.0; 4.5)	0.0; 304.7	282 (90.7)
	T1	312	8.3 ± 26.3	0.3 (0.0; 3.9)	0.0; 283.5	261 (83.6)
	T2	312	7.0 ± 20.2	0.3 (0.0; 2.9)	0.0; 170.6	251 (80.4)
Conc. of negative cells (n/mm²)						
	H0	309	1247.0 ± 678.7	1059.6 (806.7; 1518.4)	0.0; 4443.9	308 (99.7)
	H1	310	1210.8 ± 587.7	1081.4 (807.9; 1434.6)	84.6; 3607.1	310 (100.0)
	H2	299	1215.1 ± 560.2	1093.6 (807.8; 1463.7)	0.0; 3483.7	298 (99.7)
	T0	311	3716.0 ± 809.5	3739.8 (3232.3; 4173.3)	0.0; 7214.5	309 (99.4)
	T1	312	2450.5 ± 802.1	2386.4 (1907.6; 2891.8)	603.4; 6741.7	312 (100.0)
	T2	312	2045.3 ± 755.7	1919.9 (1476.8; 2499.3)	711.5; 6044.4	312 (100.0)
Conc. of detected cells (n/mm²)						
	H0	309	1251.6 ± 683.3	1059.6 (810.2; 1536.3)	0.0; 4465.3	308 (99.7)
	H1	310	1215.9 ± 592.3	1084.4 (810.1; 1443.4)	84.6; 3654.4	310 (100.0)
	H2	299	1219.4 ± 562.1	1093.7 (814.3; 1475.3)	0.0; 3533.7	298 (99.7)
	T0	311	3725.4 ± 814.3	3746.3 (3233.4; 4180.6)	0.0; 7282.9	309 (99.4)
	T1	312	2458.8 ± 803.8	2412.2 (1914.1; 2910.0)	603.4; 6787.4	312 (100.0)
	T2	312	2052.3 ± 756.7	1919.9 (1478.1; 2510.4)	711.5; 6102.9	312 (100.0)

Positive rate: number of eosinophils/total number of cells detected, in %; MBP: major basic protein. MBP1/2/3: eosinophils with weak/intermediate/strong staining for MBP. Total MBP: eosinophils detected at any MBP intensity level. Negative cells: non-eosinophilic cells. Detected cells: eosinophils + negative cells. T0/T1/T2 and H0/H1/H2: refer to the zone on the tumor (T) and paired healthy (H,) slides; see material and methods. N: number.

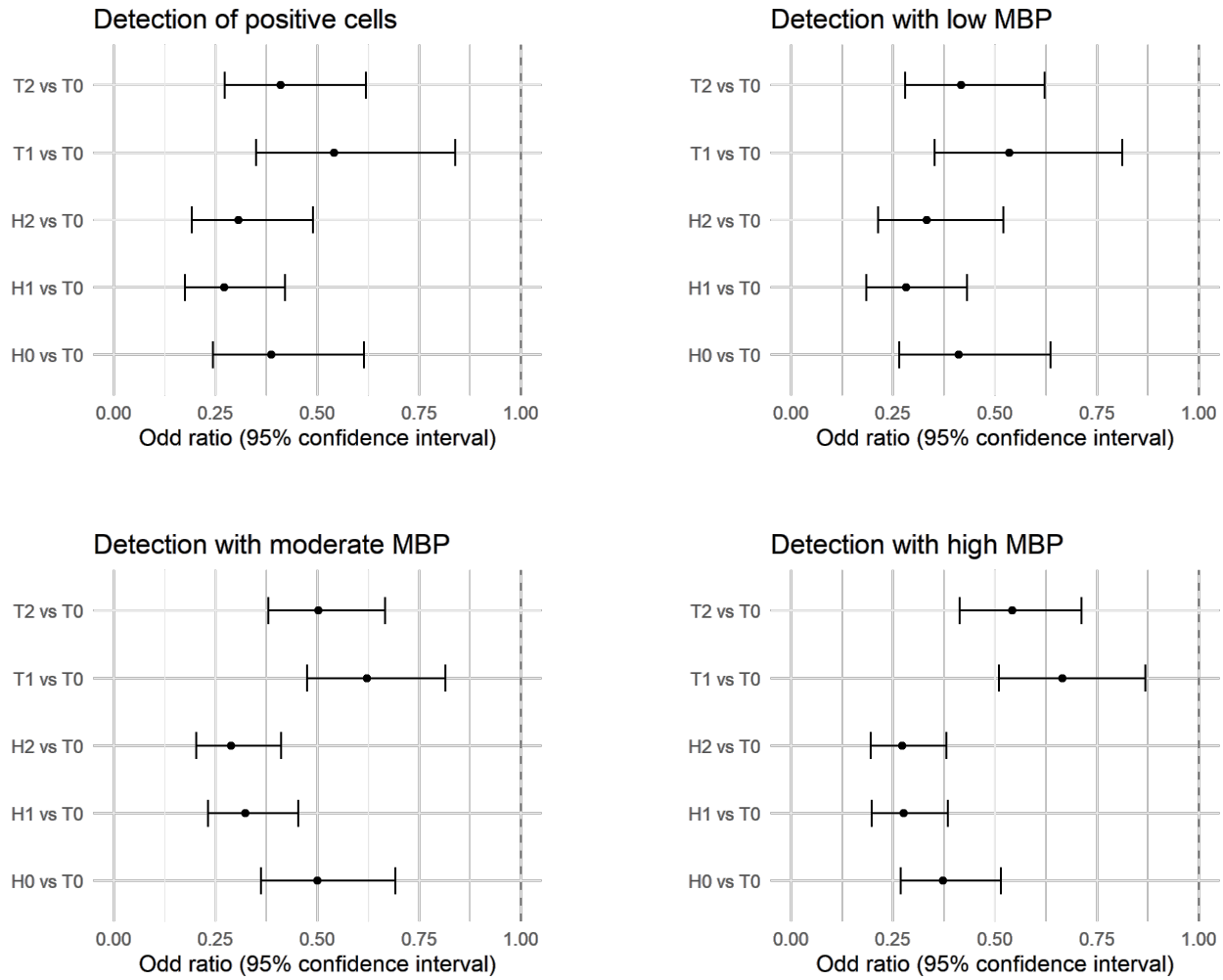


Figure 2. Odd ratios for eosinophil detection according to the zone. Forest plots expressing the adjusted odd ratios of eosinophil detection (expressed as rates) in each zone as compared to T0 (reference), for the entire study samples (a) and according to MBP staining intensity (b to d). All zones (H0/H1/H2/T1/T2) show a lower probability of including eosinophils than T0. All results are statistically significant (see Supplemental data for detailed p-values). Positive cells: eosinophils from all MBP intensity levels. MBP: major basic protein. T0/T1/T2 and H0/H1/H2: refers to the zone on the tumor (T) and control (H, healthy) slides; see material and methods.

B-Eos :

245 of the 316 patients (77,5%) in our cohort had available WBC data up to 30 days before surgery. The mean and median values are shown in **Table S7**. Of note, the median AEC was 0.11 cell/mL, and the median REC was 1.60%. No significant differences were noted between WBC values, in particular Eos, of patients using or not using corticoids at the time of surgery (**Table S5**). AEC and REC of patients with neoadjuvant chemotherapy were lower compared to those without ($p<0.0001$ for both). Higher REC and AEC were associated with a higher probability of T-Eos in the T2 zones (**Table S8**).

Survival :

With a median follow-up of 44.9 mo., the median OS in our cohort was not reached (NR)(54.9 mo. – NR). The median DFS was 92 mo. (26.1 mo. – NR). Clinical and pathological factors associated with both OS and DFS were the pathological stage (HR for death=1.64/3.47/4.07/7.28 for stages IB/IIA/IIB/IIIA vs IA, p<0.0001; HR for recurrence=1.71/3/02/3.10/7.51 for stages IB/IIA/IIB/IIIA vs IA, p<0.0001), male gender (HR for death=2.13, p=0.0030; HR for recurrence=1.48, p=0.042) and a PS >0 (HR for death=1.91, p=0.0038; HR for recurrence=1.56, p=0.0015) (**Table S9** for detailed CI). Patients with squamous cell carcinoma histology had an increased risk of dying as compared to patients with adenocarcinoma (HR=1.93, p=0.024).We found a slight significant association only between T-Eos in the T2 zone and DFS (HR progression=0.62, p=0.023) (**Table 4**). Regarding OS, no significant association was noted between T-Eos and the different zones (**Table 4**) or slides (T vs H, **Table S10**). Considering biological data, higher WBC were associated with a worse prognosis and a shorter DFS (HR for death=2.83, p=0.0011; HR for recurrence=1.84, p=0.025) while the ANC correlated with a worse prognosis (HR for death=1.88, p=0.031). B-Eos were not associated with OS or DFS (**Table S8**).

Table 4. Association between T-Eos and survival.

			OS			DFS		
Variable	**N**	**Categories**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Eosinophils								
H0	309	Yes vs no	1.18	0.69; 2.04	0.55	1.07	0.68; 1.69	0.76
H1	310	Yes vs no	0.96	0.59; 1.57	0.87	0.84	0.57; 1.25	0.40
H2	299	Yes vs no	1.74	0.95; 3.19	0.071	1.28	0.81; 2.03	0.29
T0	311	Yes vs no	0.79	0.41; 1.54	0.49	0.62	0.37; 1.05	0.078
T1	312	Yes vs no	1.89	0.94; 3.79	0.074	1.08	0.66; 1.77	0.75
T2	312	Yes vs no	0.80	0.48; 1.32	0.37	0.62	0.42; 0.94	0.023

Univariate logistic regression analysis. T0/T1/T2 and H0/H1/H2: refer to the zone on the tumor (T) and paired healthy (H) slides; see material and methods; N: numbers; HR: hazard ratio; OS: overall survival; DFS: disease-free survival.

Discussion :

We successfully quantified T-Eos in a large, monocentric cohort of eNSCLC with the use of QuPath®. T-Eos were present in most (80.8%) of the samples, although at low rates and concentrations. T-Eos were highest in the tumor and, to a lesser extent, at its direct periphery, compared to other zones. Squamous cell carcinomas showed more T-Eos than other histologies and had an increased risk of dying compared to patients with other histologies. Other prognostic factors were the pathological stage, PS, and male gender, both in terms of OS and DFS. There was no significant association between T-Eos and stage of disease or OS but Eos in the T2 zones were associated with a decreased risk of disease recurrence.

The paucity of T-Eos is a fact for most of human tissues (Kato et al., 1998). In NSCLC, so far, only one study had reported on numbers of T-Eos in a limited cohort (63 samples) (Tataroğlu et al., 2004). Authors quantified the tissues manually after staining with hematoxilin-eosin and did not mention the area used for quantification. Interestingly, though, in the reported advanced stages, the mean number of Eos was markedly lower than in the earlier stages (mean 21.6/20.3 /8.6/3.6 for stages I, II, III and IV, respectively), a trend that we also observed in our cohort where Eos rates in stage I were higher than in stage III.

The higher detection of T-Eos in tumoral vs non-tumoral tissue in our series aligns with the results of Ye and colleagues where tumors contained higher eosinophil peroxidase (EPO) concentrations than paired, non-tumoral tissues ($p<0.001$) (Ye et al., 2019). Additionally, we found higher Eos in the tumor than further on the T slide. Similarly, Cormier and colleagues noted that Eos were concentrated in the necrotic (tumor core) and fibrotic (capsule) regions of a melanoma mouse model and identically after the injection of a lung cancer cell line in mice (Cormier, 2006). We now know that dying tumor cells release high mobility group box-1 (HMGB-1) protein and interleukin (IL)-33, both chemoattractants for Eos (Lotfi et al., 2009; Lucarini et al., 2017). We, on the contrary, did not observe higher concentrations of Eos in necrotic zones. Lastly and interestingly, we noted a higher Eos concentration in squamous cell carcinomas than in other histologies. There is no comparator on that point in the literature.

We found no association between T-Eos and OS. It is noteworthy, however, that the median OS in our cohort was not reached at the time of this analysis. Further censoring of OS data is planned and might change this data. Although the Regarding the DFS, we saw an association between higher Eos in the T2 zones and a lower risk of recurrence. It may be that the functional status of Eos differs according to the zone where they are located, and that the localization is more important that the concentration. Yet, our study does not allow to draw any conclusion

on the function(s) of the detected Eos. Hu et al. found that, mainly in colorectal cancer, T-Eos were a prognostic favorable factor (HR for death=0.82, 95% CI 0.68-0.99, p=0.041) (Hu et al., 2020). In Ye et al, the EPO intensity level was an independent negative prognostic marker in lung adenocarcinoma (HR death=3.145, 95% CI 2.016-5.519, p=0.018) (Ye et al., 2019). Conversly, Tataroglu et al, although not formally reporting on OS, noted an inverse correlation between T-Eos infiltration and pTNM stage, suggesting that T-Eos were associated with better outcomes (Tataroğlu et al., 2004).

Interestingly, our study shows an association between squamous cell histology and a worse prognosis. This is already known in later stages of disease where several factors can explain this: tumors that are more central, thus more prone to complications (bleeding and retro-obstructive infections); lower response rate and duration of response to systemic therapy; and less therapeutic options, owing to the lower sensitivity to some chemotherapy regimens and to the virtual absence of actionable oncogenic drivers (Garassino et al., 2023; Hendriks et al., 2023; Novello et al., 2023). In early stages, studies have shown a different gene expression profile for squamous than for adenocarcinoma and a low rate of programmed death-ligand 1 (PD-L1) high expression level (Jin et al., 2018; Yu et al., 2019). The worse prognosis observed here may thus relate to histology-specific gene expression but perhaps also to correlated factors, like cardiovascular comorbidity or higher stages of disease, explaining higher mortality and recurrence rates.

In the present data set, T-Eos infiltration was not correlated with the stage of disease. This is discordant to what Ye and Tataroglu found, bearing in mind their small sample size (Tataroğlu et al., 2004; Ye et al., 2019). This lack of association might be due to the low number of higher stages, e.g., stages III. Preoperative B-Eos and T-Eos did not correlate, and B-Eos were not prognostic. Contrary to our findings, Dai et al published that preoperative low and postoperative normal B-Eos had a favourable prognostic value in a retrospective study on 414 eNSCLC (Dai et al., 2023). Our biological data further support the prognostic value of the total WBC count and of the ANC, as already demonstrated in early and in advanced stages of NSCLC (Mezquita et al., 2018; Park et al., 2020; Sulibhavi et al., 2020; Yuan et al., 2017). Finally, T- and B-Eos rates were independent of corticoid exposure before surgery.

We acknowledge limitations in our study. QuPath® quantification is limited by batch effects, slide artefacts and staining variability (Viswanathan et al., 2022). However, solutions exist and have been described to allow its use (Janowczyk et al., 2019; Kothari et al., 2014; Schömig-Markiefka et al., 2021). Based on the ICC shown and on a careful review of the samples, we recommend using MBP levels 2 & 3 for T-Eos quantification. On the other hand, QuPath®

offers the following advantages: handling many samples at high speed; potentially quantifying several cell types in parallel; benefitting from users' input for performance improvement of the software. We quantified Eos through visualization of their granules, which may create a bias in cell quantification if the cells had degranulated. However, we only observed this phenomenon in rare cases and in cells with low MBP signal intensity, a supplementary reason to preferentially quantify the MBP2 and 3 positive cells. Comforting our choice, Weller and Spencer describe the low sensitivity of the hematoxilin-eosin stain for the study of Eos, suggesting alternative stains like MBP or EPO-directed stains (Weller & Spencer, 2017). Hence, we are confident that our data can serve as reference quantification data for future research. The retrospective nature of the study implies limitations such as incomplete data with subsequent limited representativeness of the studied population. Lastly, our study is descriptive in nature and does not allow to affirm which functions T-Eos fulfill. Future directions for research on this topic should therefore include a combination of both spatial and functional information on T-Eos and on other myeloid cells to unravel their roles in the TME of resected, eNSCLC. Spatial transcriptomics allows to gather both information: imaging data to locate the constituents in a specific disease state and to observe their spatial arrangements with one another, and functional data (at the genomic, transcriptomic, or proteomic level) to reveal their activation state and suspected role(s) (Rao et al., 2021).

Interpretation :

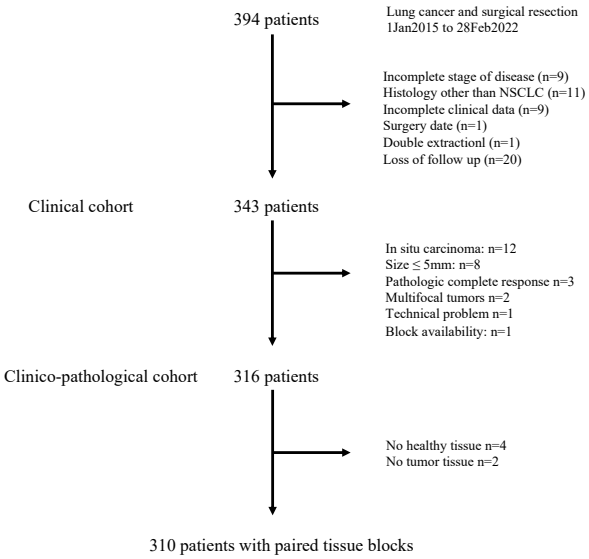
Detecting and quantifying T-Eos with QuPath® after staining with an anti-MBP antibody coupled to the alkaline phosphatase red chromogen is feasible and reliable. It allows for high throughput tissue analysis. T-Eos in eNSCLC are mostly located in the tumor and at its direct periphery. So far, T-Eos and B-Eos do not seem to have a prognostic value. Additional work is needed to specify the roles of T-Eos in eNSCLC.

Supplemental data:

Patient population:

Figure S1. Consort diagram of study patients.

Figure 1. Flow diagram of patients included in the analysis.



Incomplete clinical data: as pre-defined, see Table 1a (main manuscript). Loss of follow-up: follow-up shorter than 5 years post-resection. Pathological complete response: after induction chemotherapy. No healthy tissue: no paired healthy tissue; no tumor tissue: no paired tumor tissue.

Staining protocols:

Per patient, one slide was stained with hematoxylin-eosin (**Table S1**) and one slide with an antibody (AB) against the eosinophil Major Basic Protein (MBP), followed by a secondary AB coupled to a chromogenic substrate (alkaline phosphatase) to identify the Eos granules, emitting a DAB (diaminobenzidine) signal (**Table S2**). This "MBP" slide was then counterstained by an hematoxylin stain at a lower concentration in order to localise the observed DAB signal (**Table S3**).

Table S1. Hematoxylin-eosin staining protocol.

Product	**Incubation time**
Xylene	2'
Isopropanol 80%	2'
Isopropanol 60% 2'	2'
Demineralized water2'	2'
Carazzi's hematoxylin 2'	2'
Wash station	
Eosin 30"	30"
Wash station	
Isopropanol 100%	30"
Xylene	2'

Table S2. Major basic protein (MBP) staining.

Immunohistochemical steps	**Product**	**Procedure**
Paraffin removal	Xylene > isopropanol 80% > isopropanol 60% > demineralized water	2' for each bath
MBP demasking	Pepsin (Ab64201; ABCAM)	20' @ 37°C
Endogenous peroxidase inhibition	H202	20'
Primary antibody: Mouse anti-human eosinophil MBP	MCA 5751 (Bio-RAD)	1/50 dilution, overnight incubation
Secondary antibody + chromogen	Antibody: Polyview Plus AP - Rabbit (ENZ-ACC110-0150)* Chromogen: ADI 950-141-0030 (High Def. Red Chromogen)**	15'

*for more details, please see: https://www.enzolifesciences.com/ENZ-ACC110/polyview-plus-ap-anti-rabbit-reagent/

** https://www.enzolifesciences.com/ADI-950-140/highdef-red-ihc-chromogen-ap/

Table S3: Hematoxylin counterstain on MBP stained slides.

Product	**Incubation time**
Demineralized water	30’’
Carazzi’s hematoxylin	2’
Wash station	2’
Demineralized water	30’’
Isopropanol 100%	30’’
Isopropanol 100%	30’’
Isopropanol 100%	30’’
Xylène	2’
Xylène	2’

Detailed eosinophil quantification protocol:

Eos quantification was achieved using the **QuPath®** version 0.4.1 software. The scripts are available at: https://github.com/AlexHego/Eosino_Tumor. Prior to quantification, we delineated three zones on each slide.

Zone delineation: The tumor zone (T0; named "central margin" in the script) was manually delineated on the T slides by a certified pathologist dedicated to the study of lung cancer at our institution and limited to invasive tumor only in lesions of at least 5mm diameter. A healthy zone (H0) was delineated on the H slides, with a surface equalling the average surface of the T0 zones. To analyse the localisation of eosinophils in the tumor, we created 2 other zones of analysis: zone 1 (T1/H1, corresponding to an expand of 1200µm around the T0/H0 margin) and zone 2 (T2/H2, corresponding to an expand of 1200µm around the T1/H1 margin).

Eosinophil quantification:

First, anthracosis was removed from the quantification area to avoid false positive signal detection. For this purpose, a pixel classifier was created to detect and subtract anthracosis prior to MBP signal detection. White, blue (hematoxilin), red (MBP signal), and black (anthracosis) colors were next defined separately for each staining batch. Cells were detected, and red signal corresponding to MBP was quantified in the 6 zones described above. Both script (Script_Eosino_Tumor_expandonly.groovy) and classifier (anthracose.zip) are provided on AlexHego github. For each slide, the following data on T-Eos were available: positive rate (number of MBP positive cells/total number of cells, expressed as a percentage); concentration of positive cells (number/area in mm^2) for each MBP intensity level and for the total number of positive cells, and concentration of MBP negative cells.

Influence of the staining batch on T-Eos detection:

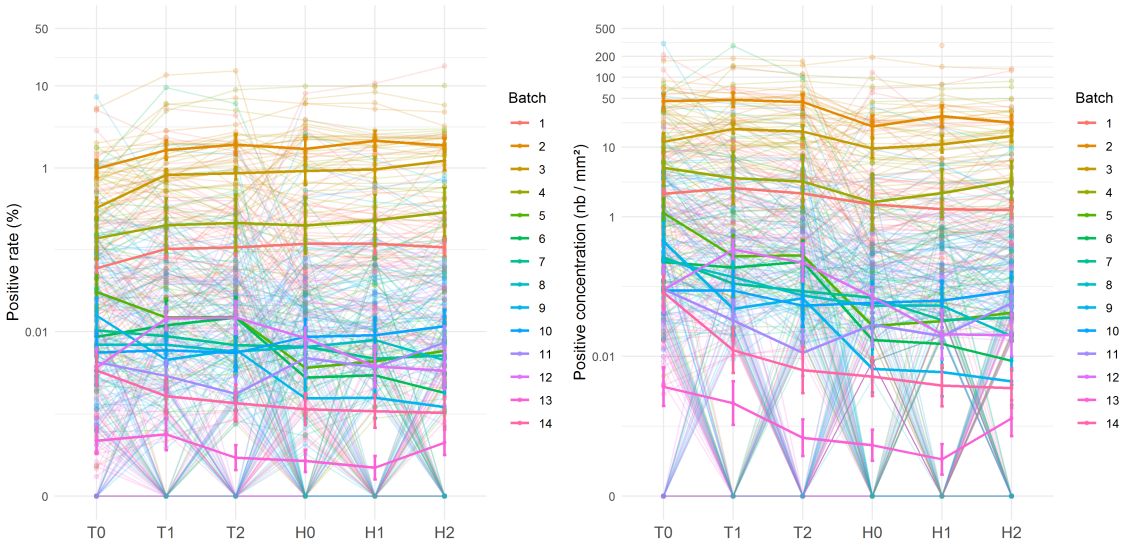


Figure S2. Eosinophil rates (left) and concentrations (right) according to the batch number.
Thick lines represent the mean values of positive rates (left) and concentrations (right) of T-Eos for each batch, coded with a different color. Higher mean rates and higher mean concentrations were noted in the first 4 batches as compared to other staining batches. T0/T1/T2 and H0/H1/H2: refer to the zone on the tumor (T) and paired healthy (H) slides; see material and methods. Y-axis: logarithmic scale.

T-Eos quantification per zone:

Table S4. Probability of detecting eosinophils in the different zones.

Model	Effect	Adjusted OR	95% CI	p-values
Positive cells				
	Batch			<0.0001
	Zones (ref.= T0)			
	H0 vs T0	0.39	0.24; 0.61	<0.0001
	H1 vs T0	0.27	0.17; 0.42	<0.0001
	H2 vs T0	0.31	0.19; 0.49	<0.0001
	T1 vs T0	0.54	0.35; 0.84	0.0059
	T2 vs T0	0.41	0.27; 0.62	<0.0001
MBP1				
	Batch			<0.0001
	Zones (ref.= T0)			
	H0 vs T0	0.41	0.27; 0.64	<0.0001
	H1 vs T0	0.28	0.18; 0.43	<0.0001
	H2 vs T0	0.33	0.21; 0.52	<0.0001
	T1 vs T0	0.53	0.35; 0.81	0.0033
	T2 vs T0	0.42	0.28; 0.62	<0.0001
MBP2				
	Batch			<0.0001
	Zones (ref.= T0)			
	H0 vs T0	0.50	0.36; 0.69	<0.0001
	H1 vs T0	0.32	0.23; 0.45	<0.0001
	H2 vs T0	0.29	0.20; 0.41	<0.0001
	T1 vs T0	0.62	0.47; 0.81	0.0006
	T2 vs T0	0.50	0.38; 0.67	<0.0001
MBP3				
	Batch			<0.0001
	Zones (ref.= T0)			
	H0 vs T0	0.37	0.27; 0.51	<0.0001
	H1 vs T0	0.28	0.20; 0.38	<0.0001
	H2 vs T0	0.27	0.19; 0.38	<0.0001
	T1 vs T0	0.67	0.51; 0.87	0.0028
	T2 vs T0	0.54	0.41; 0.71	<0.0001

Adjusted odd ratios (OR) showing the probability of finding eosinophils in each zone as compared to the T0 zone, after correction for the staining batch effect. Positive cells: eosinophils, expressed as a rate, for all MBP staining intensity levels and per MBP intensity level (MBP1/2/2: eosinophils with weak/intermediate/strong staining for MBP). MBP: major basic protein. Ref.: reference. T0/T1/T2 and H0/H1/H2: refer to the zone on the tumor (T) and healthy (H) slides; see material and methods.

Eosinophils and corticoids:

Table S5. Blood and tissue eosinophils in patients with or without corticoids.

Variable	Without corticoid*			With corticoid*			p-value
	N	Mean ± SD	Med. (Q1; Q3)	N	Mean ± SD	Med. (Q1; Q3)	
AEC	197	0.15 ± 0.15	0.11 (0.06; 0.19)	48	0.16 ± 0.13	0.14 (0.06; 0.26)	0.50
REC	197	1.95 ± 1.54	1.60 (1.00; 2.70)	48	2.14 ± 1.67	2.25 (0.85; 2.95)	0.38
Rate (%) of positive cells by zone							
H0	249	0.39 ± 1.19	0.01 (0.00; 0.09)	60	0.26 ± 0.86	0.01 (0.00; 0.07)	0.78
H1	250	0.41 ± 1.36	0.01 (0.00; 0.12)	60	0.29 ± 0.91	0.01 (0.00; 0.09)	0.76
H2	243	0.41 ± 1.48	0.01 (0.00; 0.13)	56	0.21 ± 0.57	0.01 (0.00; 0.08)	0.35
T0	251	0.23 ± 0.72	0.01 (0.00; 0.14)	60	0.23 ± 0.77	0.01 (0.00; 0.09)	0.59
T1	251	0.30 ± 0.95	0.01 (0.00; 0.17)	61	0.53 ± 1.90	0.01 (0.00; 0.19)	0.79
T2	251	0.32 ± 0.98	0.02 (0.00; 0.15)	61	0.59 ± 2.09	0.02 (0.00; 0.12)	0.75
Concentration (n/mm²) of positive cells by zone							
H0	249	5.10 ± 18.25	0.13 (0.01; 1.15)	60	2.47 ± 6.25	0.16 (0.01; 0.97)	0.83
H1	250	5.68 ± 22.85	0.12 (0.00; 1.21)	60	2.24 ± 6.16	0.11 (0.02; 0.91)	0.92
H2	243	4.85 ± 15.63	0.14 (0.02; 1.37)	56	1.81 ± 4.46	0.12 (0.00; 0.64)	0.39
T0	251	9.62 ± 29.87	0.54 (0.05; 4.89)	60	8.13 ± 26.49	0.43 (0.06; 3.64)	0.54
T1	251	7.79 ± 25.07	0.31 (0.04; 3.74)	61	10.38 ± 30.95	0.35 (0.03; 4.11)	0.78
T2	251	6.45 ± 17.98	0.27 (0.03; 3.41)	61	9.17 ± 27.56	0.45 (0.02; 2.49)	0.70

Non-parametric, Kruskal-Wallis test. *: with/without corticoids: n=255 and 61, respectively, for the tissue analysis; n=197 and 48, respectively, for the blood analysis. B-Eos quantification: reflected by the AEC (absolute eosinophil count, cell/mL) and REC (relative eosinophil count, %); T-Eos quantification: reflected by the rate and concentration; positive rate: number of eosinophils/total number of cells detected, in %; positive cells: major basic protein (MBP)-positive staining cells, i.e., eosinophils. N: number. SD: standard deviation. Med: median. Q1-Q3: first-third quartile. T0/T1/T2 and H0/H1/H2: refer to the zone on the tumor (T) and healthy (H) slides; see material and methods.

Eosinophils and neoadjuvant chemotherapy:

Table S6. Tissue and blood eosinophils according to the neoadjuvant chemotherapy status.

Variable		No chemo N=266		Chemo N=50		p-value
		N (%) Mean ± SD	Med (Q1 ; Q3)	N (%) Mean ± SD	Med. (Q1 ; Q3)	
Detected positive cells						
	H0	210 (80.5)		36 (75.0)		0.39
	H1	191 (73.2)		39 (79.6)		0.35
	H2	193 (76.6)		35 (74.5)		0.75
	T0	239 (91.2)		43 (87.8)		0.44
	T1	219 (83.3)		42 (85.7)		0.67
	T2	213 (81.0)		38 (77.6)		0.58
Conc. positive cells (n/mm²)						
	Healthy	5.07 ± 16.27	0.18 (0.02 ; 1.24)	2.49 ± 8.64	0.10 (0.02 ; 0.65)	0.37
	Tumoral	9.29 ± 24.40	0.60 (0.06 ; 5.01)	3.52 ± 8.22	0.40 (0.12 ; 2.48)	0.70
	Both	7.10 ± 17.53	0.56 (0.08 ; 3.95)	2.78 ± 6.37	0.37 (0.14 ; 1.65)	0.52
WBC		8.16 ± 5.24	7.51 (6.26 ; 8.82)	7.67 ± 3.60	6.85 (5.62 ; 8.90)	0.17
REC (%)		2.12 ± 1.54	1.80 (1.10 ; 2.90)	1.39 ± 1.53	1.00 (0.30 ; 1.70)	0.0002
AEC (cell/mL)		0.16 ± 0.14	0.13 (0.08 ; 0.21)	0.10 ± 0.13	0.07 (0.02 ; 0.11)	<.0001
ALC (cell/mL)		1.93 ± 1.06	1.78 (1.36 ; 2.37)	1.87 ± 0.77	1.82 (1.41 ; 2.24)	0.89
RLC (%)		25.11 ± 9.84	25.60 (18.65 ; 31.00)	26.60 ± 9.51	26.90 (20.70 ; 32.10)	0.40
ANC (cell/mL)		5.15 ± 2.34	4.56 (3.63 ; 6.10)	4.91 ± 3.23	4.19 (3.12 ; 5.50)	0.17
RNC (%)		63.70 ± 12.10	62.90 (57.05 ; 70.75)	60.95 ± 11.51	60.90 (54.80 ; 68.10)	0.18

Chi-square test for binary variables and non-parametric, Kruskal-Wallis test for continuous variables. SD: standard deviation. Med: median. Q1-Q3: first-third quartile. Positive cells: major basic protein (MBP)-positive staining cells, i.e., eosinophils. N: number. T0/T1/T2 and H0/H1/H2: refer to the zone on the tumor (T) and healthy (H) slides; see material and methods. Nr: number. WBC: white blood cells; ANC: absolute neutrophil count (cell/mL), ALC: absolute lymphocyte count (cell/mL), AEC: absolute eosinophil count (cell/mL), RNC: relative neutrophil count (%), RLC: relative lymphocyte count (%), REC: relative eosinophil count (%), NLR: neutrophil-to-lymphocyte ratio.

Blood eosinophils:

Table S7. Preoperative white blood cell counts.

Variable	Categories	N	N (%)	Mean ± SD	Med (Q1 ; Q3)	Extremes
WBC		245		8.07 ± 4.98	7.36 (6.15; 8.83)	2.91; 73.65
ANC		245		5.11 ± 2.52	4.48 (3.59; 5.92)	1.19; 19.73
ALC		245		1.92 ± 1.01	1.78 (1.37; 2.36)	0.19; 12.15
AEC		245		0.15 ± 0.14	0.12 (0.06; 0.20)	0.00; 1.18
	<0.15 cell/mL		150 (61.2)			
	≥0.15 cell/mL		95 (38.8)			
RNC		245		63.19 ± 12.01	62.30 (56.70; 70.30)	11.00; 93.60
RLC		245		25.39 ± 9.78	25.60 (19.60; 31.60)	2.10; 50.40
REC		245		1.99 ± 1.56	1.60 (1.00; 2.80)	0.00; 9.40
	<1.5%		109 (44.5)			
	≥1.5 %		136 (55.5)			
NLR		245		3.64 ± 4.58	2.44 (1.83 ; 3.48)	0.66; 43.68

WBC: white blood cells; ANC: absolute neutrophil count (cell/mL), ALC: absolute lymphocyte count (cell/mL), AEC: absolute eosinophil count (cell/mL), RNC: relative neutrophil count (%), RLC: relative lymphocyte count (%), REC: relative eosinophil count (%), NLR: neutrophil-to-lymphocyte ratio.

Table S8. Association of B-Eos and T-Eos.

Variable	Zone	REC Coeff. (log)	REC p-value	AEC Coeff. (log)	AEC p-value
Detection (yes vs no)					
	H0	0.23	0.50	0.03	0.87
	H1	-0.20	0.54	-0.09	0.59
	H2	0.62	0.068	0.23	0.17
	T0	0.35	0.40	0.12	0.57
	T1	-0.003	0.99	0.02	0.91
	T2	0.88	0.010*	0.37	0.027**
Conc. positive cells (n/mm²)					
	Total H	0.39	0.13	0.24	0.083
	Total T	0.48	0.078	0.26	0.070
	Both	0.43	0.076	0.24	0.059

* Detection in T2 explained by REC (log): OR= 2.42, 95% CI: 1.23; 4.76. ** Detection in T2 explained by AEC (log): OR= 1.45, 95% CI: 1.04; 2.02. N: number. REC: relative eosinophil count AEC: absolute eosinophil count; OR: odd ratio; CI: confidence interval. T0/T1/T2 and H0/H1/H2: refer to the zone on the tumor (T) and healthy (H) slides; see material and methods. Total H: pooled H0/1/2. Total T: pooled T0/1/2. Coeff.: regression coefficient: the sign (+ vs -) indicates the association.

Survival analysis:

Table S9. Clinical and biological factors associated with prognosis.

Variable	Categories	Death HR	Death 95% CI	Death p-value	Recurrence HR	Recurrence 95% CI	Recurrence p-value
Demographics							
Diagnosis during surgery	Yes vs no	0.74	0.38; 1.44	0.38	0.51	0.27; 0.94	0.032
Surgery delay (months)		1.10	1.00; 1.20	0.039	1.09	1.02; 1.17	0.018
Age (10 years)		1.37	1.05; 1.78	0.019	1.13	0.92; 1.39	0.26
Gender	Male vs female	2.13	1.29; 3.50	0.0030	1.48	1.01; 2.17	0.042
Smoking	Yes vs no	1.49	0.69; 3.24	0.31	1.25	0.69; 2.27	0.47
Atopy	Yes vs no	0.75	0.33; 1.72	0.50	0.77	0.40; 1.47	0.42
Airway disease	Yes vs no	1.12	0.73; 1.74	0.60	0.94	0.66; 1.34	0.74
Corticoids	Yes vs no	1.08	0.61; 1.89	0.80	0.91	0.57; 1.46	0.71
PS	1 vs 0	1.91	1.23; 2.95	0.0038	1.56	1.09; 2.23	0.0015
History of cancer	Yes vs no	1.82	1.14; 2.92	0.013	1.05	0.69; 1.62	0.81
Surgery and tumor							
Resection type				0.22			0.60
	Lobectomy	1.00			1.00		
	Others	1.57	0.76; 3.27		0.84	0.42; 1.65	
Histology				0.024			0.21
	Adenocarcinoma	1.00			1.00		
	Squamous cell carcinoma	1.93	1.20; 3.10		1.43	0.95; 2.14	
	Neuroendocrine NSCLC	0.52	0.13; 2.15		0.44	0.14; 1.40	
	Mixed type	1.46	0.20; 10.57		0.83	0.12; 6.01	
	NOS	3.12	0.97; 10.05		1.57	0.50; 4.99	
Neoadjuvant treatment	Yes vs no	1.26	0.74; 2.16	0.39	1.72	1.13; 2.63	0.012
pTNM				<.0001*			<.0001*
	IA	1.00			1.00		
	IB	1.64	0.74; 3.63		1.71	0.92; 3.17	
	IIA	3.47	1.94; 6.23		3.02	1.86; 4.88	
	IIB	4.07	1.95; 8.52		3.10	1.66; 5.76	
	IIIA	7.28	3.87; 13.68		7.51	4.54; 12.44	
	IIIB	2.07	0.12; 36.20		1.36	0.08; 23.13	
Stage				<.0001			<.0001
	I	0.30	0.19; 0.50		0.37	0.24; 0.55	
	II	1.00			1.00		
	III	1.81	1.00; 3.28		2.23	1.35; 3.68	
PD-L1				0.82			0.79
	≥50%	1.00			1.00		
	1-49%	0.84	0.42; 1.68		0.91	0.53; 1.56	
	<1%	0.89	0.47; 1.70		0.98	0.58; 1.63	
	Unknown	0.75	0.42; 1.36		0.79	0.49; 1.29	
Oncogenic driver				0.43			0.20
	No	0.65	0.38; 1.13		0.78	0.48; 1.25	
	Yes	0.85	0.47; 1.51		1.29	0.81; 2.04	
	Unknown	1.04	0.49; 2.22		1.01	0.51; 1.99	
	NA	1.00			1.00		
White blood cell count							
WBC		2.83	1.51; 5.29	0.0011	1.84	1.08; 3.16	0.025
ANC		1.88	1.06; 3.33	0.031	1.47	0.91; 2.36	0.11
ALC		0.95	0.55; 1.67	0.87	1.02	0.66; 1.58	0.93
AEC		1.07	0.81; 1.42	0.61	0.90	0.73; 1.10	0.30
RNC		1.00	0.98; 1.03	0.72	1.00	0.98; 1.02	0.83
RLC		0.98	0.95; 1.00	0.10	0.99	0.97; 1.01	0.26
REC		0.93	0.56; 1.55	0.78	0.75	0.50; 1.12	0.16
NLR		1.38	0.94; 2.03	0.099	1.17	0.86; 1.60	0.31
AEC (cell/mL)	≥0.15 vs <0.15	1.44	0.87; 2.38	0.16	1.04	0.70; 1.57	0.83
REC (%)	≥1.5% vs <1.5	0.90	0.55; 1.49	0.69	0.91	0.61; 1.35	0.63

HR: hazard ratio; CI: confidence interval ; surgery delay: ≤ vs >1 month after index date of diagnosis; PS: performance status; neuroendocrine NSCLC: carcinoid tumors and large cell neuroendocrine carcinomas; NOS: not otherwise specified; pTNM: pathological Tumor, Node, Metastasis, according to the TNM stage 7th classification; NA: not applicable; WBC: white blood cells (log); ANC: absolute neutrophil count (log, cell/mL), ALC: absolute lymphocyte count (log, cell/mL), AEC: absolute eosinophil count (log, cell/mL); RNC: relative neutrophil count (%), RLC: relative lymphocyte count (%), REC: relative eosinophil count (log, %); NLR: neutrophil-to-lymphocyte ratio (log). *Other significant differences for pTNM on the risk of death: IB vs IIIA (HR=0.23, 95% CI: 0.10; 0.52) and IIA vs IIIA (HR=0.48; 95% CI 0.25; 0.9) and on the risk of recurrence: IB vs IIIA (HR=0.23; 95% CI 0.12; 0.45), IIA vs IIIA (HR=0.40; 95% CI 0.23; 0.69) and IIB vs IIIA (HR=0.41; 95% CI 0.21; 0.81).

Table S10. T-Eos and survival according to the zone.

Variable	OS HR	OS 95% CI	OS p-value	DFS HR	DFS 95% CI	DFS p-value
Conc. positive cells (no./mm²)						
Healthy	1.02	0.94 ; 1.12	0.59	1.00	0.93 ; 1.08	0.91
Tumoral	1.01	0.92 ; 1.10	0.86	0.96	0.90 ; 1.03	0.29
Both	1.03	0.94 ; 1.13	0.49	0.98	0.91 ; 1.06	0.67

Cox regression analysis for the overall (OS) and disease-free survival (DFS) according to the type of slide.Concentration of positive cells: eosinophil concentration, expressed in logarithmic transformation. Healthy: H0+H1+H2. Tumoral: T0+T1+T2.

DISCUSSION AND PERSPECTIVES

Treatment strategy for LC has dramatically improved since the “one-size-fits-all” option in the 1980’s where platinum-doublet CT was prescribed to nearly all patients. The aim for an oncology practitioner, is to add quality and, if possible, time, to their patients’ life (Booth & Tannock, 2008). For this, in order to better select treatments, physicians need prognostic and predictive disease indicators, i.e., biomarkers of future disease behavior and of treatment efficacy. Prognostic biomarkers could help define the best follow-up intervals while predictive biomarkers would, ideally, guide the treatment choice.

Our work explored the value of eosinophils as biomarkers in NSCLC in two different clinical settings. The first part investigated the predictive value of blood eosinophils (B-Eos) in patients treated with an ICI for advanced disease. The need for biomarkers of efficacy is high for patients treated with ICI, knowing that, until now, the only widely accepted “predictive” biomarker is the PD-L1 expression level with the limitations we discussed at the beginning of this manuscript. This, while there is an increasing number of indications for ICI in NSCLC and while atypical radiological patterns may complicate the interpretation of ICI efficacy, underlines an unmet need for clinicians.

Our first cohort retrospectively included 117 patients with an advanced stage NSCLC consecutively treated with an ICI at the CHU de Liège between August 1, 2015 and April 30, 2018. We collected B-Eos data before and under treatment (i.e., at the first and second clinical and radiological evaluation) together with response data as per RECIST at the same time points. We noted not association between baseline B-Eos and response to ICI. We observed that B-Eos were higher in non-progressive patients (i.e., with stable disease and objective response according to RECIST) at the second evaluation. Considering the biases inherent to the retrospective nature of the study, we found that this potential predictive value was independent of the PD-L1 expression, histology, and smoking history. The kinetic study of the B-Eos levels showed an early (at the first time point, i.e., 2 to 3 months after treatment initiation) and a significant increase of both AEC and REC.

Based on these results, we sought to confirm the predictive value of B-Eos in a larger cohort of patients and investigated their prognostic value. The total white blood cells, neutrophils, lymphocytes, and their ratio were included to explore the potential prognostic and predictive value of other myeloid cells. Potential confounders for blood eosinophilia (atopy, use of corticosteroids, immune-related toxicity) were registered. In our 191 patients cohort, a higher

REC was associated with more objective responses at the first evaluation. A higher REC and a lower ANC at the time of the first evaluation were associated with a longer duration of response. Again, the predictive value of REC seemed independent of the PD-L1 status, smoking history, and immune-related toxicity. At the second evaluation, all myeloid cells showed a significant association with the duration of response. Baseline, i.e., pretreatment B-Eos were not predictive of response. A cutoff of 5.3% REC yielded significant association with response to ICI, with a good specificity (81.9%) but a poor sensitivity (32.8%), not allowing to use the REC as a trustable response biomarker. Neutrophils, lymphocytes, and their ratio were associated with OS. The study of the WBC kinetics confirmed an early and significant increase at the first evaluation which was restricted to the AEC and REC.

We acknowledge several limitations in these two studies. First, as retrospective studies, patients were excluded based on post-hoc criteria (mostly, death before the second evaluation time point), inducing biases in the numbers of responders or non-responders and, hence, in the potential predictive value of B-Eos. Second, the sample size may have been too small to show an association of some variables with outcomes. Indeed, in the second study, the PS did not appear to be associated with prognosis, while it is known as a strong prognostic factor. When investigating this in the initial group of patients (i.e., automated computer search before application of the exclusion criteria, n=346), we found a significant association between PS and OS. This illustrates that higher numbers of patients are more representative and more reliable. Third, the criterium “duration of treatment” was chosen in a real-life setting to reflect the duration of response. Yet, it does not truly reflect this, as some clinicians continued treatment beyond radiological progression, for presumed clinical benefit. Also, as a real-life study, the evaluation time points were variable, potentially leading to a longer duration of treatment than it would have been the case if CT scans had been performed earlier in the treatment course.

With these limitations in mind, we designed a prospective, single-center study investigating Eos in the context of NSCLC, both before and under treatment with ICI. We initiated the **PROTEON** (PROspective Trial on Eosinophils in Non-small cell lung cancer) study in November 2022 (registration reference NCT 05602259). With this trial, we want to gather qualitative clinical data: a large cohort of patients, a prospective data collection with upfront definition of in- and exclusion criteria, and an independent radiological evaluation, blinded to other results. We also aim at comparing the potential predictive value of B-Eos with Eos in

alternative bodily material: sputum, bronchoalveolar lavage and tissue. Indeed, the lack of sensibility of B-Eos as a biomarker of response to ICI may be due to the type of material to quantify Eos. We know from chronic obstructive lung diseases such as COPD and asthma that B-Eos show day-to-day but also circadian variations (Van Rossem et al., 2021). Furthermore, looking at literature data on this topic in asthma, we see that the predictive value of sputum Eos for response to anti-IL-5 is superior to that of B-Eos (AUC sputum Eos=0.76, p=0.008 vs AUC B-Eos=0.60, p=0.33) (Moermans et al., 2023). Interestingly, in this work, Moermans and colleagues found a similar area under the receiver operating characteristic curve (AUC) for the predictive value of sputum Eos, sputum IL-5 (AUC=0.80), and sputum eosinophil peroxidase (EPX) (AUC=0.81), suggesting that Eos as such are not inferior to related biomarkers (IL-5, EPX) but that the material to quantify them is of importance, at least in asthma. The PROTEON study will, however, remain a study carried out in routine clinical practice, meaning that, for instance, post-progression treatment continuation may occur.

The prognostic and predictive value of B-Eos have also been the focus of research in other tumor types. In breast cancer, baseline B-Eos were associated with DFS and breast-cancer specific survival (BCSS) in three large, retrospective studies (Onesti et al., 2018, 2020; Ownby et al., 1983). One study with 419 patients did not find any association with OS (Zenan et al., 2019). In Onesti et al., the significant association for B-Eos was found based on the REC and on a composite biomarker, the eosinophil-lymphocyte product (ELP), with a cutoff at 35.8 indicating higher chances of longer breast cancer-specific survival BCSS and DFS (Onesti et al., 2018). The REC under CT for breast cancer was positively associated with response and a lower REC on treatment was associated with a higher risk of relapse (Onesti et al., 2020). Additionally, lower REC at diagnosis was associated with higher chances of having a breast cancer instead of a benign lesion. In a recent publication, Willems and colleagues investigated the value of pretreatement B-Eos in malignant pleural mesothelioma (MPM) (Willems et al., 2023). In their retrospective cohort of 230 patients, a baseline AEC ≥200 cells/µL was associated with a higher OS and PFS. It should be noted, however, that the AUC for this cutoff was not optimal (0.65), although statistically significant (p=0.0006). Although the authors described both a prognostic and a predictive effect of the pretreatment AEC, we believe that the value, if confirmed in a prospective, perhaps larger cohort of patients, is only prognostic because the association between B-Eos and response (ORR and duration of response (DoR)) was independent of the type of treatment (CT vs IT). In colorectal cancer too, B-Eos have been a focus of research. Two studies, one retrospective and one prospective, collected data on

preoperative B-Eos values and identified higher B-Eos as a negative prognostic marker (Alsalman et al., 2022; Wei et al., 2018). On the contrary, Gao and colleagues combined clinico-pathological and inflammatory markers (based on the preoperative complete blood count) to define a risk score for death in stages II & III colorectal cancer patients (Gao et al., 2023). Raised eosinophils were significantly associated with longer OS in their cohort. It should be noted that confounding factors like mismatch repair deficiency and microsatellite instability were not considered in this retrospective study, constituting potential biases.

Numerous studies on the association between B-Eos and clinical outcomes in patients treated with ICI were published across a variety of cancer types (Bernard-Tessier et al., 2017; Chu et al., 2020; Delyon et al., 2013; Gebhardt et al., 2015; Huai et al., 2023; Krishnan et al., 2020; Lo Russo et al., 2023; Martens et al., 2016; Moreira et al., 2017; Okauchi et al., 2021; Sibille et al., 2021; Tanizaki et al., 2018; Weide et al., 2016). This led to the assumption that B-Eos could serve as a predictive and/or prognostic biomarker to this class of drugs. There exists indeed a rationale to explain raised Eos under ICI. In a breast cancer mouse model, CTLA-4 blockade led to increased $CD4^+$ and $CD8^+$ T lymphocytes activity with an increase in CCL11, CCL5 and IL-5, all leading to the recruitment of Eos and to their accumulation in the TME (Zheng et al., 2020). There is also positive feedback on lymphocytes and Eos recruitment through tumor blood vessel normalization via lymphocytes- and Eos-production of interferon (IFN)γ. Of note, the rise in Eos in this experiment was seen in the blood, not at tissue level. Jacquelot and colleagues described that the tumor shrinkage in a melanoma mouse model after administration of an anti-PD-1 was associated with Eos infiltration at the tumor site (Jacquelot et al., 2021). They showed that it was the GM-CSF production of group 2 innate lymphoid cells (ILC2) that mediated this effect. ILC2 can express PD-1 receptors. When treated with a PD-1 inhibitor and with the alarmin IL-33 (ligating to their receptor, ST2, on ILC2), the tumor regressed.

Alternative explanations for blood eosinophilia under ICI treatment exist, independently of their potential predictive value. Raised B-Eos may be seen in patients displaying an immune-related adverse event (irAE). These adverse events are specific to ICI and reflect an excessive immune reactivation (Postow et al., 2018). Several case series and case reports confirm this across various tumor types (Krishnan et al., 2020). In the context of NSCLC, Chu and colleagues described an increased risk for ICI-induced pneumonitis with a baseline AEC ≥0.150 cells/mL Chu et al., 2020). Also, a non-allergic drug reaction may lead to increased

blood eosinophilia, as it was seen with IL-2 (Van Gool et al., 2014; Yamaguchi et al., 2021). IL-2, a type of IT previously used to treat melanoma, increases IL-5 plasma levels, which, in turns, promotes Eos progenitor cells differentiation to mature Eos, expands and activate them. Finally, an allergic reaction, characterised by the occurrence of a Th2-mediated immune response, can appear, as seen with various types of medication (Werner J., 2003). In that case, a skin rash and/or pruritus is (are) mostly noted but those will be hard to differentiate from a non-allergic, cutaneous irAE.

In the second part of our work, we investigated the prognostic value of tissue eosinophils (T-Eos) in early, resected NSCLC. Clinical literature on the topic is scanty, with only two studies reporting on T-Eos in NSCLC, and only one reporting on an association between survival and T-Eos (Tataroğlu et al., 2004; Ye et al., 2019). Hence, we decided to investigate this in a cohort of 316 patients and to gather data on preoperative WBC counts. We had to quantify T-Eos in a large number of samples (1853 zones, see below) and used for this the QuPath® software, an image analysis software allowing to quantify cells, for instance Eos, in a(n) (semi-)automated way with high efficiency. Slides were stained with hematoxilin-eosin and with an antibody (AB) against the Major Basic Protein (MBP) in eosinophil granules. To evaluate T-Eos distribution across different zones in tumoral and healthy slides, we delineated 3 zones on the tumor and 3 zones on the healthy slides. Our results indicate that Eos were mostly rare in early, resected NSCLC, but that they were present in approximately 80% of the cases. We observed a significant effect of the batch on T-Eos detection. According to our findings, T-Eos concentrate in the tumor itself and, to a lesser extent, at the direct periphery of the tumor, with a meaningful difference with paired, non-tumoral tissue. T-Eos did not differ according to the stage of disease, but higher concentrations were noted in squamous cell carcinoma than in other histologies. We further found no association between T-Eos and OS. Regarding DFS, we saw a statistically significant decrease in the risk of recurrence when more Eos were present in the adjacent, non-tumoral lung parenchyma. Finally, with higher B-Eos, the probability of finding T-Eos in the normal, adjacent lung parenchyma was higher, with a marginally positive p-value.

We had hypothesized that T-Eos would show a prognostic value given the results of the preclinical models on the roles of Eos and because we extrapolated the many reports of a positive association of B-Eos and clinical outcomes in advanced stages of NSCLC on the other hand. Our results show the lack of an association between T-Eos and OS. We therefore first questioned our methodology. We chose to use a stain directed at the Eos granules as literature

suggests that there are more sensitive than HE stains and because they can show Eos activity even after cell degranualtion (Weller & Spencer, 2017). We ascertained the quality of our stain directed at the MBP from the Eos granules by staining an Eos-rich tissue, i.e., nasal polyps, concomitantly to our resection specimen (1 polyp per staining batch) and observed a good performance of the immunostaining. We did observe variations in the level of staining intensity, but, as described in our last work, this did not impact the significance of the Eos quantification results. Although our staining was directed at the Eos granule proteins, direct light microscopy taught us that, to a few exceptions, what we quantified were entire and not degranulating Eos. Most importantly, the accuracy of our quantification was verified by an intraclass correlation coefficient calculation between QuPath® and the manual count by our certified pathologist, blinded to survival outcomes. The accuracy was rated as moderate to good according to the criteria described be Koo & Li, when using the MBP intensity levels 2 and 3 (Koo & Li, 2016). Based on these elements, we recommend using the MBP intermediate or high levels for T-Eos quantification with QuPath®. We conclude that the specificity of QuPath® in the T-Eos quantification is high, with a lower sensitivity that can be improved by using higher levels of MBP staining intensity. In our view, as accuracy is defined by the ratio between true positive plus true negative detections over the total number of assessments (cell detections, in our case), the accuracy of QuPath® remains acceptable (Cagney et al., 2018).

We already alluded to the fact that our data on T-Eos are innovative in the way we quantified T-Eos and in the sample size. We summarize the current knowledge on TATE in NSCLC in **Table 1** and conclude that the prognostic value of TATE in NSCLC remains uncertain at present, certainly given the fact that, in our series, the median overall survival had not been reached at this first censoring time point. In a second step, we looked at literature data on T-Eos in other tumor types to see whether a lack of association between TATE and survival was also described in other series. In breast cancer, TATE remains rare (3.7% in a series of >11000 specimens) (Ali et al., 2016; Chouliaras et al., 2021). In their very large series, Ali and colleagues found a significant association between the presence of T-Eos detected by CIBERSORT analysis and OS, in a subset of patients (estrogen receptor positive breast cancers). Another group, however, described no association between TATE and DFS or OS with a smaller cohort of patients (n=1069, whereof 40 with TATE) (Chouliaras et al., 2021). An interesting finding noted by Samoszuk already some 30 years ago is that, similarly to some of our data and to those of Cormier and colleagues, T-Eos in breast cancer specimen was almost exclusively restricted to the stroma, as opposed to the tumor core (Cormier, 2006; Samoszuk

et al., 1996). In colorectal cancer (CRC), authors of a large study (n=934 adenocarcinomas) quantified lymphocytes, plasma cells, Eos, and neutrophils by QuPath® in a way that was similar to ours (Vayrynen et al., 2020). They noted an association between higher T-Eos, lymphocytes and plasma cells in the stroma and longer survival, independently of known prognostic confounders like microsatellite instability or tumor stage. This beneficial effect of TATE in CRC was confirmed in a meta-analysis (note, with an overwhelming representation of CRC data) where OS and DFS benefit was noted in association with TATE, as was the case in esophageal cancer (Hu et al., 2020).

Table 1. Studies reporting tissue eosinophils in NSCLC.

	Tataroglu et al. 2004	**Ye et al. 2019**	**Sibille et al.**
Samples			
Patients, n	63	30	316
Samples, n	63	60[$]	1853
Histology	Mixed*	ADC	Mixed[#]
Paired, yes/no	no	yes	yes[##]
pStage	I-IV	I-III	I-III
Methods			
IHC type	HE	EPO	MBP
IHC grading	no	yes[$$]	yes
IHC quantification method	manual	manual [$$$]	semi-automated[###]
Results			
Eos – primary results	More Eos in lower pTNM[**]	EPO mRNA T>H ($p<0.05$) EPO IHC T>H (P=0.001)	Eos T0>H, T1, T2 ($p<0.0001$) Eos detected in 80.2%
Correlation with OS	NR	in EPO >4 HR death=3.145	No
Correlation with DFS	NR	NR	In T2 HR recurrence=0.62, p=0.0023
Other	Eos > vs ≤3 not correlated with pTNM	Eos correlated with: - pTNM (p=0.017) - N-status (p=0.027)	Eos not correlated with pTNM

P: pathological; ADC: adenocarcinoma SCC: squamous cell carcinoma; NE:neuroendocrine; HE: hematoxilin-eosin; NR: not reported; [*]: 32 SCC, 19 ADC, 2 adeno-squamous carcinomas, 1 large cell NE carcinoma; **: see table hereunder where n=number, Eos=eosinophils, stages according to the TNM[7th] classification, means ± standard deviation; [$]: + 6 tumoral cell lines (4 ADC, 1 bronchial epithelial cells, 1 SCC); [$$]: according to staining intensity (0-4 scale) and extent of involvement on the slide (0-25/26-50/51-75/76-100%), leading to a composite score, ≤4 vs >4; [$$$]: additional qRT-PCR for EPO mRNA and Western blot for EPO (on cell lines only); [#]69.6% ADC, 22.5% SCC, 8% other histologies; [##]: 9 patients had unpaired tissue (6 no healthy tissue and 3 no tumor tissue); [###]: QuPath® software, trained before automated count.

Addendum: Eosinophil counts according to TNM stage in Tataroglu et al.

	n	**Eos**
Stage I	15	21.6 ± 23.5
Stage II	12	20.3 ± 47.7
Stage III	33	8.6 ± 12.7
Stage IV	3	3.6 ± 3.6

The lack of uniformity in clinical data calls out for a more detailed description of the T-Eos. We learned from asthma mouse models that, in the lungs, Eos undergo functional changes between their recruitment from the blood compartment and their positioning in the airway lumen, reflecting a plasticity that has previously been observed with other myeloid cells (Abdala Valencia et al., 2016; Biswas & Mantovani, 2010; Jaillon et al., 2020). Also in mice, house dust mite challenge allowed Mesnil and colleagues to discern resident lung Eos from their inflammatory, allergy-induced, counterparts (Mesnil et al., 2016). Importantly, the last study showed identical findings in transbronchial biopsies from non-asthmatic controls vs sputum from asthmatic patients, suggesting that the changes seen in mice are applicable to humans, too. A third group reported distinct Eos subgroups in the same conditions of allergy challenge that could be differentiated with flow cytometry, while light microscopy showed identical Eos (Percopo et al., 2017). Those experiments reflect the plasticity of Eos, depending on their microenvironment. One of the questions raised by our study on T-Eos in NSCLC is why Eos concentrating in the tumor core do not reflect either a negative or a positive prognostic value. A **functional assessment** of T-Eos detected in lung tumors and in non-tumoral lung parenchyma would allow us to define more precisely the role(s) of the, perhaps functionally different, T-Eos in this context. The function of a cell is defined by its genetic content that will undergo the translational process to deliver proteins as effector units of the cells (Buccitelli & Selbach, 2020). Over the last years, the technique of single cell (sc) ribonucleic acid (RNA) sequencing (seq) has become a widely used technique for transcription analysis of myeloid cells. It allows for a detailed information mapping at the cellular level, fine-tuning our knowledge on cells that, until then, were believed to be terminally differentiated. However, a detailed information on each cell does not allow to consider its interaction with other cells. In this prospect, the spatial distribution of cells also carries useful information. Spatial transcriptomics is a scRNA seq technique applied to a whole tissue slide, combining both approaches to uncover as best as it can the function of a cell within its environment. Studying Eos with scRNA seq technique, however, remains a challenge as, for transcriptomic analysis, cells must be lysed, which causes the release of the ribonucleases (mainly RNAse 2, eosinophil-derived neurotoxin) contained in the granules, into the cytoplasm, “cutting” the RNA before its reading (Van Hulst et al., 2020). Alternative techniques, however, are being used and could leverage important information soon.

CONCLUSION

Our work explored the value of blood (B) and tissue (T) eosinophils (Eos) as biomarkers in the context of non-small cell lung cancer (NSCLC). First, we studied B-Eos in the context of advanced stages of disease, in patients treated with immune checkpoint inhibitors (ICI). Then, we focused on early stages of disease and studied, on resection specimens, the presence, and the distribution of T-Eos across both tumoral and paired, non-tumoral tissue before exploring their association with survival outcomes.

A first study suggested a prognostic and predictive role of B-Eos in patients treated with second or later line ICI for advanced NSCLC (Tanizaki et al., 2018). Authors found that a composite score including the pretreatment ANC, ALC and AEC was associated with better PFS and OS. Following this publication, we conducted our first study in a similar patient population. We demonstrated an early, i.e., two to three months after the start of treatment, rise in B-Eos under ICI, that persisted at the second evaluation, i.e., four to six months after treatment initiation. We further saw a significant rise in both AEC and REC in patients deriving clinical benefit, i.e., with objective response or stable disease according to the RECIST, compared to baseline and compared to patients whose disease progressed on treatment.

Next, we demonstrated in another cohort that Eos were the only WBC subtype to show such an increase on treatment with ICI. Our data confirmed the role of neutrophils, lymphocytes, and their ratio in the prognosis of such patients, as demonstrated by other authors (Mezquita et al., 2018; Park et al., 2018, 2020). We further confirmed the association between higher B-Eos (REC) and response to treatment early in the treatment course and between higher REC and the duration of treatment. Yet, we could not find a cutoff for the REC with a sensitivity sufficient to suggest that this parameter may be used as a predictive biomarker. Indeed, looking back at the few studies on this topic, it appears clearly that there is no standardized, well described method for sample collection, and no clear definition of a threshold outlining the effect of raised blood eosinophils, which are prerequisites for a qualitative biomarker.

The last part of our work focused on early stages of disease. Indeed, clinical data on T-Eos are scanty and the series published were small in size, making it difficult to draw conclusions (Tataroğlu et al., 2004; Ye et al., 2019). We demonstrated in our series including more than 300 patients that TATE in NSCLC is more common (80.2% of the samples were positive for at least one T-Eos) than in other cancer types like breast cancer, although we acknowledge the use of a specific staining and of an image analyzing scoring system (QuPath®), which, however, is

unique and reliable for the study on NSCLC specimen. We described that Eos concentrated in the tumor core and, to a lesser extent, at the direct periphery of the tumor, with significant differences compared to other regions of the tumoral or the non-tumoral slide. Regarding clinical outcomes, we found a lower risk of recurrence in patients showing higher T-Eos in the adjacent lung parenchyma, at a distance from the tumor. This latter finding highlights the need for functional studies complementing the useful information gained by histological, human data.

We conclude here, stating that blood eosinophils cannot, for now, be called biomarkers of response in advanced stage non-small cell lung cancer patients treated with immune checkpoint inhibitors. We are confident that our prospective, comprehensive, and comparative data collection on eosinophils and clinical parameters addresses this question in a methodologically more correct way. Regarding tissue eosinophils, our work lay the basis for an up-to-date, large-scale, and modern quantification method. We hope to raise sufficient interest in the oncology community to further develop this and, hopefully, answer the question of their prognostic significance in the context of early stage, resected, non-small cell lung cancer.

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APPENDIX

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Review

Eosinophils and Lung Cancer: From Bench to Bedside

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Abstract: Eosinophils are rare, multifunctional granulocytes. Their growth, survival, and tissue migration mainly depend on interleukin (IL)-5 in physiological conditions and on IL-5 and IL-33 in inflammatory conditions. Preclinical evidence supports an immunological role for eosinophils as innate immune cells and as agents of the adaptive immune response. In addition to these data, several reports show a link between the outcomes of patients treated with immune checkpoint inhibitors (ICI) for advanced cancers and blood eosinophilia. In this review, we present, in the context of non-small cell lung cancer (NSCLC), the biological properties of eosinophils and their roles in homeostatic and pathological conditions, with a focus on their pro- and anti-tumorigenic effects. We examine the possible explanations for blood eosinophilia during NSCLC treatment with ICI. In particular, we discuss the value of eosinophils as a potential prognostic and predictive biomarker, highlighting the need for stronger clinical data. Finally, we conclude with perspectives on clinical and translational research topics on this subject.

Keywords: eosinophils; non-small cell lung cancer; immunotherapy; biomarkers; predictive value; prognostic value

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1. Introduction

Paul Ehrlich first described eosinophils more than a century ago and already suggested that their alpha-granules contain secretory products [1]. Eosinophils are multifunctional white blood cells (WBC) whose functions have been intensely studied in both physiological and pathological conditions. Their role in non-oncological pulmonary diseases such as asthma and chronic obstructive pulmonary disease (COPD) has been emphasised by major therapeutic developments in the field, more specifically inhaled corticosteroids (ICS) and agents targeting the interleukin (IL)-5 pathway that is essential for the expansion, recruitment, and migration of eosinophils in both physiological and pathological (inflammatory) conditions [2,3]. In oncological diseases also, the study of WBC (neutrophils, lymphocytes and eosinophils) has gained interest, particularly since the advent of immune checkpoint inhibitors (ICI) [4]. In this setting, WBC counts have been studied for their potential prognostic and predictive value in various solid tumors such as non-small cell lung cancer (NSCLC) [5]. Paralleling this, a paradigm shift was observed in the study of solid tumors, highlighting the importance of the tumor microenvironment (TME), which consists of immune and non-immune cells, and of chemo- and cytokines interacting with each other (cross-talk) [6]. Here, we review in the context of NSCLC the biological properties of eosinophils in humans and their roles in homeostatic and pathological conditions, with a focus on their pro- and anti-tumorigenic effects. We also explore possible explanations for

blood eosinophilia during NSCLC treatment with ICI. In particular, we discuss the value of eosinophils as a potential prognostic and predictive biomarker, highlighting the need for stronger clinical data. Then, we conclude with suggestions for clinical and translational research topics on this subject.

2. Biology of Eosinophils

Eosinophils are granulocytes that differentiate from multipotent stem cells, called common myeloid progenitors in humans and granulocyte/macrophage progenitors in mice [7,8]. According to recent research, the lineage of myeloid cells is set early in the development of different cell subtypes [9]. Mack EA and colleagues reviewed the major transcription factors identified in the eosinophil lineage commitment [10]. They describe the central role of c/EBPα, GATA-1&2, FOG, PU.1, TRIB-1, and IRF8 (Figure 1). Not only the presence of those transcription factors seems important, but also the level and the timing of their expression for eosinophil development. Eosinophil precursors are further matured, expanded, and activated by cytokines, among which IL-5 (in physiological and pathological conditions) and IL-33 (in pathological conditions) play a central role [10]. The major importance of IL-5 has been demonstrated by several experiments where its deletion or overexpression in mice led to eosinophil depletion or excessive synthesis, respectively, and by clinical trials in severe asthma patients displaying a profound eosinophil depletion when treated with IL-5 antagonists, leading to a dramatic control of their symptoms and of the need for oral corticoids [11–13]. Interestingly, it is now believed that IL-5 orchestrates the action of other cytokines, such as IL-4, rather than acting as a sole direct trigger on eosinophil precursors via binding to its receptor, IL-5 Receptor unit α (IL-5Rα) [14]. Once triggered, eosinophils are released in a mature state in the blood where they stay for a short time (half-life of 18 h) [15]. In physiological steady-state conditions (see below), eosinophils migrate to the gastrointestinal tract [16] and, to a lesser extent, to the thymus, mammalian gland, and uterus [17,18]. This occurs under the action of chemokine eotaxin-1 (also called CCL11). In inflammatory conditions, the recruitment of eosinophils to alternative tissues such as the lungs is triggered by cytokines (IL-4, IL-5, IL-13, IL-33) [19–22], adhesion molecules (β-integrins) [23], and eotaxins-1,-2 and -3 (CCL11, CCL24, and CCL26, respectively) [24]. Thus, the expansion and survival of eosinophils depend on IL-5. Eosinophil lung infiltration depends on both IL-5 and on eotaxins. The life span of eosinophils in tissues is shorter in homeostatic conditions [2–5 days] than in inflammatory conditions (~two weeks), at least in vitro [25,26].

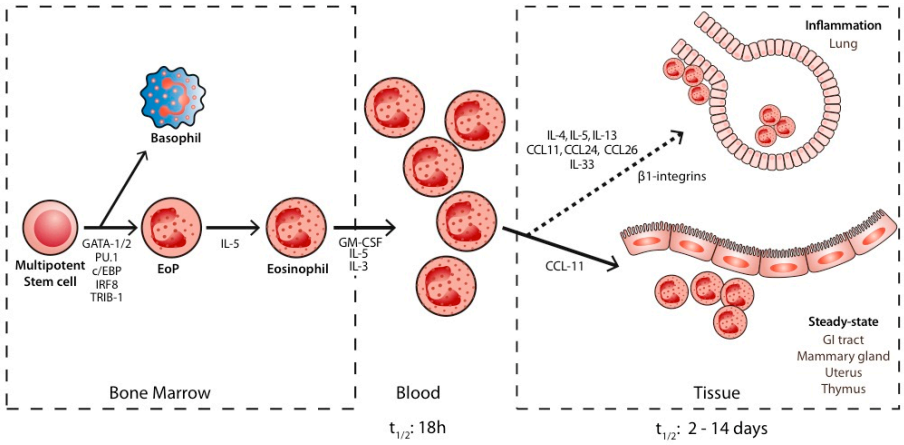

Figure 1. Biology of eosinophils. Eosinophils derive from multipotent stem cells. They proliferate, migrate, and are activated by cytokines, mainly Interleukin-5 (IL-5). They spend a short time in blood and subsequently migrate to tissues via the interplay of several chemokines. GM-CSF: Granulocyte-Macrophage–Colony Stimulating Factor. EoP: eosinophil progrenitor. IL-5: Interleukin-5. IL-3: Interleukin-3; CCL11: CC-chemokine ligand 11(=eotaxin1); CCL24: eotaxin-2; CCL-16: eotaxin-3. $T^{1/2}$: half-life. GI tract: gastrointestinal tract.

Morphologically, eosinophils can be characterized by their intracellular content and by their surface receptors (Figure 2). A bilobed acidophilic nucleus and intracellular granules are common to all species [27]. The granules can be divided into primary granules (containing Charcot–Leyden crystal proteins and lipids), secondary granules, and small granules. In human eosinophils, secondary granules contain four predominant cytotoxic proteins called cationic proteins: major basic protein (MBP)-1, eosinophil peroxidase (EPX), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN), the latest two also showing a ribonuclease activity. The granules also contain cytokines, chemokines, and growth factors that enable eosinophils to play their role in inflammation. Cell-surface receptors of eosinophils are numerous [28]. They can be classified into: adhesion molecules (selectins), chemotactic factor receptors (e.g. chemokine receptor 3 (CCR3)), cytokine receptors (e.g., IL-5Rα/β), complement receptors, immunoglobulin receptors, inhibitory receptors (e.g., sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8)), and pattern-recognition receptors (PRR; including Toll-like receptors and RAGE). The PRR recognises danger signals, also called alarmins. These can be of exogenous (infectious) origin (bacterial, fungal, or parasitic; so-called pathogen-associated molecular patterns-PAMPs) or endogenous, tumor-derived signals (so-called danger-associated molecular patterns-DAMPs). Activation of the PRR by the alarmins leads to expansion, adhesion to blood vessels, chemotaxis, degranulation, and cell-to-cell interactions of eosinophils [28], triggering the immune system [29]. IL-33 is an epithelial- and tumor-derived cytokine belonging to the IL-1 cytokine family [30]. It seems to be a crucial alarmin in host defense against tumors. Indeed, eosinophils recruited and activated through IL-33 were shown to be responsible for tumor growth control and for the prevention of pulmonary metastases development in melanoma-bearing mice. Mechanisms leading to these anti-tumorigenic effects have been deciphered and are detailed further. Andreone and colleagues underline the central role of IL-33 through in vitro experiments where induction of eosinophil degranulation by IL-33 in the context of cancer is even superior to that of IL-5 [31].

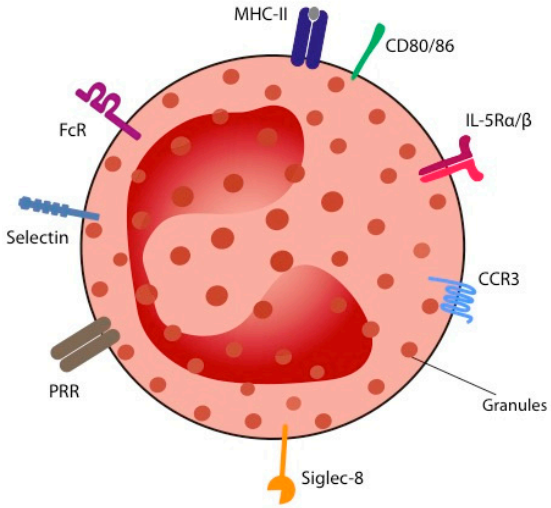

Figure 2. Structure of the human eosinophil. Eosinophils can be characterised by their surface markers and by their intracellular content. Cell-surface markers are: adhesion molecules (selectins) allowing for adhesion and endothelial transmigration; chemokine receptors (CCR) and chemotactic factors allowing for the attraction and local activation of eosinophils; cytokine and growth factor receptors (e.g., Interleukin-5 Receptor alpha subunit (IL-5Rα)); complement receptors; immunoglobulin receptors (e.g., FcR); inhibitory receptors (e.g., Sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8)) and pattern recognition receptors (PRR), e.g., Toll-like receptors whose activation is triggered by alarmins (Pathogen-associated molecular patterns (PAMPs) in case of infection and Danger-associated molecular patterns (DAMPs) in case of tumor).

3. Role of Eosinophils in Physiological Steady-State Conditions

Eosinophils are similarly found in various tissues of healthy humans and mice: bone marrow, blood, gastrointestinal tract, thymus, secondary lymphoid tissues, uterus, and adipose tissue. They are implicated in diverse processes, highlighted by the study of IL-5 overexpressing, eosinophil-deficient or cytokine reporter mice [32,33].

The first role of eosinophils is to contribute to tissue development, as is the case in the mammary glands [18], in the uterus [17,34,35], and in the gastrointestinal tract, where they contribute to the development of the Peyer's patches [16,36]. The second role of eosinophils is in tissue regeneration. As an example, the eosinophil-dependent IL-4 production proved to be crucial for the differentiation of fibrocyte-adipocyte progenitors into hepatocytes and myocytes in the context of liver or muscle injury [37,38]. Thirdly, eosinophils take part in metabolism. In adipose tissue, their IL-4 and IL-13 production leads to the differentiation of macrophages into the M2-phenotype that has greater insulin sensitivity [39] and to the increase in thermogenic, "beige" adipocytes [40]. Finally, eosinophils appear to be of great importance in immune homeostasis, playing a role as innate immune cells and as regulatory cells for the adoptive immunity. Indeed, the priming of B lymphocytes, as well as maintenance of plasma cells within the bone marrow or intestinal mucosa, are (partly) promoted by eosinophil-linked mechanisms: production of IL-4, IL-6, and the activation and proliferation-induced ligand (APRIL) cytokines [41–44]. Moreover, IgA production, microbiome composition, the integrity of the mucosal barrier, and the development of Peyer's patches are, in mice at least, all eosinophil-driven through IL-6, APRIL, and transforming growth factor (TGF)-β [36,45]. Lastly, eosinophils are mediators of T-cell tolerance: in the thymus, they participate in the destruction of self-reactive T cells via the secretion of indoleamine 2,3-deoxygenase (IDO) [46].

4. Eosinophils and Cancer: The Bench Side

The recruitment of eosinophils at tumor sites relies on tumor cells and on the inflammatory reaction (necrosis) they induce, as well as on peri- or intra-tumoral immune cells (lymphocytes, mast cells, dendritic cells) that can secrete eosinophil chemoattractants [47]. Based on in vitro models of NSCLC, Huang and colleagues demonstrated that eosinophils are attracted by type 2 cytokines (IL-5, IL-4, IL-10, and IL-13) that are produced by tumor cells [48]. GM-CSF and CCL11 (eotaxin 1), which are present in tumor tissue, contribute to the attraction of eosinophils [49,50]. Hollande and colleagues emphasised the role of CCL11 by demonstrating that dipeptidyl peptidase DPP4 (CD26) inhibitor sitagliptin led to enhanced tumor control through enhanced CCL11-mediated eosinophil recruitment at the tumor site [51]. Furthermore, the role of dying tumor cells in eosinophil recruitment was demonstrated in a mouse model for melanoma, where eosinophil concentrations were significantly higher in the capsule (fibrotic area) and in the central (necrotic) area of the lesions [52]. The following alarmins promoting eosinophil infiltration of tumors were identified: high-mobility group box-1 protein (HMGB-1) and IL-33 [30,53]. Recent data on colorectal cancer suggest that the gut microbiota may also influence eosinophil recruitment in such cancers [54].

Preclinical data reveal both anti- and pro-tumorigenic activities of eosinophils, both through direct and indirect mechanisms. As a first step in exploring the hypothetical anti-tumorigenic role of eosinophils, several authors manipulated eosinophil-linked cytokines (IL-4 or IL-33 injections, CCL11, and IL-5 depletion) [30,50,55]. They observed that tumor incidence and/or growth were inversely correlated with eosinophil infiltration. Further in vitro studies showed more precisely the mechanisms by which activated eosinophils can control tumors. In addition to a direct cytotoxic effect on cancer cells through degranulation [30,56], activated eosinophils recruit, activate, and lead to the maturation of several immune cells promoting tumor rejection [30,57–59] (Figure 3). Carretero and colleagues showed that activated eosinophils recruit cytotoxic $CD8^+$ T cells and are essential for tumor control in their melanoma mouse model [57]. They also demonstrated that eosinophils are capable of macrophage polarisation into an antitumor (M1) phenotype. A pivotal study

in colorectal cancer identified that intratumoral eosinophils exert these anti-tumorigenic effects through interferon-gamma (IFNγ) signaling [54]. Additionally, eosinophils tend to normalise tumor vasculature, a crucial factor for tumor maintenance and expansion. Indeed, depletion of eosinophils led to increased vascular leakiness, diminished perfusion, and diminished coverage by mature pericytes [57].

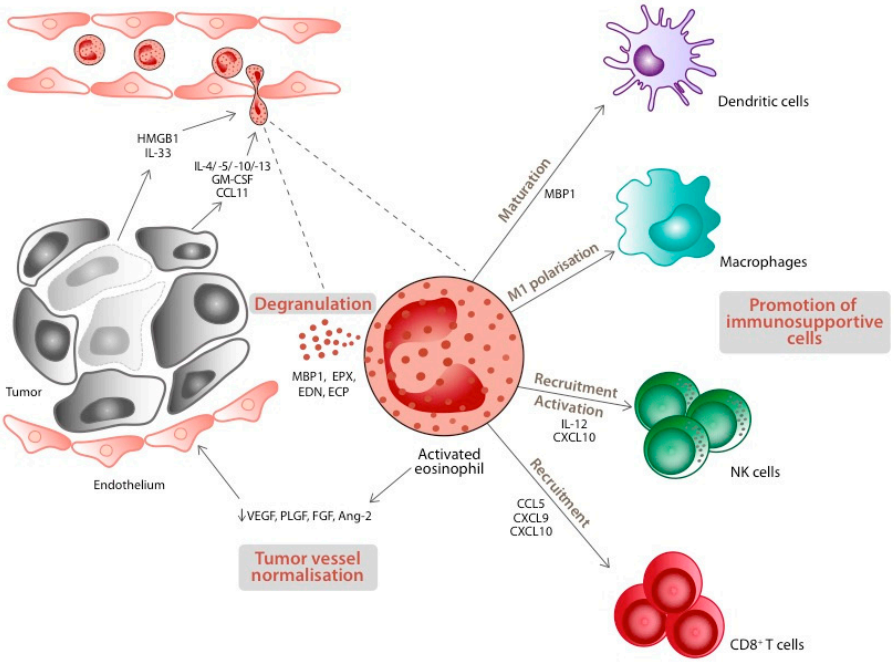

Figure 3. Eosinophil recruitment at tumor sites and anti-tumor effects of eosinophils. In response to their recruitment and activation via different cytokines and chemokines such as tumor-secreted Interleukin-5 (IL-5), or IL-33 and High Mobility Group Box-1 protein (HMGB-1), alarmins secreted by dying tumor cells, eosinophils display both direct and indirect anti-tumorigenic effects. Degranulation of eosinophils has cytotoxic and ribonucleasic effects. Moroever, activated eosinophils are capable of recruiting immune cells to engage against tumors: Natural Killer (NK) cells, cytotoxic $CD8^+$ T cells, and dendritic cells (DC). Additionally, they can polarise macrophages to an M1, anti-tumorigenic phenotype. Finally, eosinophils appear to affect tumor vasculature by increasing vascular leakiness, leading to tumor necrosis. IL: Interleukin; HMGB-1: High Mobility Group Box-1 protein; PRR: Pattern Recognition Receptor; CCL11: CC-chemokine ligand 11 = eotaxin1; CXCL9: CXC-chemokine ligand 9; MBP-1: major basic protein-1; EPX: eosinophil peroxidase; EDN: eosinophil-derived neurotoxin; ECP: eosinophil cationic protein; ↓: reduced expression; VEGF: vascular endothelial growth factor; PLGF: platelet growth factor; FGF: fibroblast growth factor; Ang-2: angiopoietin-2.

However, pro-tumorigenic effects of eosinophils have also been reported. As an example, preclinical models of oral squamous cell carcinoma showed reduced growth when eosinophil infiltration was hampered [60,61]. A model of cervix carcinoma also revealed that eosinophils, activated by tumor-generated thymic stromal lymphopoietin (TSLP), triggered tumor growth [62]. Eosinophils facilitate the recruitment of regulatory T cells (Treg) [63], inhibit cytotoxic T cells via the production of IDO [64], and induce the polarisation of macrophages into the M2, immunosuppressive phenotype through the production of IL-13 [65]. Finally, eosinophils produce many growth factors, with direct effects on tumor growth, metastatic spread, matrix remodeling, or on tumor-associated blood vessels [66].

Those seemingly opposing roles of eosinophils in tumors probably reflect their functional plasticity rather than underline contradictory findings. Firstly, eosinophils are, similar to other myeloid cells, part of the tumor microenvironment (TME), an entity where tumor cells, inflammation, and immune cells interact and evolve over time [67,68]. It

is reasonable to think that, as for macrophages and neutrophils, eosinophils' behavior could vary depending on the surrounding stimuli (cytokines, exosomes) [69,70]. Indeed, while IFNγ and IL-33 trigger an anti-tumorigenic role of eosinophils, IL-5 favors their pro-tumorigenic function [30,54,63]. Secondly, in light of the data described, a differential role for eosinophils according to the histologic subtype might be suspected: immuno-supportive in melanoma, immuno-suppressive in oral squamous or cervix carcinoma. However, it may be so that different tumor types simply reflect different TME. Thirdly, phenotypic studies of eosinophils in asthma mouse models showed eosinophils with different localisations (airway lumen vs. epithelium), morphology (ring-shaped vs. segmented nucleus), and different gene and cytokine expression profiles, reflecting different functions [71–73]. This, however, remains to be demonstrated in the context of cancer.

5. Eosinophils and Lung Cancer: The Bedside

5.1. Blood Eosinophils (B-Eos)

The first data on cancer patients showing an association between anti-neoplastic treatment and eosinophilia came from a cohort of 20 patients treated with IL-2 and lymphokine-activated killer cells for advanced cancer [74]. A study by van Haelst Pisani and colleagues further demonstrated that IL-2 administration was followed by IL-5 production and eosinophilia [75]. Some 20 years later, several authors demonstrated an association between B-Eos, anti-cytotoxic T-cell lymphocyte antigen (CTLA) 4 antibodies, or anti-Programmed Death (Ligand)(PD)-(L)1 antibodies and improved clinical outcomes across various types of cancer [4,5,76–83].

Strikingly, little data exist on the study of B-Eos in NSCLC patients treated with ICI and outcomes (Table 1). The studies are all retrospective in nature. Authors noted a correlation between raised blood eosinophils and a favorable clinical or radiological outcome. The princeps study by Tanizaki and colleagues suggests a prognostic and/or predictive role of B-Eos in patients treated with nivolumab for advanced NSCLC after the failure of previous systemic treatment [5]. Pre-nivolumab absolute eosinophil count (AEC) >0.15 cells/mL, absolute lymphocyte count (ALC) >1.0 cells/mL, and absolute neutrophil count (ANC) >7.5 cells/mL were significantly associated with a better overall and progression-free survival (OS and PFS, respectively). This was confirmed in the tumors with PD-L1 expression ≥50% but was not significant for tumors with PD-L1 expression <50%. For patients with an AEC > 0.15 cells/mL, the risk of death was reduced by 76% and the risk of progression by 47%. Two other studies looking at leucocytes under ICI treatment comforted those results on a slightly higher number of patients and in a similar therapeutic context [82,83]. In our cohort of patients, none of the pre-treatment B-Eos values were predictive nor prognostic [82]. The relative eosinophil count (REC) was predictive of objective response according to the Response Criteria In Solid Tumor (RECIST) at the first evaluation [8–12 weeks after the first treatment] and at the second evaluation (+8–12 weeks) ($p = 0.0019$, OR = 0.54, and $p = 0.0014$, OR = 0.53, respectively). The duration of treatment, an indirect reflection of the clinical benefit, was significantly longer with a lower ANC ($p = 0.0096$) and a higher REC ($p = 0.0021$) at the first RECIST evaluation. Notably, no association was found between B-Eos and toxicity. Neutrophils, lymphocytes, and their ratio were prognostic in this treatment setting. Okauchi and colleagues concentrated on the study of B-Eos only [83]. They showed that pre-treatment AEC was lower in patients that would later progress under ICI ($p = 0.002$). Under treatment, AEC and REC were lower in progressive patients ($p = 0.002$ and <0.0001, respectively). The time to treatment failure was longer in patients with an AEC > 0.15 cells/mL and a REC > 3% before ICI initiation ($p = 0.046$ and 0.003, respectively) and with an AEC > 0.3 and >0.5 cells/mL ($p < 0.001$ for both) and a REC > 3 and >5% on treatment ($p < 0.001$ for both). The two latest studies further suggest, based on Receiver Operator Curves (ROC) analysis, that a REC > 5% is predictive of disease control, although with disputable sensitivity and specificity (81.9% and 32.8%, respectively [82]; 60.7% and 27.3%, respectively [83]). In the last study, Chu and colleagues analysed data from 300 NSCLC patients treated with ICI for advanced

disease and looked at pre-treatment peripheral blood characteristics that may predict the occurrence of immune-related pneumonitis and predict general outcomes (survival and response rates) [81]. They demonstrated a link between pre-treatment AEC (cut-off value of 0.125 cells/mL) and [1] a higher objective response rate (ORR) [40.9% vs. 28.8%, $p = 0.029$] and [2] a longer PFS [8.9 vs. 5.9 months, $p = 0.038$].

Table 1. Studies on the association between outcomes of NSCLC patients treated with ICI and blood eosinophils. This table illustrates the heterogeneity of study objectives and of evaluation criteria for eosinophilia: continuous/categorical variable; timing of evaluation; biomarker used alone (simple) or in combination with others (composite).

Study	N	Stage of Disease	ICI	Eosinophils	Outcome	Effects	*p* Value
Tanizaki 2017 [5]	134	IIIB-IV	nivolumab	AEC t0; categorical; simple & composite biomarker	OS PFS	HR = 0.24 [95% CI 0.09−0.62] HR = 0.53 [95% CI 0.31−0.91] if AECt0 ≥ 0.15 cells/mL	0.003 0.02
Chu X 2020 [81]	300	IIIB-IV	PD-1i +/− CT +/− AAG	AEC t0; categorical; simple	ORR PFS	40.9 % vs 28.8 % med. = 8.93 vs 5.87 mo HR = 0.744 [95% CI 0.56−0.99] if AECt0 ≥ 0.15 cells/mL	0.029 0.038
Sibille 2021 [82]	191	IIIA-IV	pembrolizumab nivolumab atezolizumab durvalumab	AEC & REC t1; continuous	ORR	OR = 0.53 [95% CI 0.32−0.88] if RECt1 > 5.3%	0.014
Okauchi 2021 [83]	190	IIIA-IV	nivolumab pembrolizumab atezolizumab +/− CT	AEC & REC t0 & q2–3 wk; RECmax. *; categorical	TTF	OR = 0.39 [95% CI 0.26−0.60] if RECmax. > 5%	<0.001

ICI: immune checkpoint inhibitor; NSCLC: non-small cell lung cancer; PD-1i: Programmed death-1 inhibitor; AAG: anti-angiogenics; CT: chemotherapy (platinum-based doublet); AEC: absolute eosinophil count; REC: relative eosinophil count; categorical: studied as a categorical variable; continuous: studied as a continuous variable; t0: value before ICI treatment; t1: timing of the first RECIST evaluation under ICI treatment (at 8–12 weeks after initiation); q2–3 wk: every 2–3 weeks; * REC max.: maximal REC value noted under ICI; OS: overall survival; PFS: progression-free survival; ORR: objective response rate; TTF: time to treatment failure; CI: confidence interval; HR: hazard ratio; OR: odds ratio.

As these data come from retrospective studies, the quality of the observations is clearly poorer. For instance, registration of medical conditions (allergy, asthma, COPD) and concomitant medications (corticoids) interfering with eosinophilia were only completely mentioned in one out of the four studies on NSCLC patients [82]. Additionally, the overview given in Table 1 allows considering the heterogeneity of the studies regarding the number of patients included and the evaluation criteria for B-Eos (studied as continuous vs. categorical variables; inconsistent evaluation time points; single vs. composite biomarker). However, there is a consistent correlation between raised B-Eos under treatment with ICI and better outcomes (OS, PFS, ORR).

Voorwerk and colleagues addressed the question of the specificity of ICI in inducing eosinophilia in their melanoma mouse model and demonstrated [1] that the rise in B-Eos after ICI was specific to this type of anti-neoplastic drug, as compared to chemotherapy, and that it also occurred when combining chemotherapy and ICI; [2] that the improved survival of mice treated with ICI relied upon eosinophils, as depletion of these cells by anti-Siglec8-antibodies resulted in survival that paralleled the survival of mice not treated with ICI. The results concerning raised B-Eos and clinical response were confirmed for metastatic bladder and lung cancer, as well as for early-stage mismatch repair proficient

colon cancer [84]. To the best of our knowledge, there is also no clinical report pointing at a link between B-Eos or T-Eos and the efficacy of chemotherapy or tyrosine kinase inhibitors.

Blood eosinophilia has also been reported in cancer patients who display toxicity to ICI. So-called immune-related adverse events (irAE) are specific to these drugs and reflect excessive immune activation [85]. There are case reports as well as (mostly retrospective) studies showing an association between the occurrence of irAE and eosinophilia. In the context of NSCLC, the series of Chu et al revealed a correlation between baseline AEC and the occurrence of pneumonitis [27.7% if AEC $\geq$ 0.125 cells/mL vs. 9.8% if AEC < 0.125 cells/mL, $p < 0.0001$] [81].

Some authors advocate for the existence of a drug-driven, irAE-independent eosinophilic syndrome in the context of ICI [80,86]. Both groups demonstrated the existence of B-Eos (>0.5 cells/mL in Bernard–Tessier, >1.0 cells/mL in Scanvion) in the absence of irAE, although the retrospective nature of the study may not allow for a complete recording of toxicity events. However, the correlation between various drugs and eosinophilia is already well known and as such there is no reason that ICI could not lead to a similar phenomenon. In that case, the rise in eosinophils can be the consequence of increased production of these cells, e.g., IL-2 triggering IL-5 production, leading to increased eosinophilopoïesis, as observed in mouse models [87,88]. It can also be the result of a type IVb allergic reaction characterised by the occurrence of a Th2-mediated immune response, as seen in some patients taking various types of medication [89]. Given the wide clinical spectrum of medication-induced eosinophilia and the possible overlap of clinical signs with irAE (such as a rash), this drug-induced eosinophilia may, in fact, be underestimated.

5.2. Tissue Eosinophils (T-Eos)

To date, these data are scarce in NSCLC. In advanced disease, we found no report on tissue eosinophils (T-Eos) for this tumor type. In the early stages, two studies described eosinophils and their value in this setting. Ye and colleagues studied the expression of EPX, one of the four proteins contained in eosinophil granules, on 30 resection specimens of adenocarcinoma of the lung and on adjacent, normal lung tissue [90]. The expression level of EPX was rated by the degree (negative/weak/medium/strong staining) and the extent [0/1–25/26–50/51–75/76–100%] of the protein expression. A score was then defined for high vs. low EPX expression. Univariate analysis revealed a higher EPX expression in the cancer areas as compared with normal tissue ($p < 0.05$) and a correlation of high levels of EPX with higher pathological Tumor Node Metastases (pTNM) stage ($p = 0.017$) and with lymph node involvement ($p = 0.027$). T-Eos here was associated with a worse prognosis with a calculated hazard ratio (HR) for death of 3.1 ($p = 0.018$) in the EPX high group. Tataroglu and colleagues published a study on the presence of mast cells, macrophages, and eosinophils and their association with tumor vasculature and TNM stage NSCLC samples [91]. No significant association was noted between eosinophils and tumor stage or between tumor-associated vasculature and eosinophils. It should be noted, however, that eosinophils were evaluated by light microscopy after staining with hematoxylin-eosin. Weller and Spencer described the difficulties in detecting eosinophils in tissue thoroughly and suggested that electron microscopy or the use of antibodies directed at eosinophil granule proteins are useful tools to optimise the count of these cells in tissue [92]. In addition to technical issues, TATE could vary according to the degree of activation of the immune cascade, i.e., according to the interplay of cytokines, chemokines, and immune cells shaping the tumor microenvironment.

6. Perspectives

While clinical data suggest potential roles for eosinophils in NSCLC in the context of ICI treatment, preclinical models offer strong evidence that these myeloid cells do play an important role in the immune response against (lung) cancer. Furthermore, in vitro and animal models have revealed the complex interplay of different cells, whereof

eosinophils, and components of the tumor microenvironment, leading to a priori opposed roles for eosinophils.

In order to further unravel the role of eosinophils in this context and, hence, to explore their possible predictive and/or prognostic value as biomarkers, it appears of fundamental importance to go over from descriptive findings, relying on the sole eosinophil count, to functional studies that will clarify what role(s) eosinophils fulfill in this setting. In asthma, those studies led to important advances in understanding their diversity and plasticity [71,73]. They showed that the role of resident eosinophils differs from those of inflammatory, allergy-induced eosinophils. Such functional studies, however, face technical challenges in humans. First, eosinophils are a numerically poorly represented myeloid cell population. Second, available techniques to access the functional repertoire of these cells, i.e., DNA, RNA, or proteins, all have their limitations and, until recently, rendered poor results, explaining the lack of functional characterisation data on human eosinophils, and in particular in lung cancer [93]. However, techniques are advancing fast and refinements have already made possible functional studies of mouse eosinophils [94].

Another issue that is yet to be solved is to strengthen the evidence from patient cohorts. Clearly, prospective data are needed to erase the biases inherent to the retrospective studies: incomplete data collection and the exclusion of patients based on a posteriori criteria. In particular, upfront registration of confounding factors such as concomitant medications (inhaled and systemic corticoids), known predictive factors of ICI efficacy (tumor PD-L1 and mutational status, smoking history, immune-related toxicity), or medical conditions (parasitic infections, atopy, asthma, COPD) is of paramount importance to ascertain (a) role(s) of eosinophils in lung cancer patients treated with ICI. Those roles, for now, can only be suggested based on the available data.

The variability of blood eosinophils is a well-known problem that may, at least in part, explain their lack of sensitivity in predicting clinical outcomes. It was formerly illustrated in the context of asthma and chronic obstructive pulmonary disease (COPD), where intra-patient, day-to-day variability but also circadian variability were demonstrated [95,96]. Given the lack of satisfying sensitivity in the two attempts to define a cut-off value for B-Eos to predict disease control in patients treated with ICI for NSCLC, the study of B-Eos should at least be challenged by studies on alternative materials. As lung cancer remains an air-borne disease, sputum, bronchoalveolar lavage, or exhaled air from lung cancer patients could provide useful information. Furthermore, although biopsies in lung cancer patients can be challenging and, in a substantial proportion of cases, will need invasive techniques, we feel that a baseline, i.e., pre-treatment, comparative assessment of eosinophils in tissue vs. other material would be valuable.

Once available, tissue should also be analysed with techniques offering the highest chance of locating (qualitative analysis) and counting (quantitative analysis) eosinophils as a first step. Such data are, at the present time, unavailable for advanced stages of NSCLC and are scarce for early stages. In any case, B-Eos and T-Eos potentially differ in terms of their ability to function, as they evolve in different conditions (such as the oxygen content). Therefore, a comparative study might be interesting.

Arguably, one could wonder whether, given the difficulties summed up here, looking for the trigger of eosinophil activation (alarmins) would not be preferable to looking for the eosinophils themselves.

Another unexplored area in the clinical research described here is the study of the kinetics of blood eosinophilia. So far, only one study reported results for patients treated for multiple oncological indications with ICI [80]. While the rise in blood eosinophils is noted early in the treatment course, the study of their evolution over time could provide valuable observations to guide further clinical and/or translational research.

7. Conclusions

Preclinical models have established a role, although not unique, for tissue eosinophils in cancer. Despite their questionable quality, clinical data suggest that raised blood

eosinophils may reflect a favorable outcome in patients treated with immune checkpoint inhibitors for advanced NSCLC. Functional studies and more stringent clinical research are needed to further elucidate the role of eosinophils in lung cancer and their potential value as a biomarker.

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LETTER TO THE EDITOR

Clinical benefit to programmed death-1 inhibition for non-small-cell lung cancer is associated with higher blood eosinophil levels

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Introduction

The use of immune checkpoint inhibitors (ICI) for non-small-cell lung cancer (NSCLC) is increasing. Currently validated indications include advanced and locally advanced disease [1]. Classically, response evaluation relies on radiological assessment with the REsponse Criteria In Solid Tumours (RECIST) version 1.1 [2]. However, in the setting of ICI these criteria seem imperfect. Indeed, atypical response patterns have been observed that make radiological evaluation less clear than it is with chemotherapy [3]. Pseudoprogression, one of these atypical radiological responses, is defined as radiological progression in the absence of clinical deterioration. It correlates with immune cell infiltration and/or transient tumour growth before response to ICI [3]. Discontinuing ICI in this case would mean stopping an efficient therapy. Alternative or additional tools for the evaluation of response could help to effectively and more accurately evaluate the efficacy of ICI. Blood eosinophil counts are routinely available at a negligible cost. Most of the objective responses to PD-1 inhibitors occur within the first two months of PD-1 inhibitor use [4, 5]. Here, we report on blood eosinophil evolution during the first months of treatment with anti-Programmed Death (PD)-1 antibodies and on their value as early indicators of response in patients treated for advanced stage NSCLC.

Materials and methods

Medical records from patients consecutively treated at our institution with any anti-PD-1/anti-PD-L1 in monotherapy for advanced stage NSCLC between 1/8/2015 and 30/4/2018 were investigated. In this time frame only two agents were used: pembrolizumab, given at the dose of 2 mg/kg every 3 weeks during the early access programm then at the fixed dose of 200 mg every 3 weeks, and nivolumab, given at 3 mg/kg every 2 weeks. We collected the following data: (i) patients characteristics (age at the start of immunotherapy, gender, smoking status, concurrent airway disease), (ii) lung cancer characteristics (histological subtype, stage, line of treatment of the anti-PD-1, PD-L1 status), (iii) treatment characteristics (agent, response at t1 (time of first evaluation, i.e. after three (for pembrolizumab) to four (for nivolumab) cycles of immunotherapy) and t2 (time of second evaluation, i.e. after four (for pembrolizumab) to six (for nivolumab) additional cycles of immunotherapy) using REsponse Criteria for Solid Tumours (RECIST) v1.1), (iv) eosinophil counts (absolute and relative) at t0 (before treatment), t1 and t2 . Of the 191 patients identified the following patients were excluded: loss of follow-up ($n = 8$), treatment discontinuation before t2 due to toxicity ($n = 2$), progressive disease ($n = 4$), patient's will ($n = 3$) or death ($n = 57$). Response was assessed according to the RECIST criteria version 1.1 [2]. We further describe patients as responders (complete (CR) or partial (PR) response), stable or progressive. We focussed on the first two radiological evaluations as the majority of objective responses (i.e. CR and PR) occur in the first two months of treatment with anti-PD-1/-PD-L1 in monotherapy for NSCLC, corresponding to the first time point (t1) in our study [4,5]. We extended the evaluation period to the second radiological evaluation (t2) in order to include the patients showing a non-significant response at t1 further evolving towards PD or PR. Blood eosinophils were expressed as median number of cells/mL for the absolute eosinophil count (AEC) and in percentage of the total white blood cell count for the relative eosinophil count (REC) with interquartile range (IQR).

Regarding the statistical analyses paired comparisons of eosinophil values between the three visits of patients were performed with a non-parametric test: Wilcoxon's signed rank test. The comparison of eosinophil levels of the 3 groups of patients ranked according to the response to the treatments were performed by an unpaired test for non-parametric continuous variables: Kruskal-Wallis test followed by the Dunn's post-hoc testing if Kruskal-Wallis tests were positive. A p value $<.05$ was considered statistically significant. Analyses were conducted by the statisticians of the Pneumology laboratory Unit of the CHU de Liège using GraphPad Prism V.7.03 (GraphPad Software, La Jolla, California, USA) for the statistical analyses and for the figures.

Results

In the 117 patients analysed baseline blood eosinophils were not statistically different in responders, stable or progressive patients. For the whole study population the AEC and REC were significantly raised at t1 compared to t0 ($p < .01$ for both AEC and REC) (Figure 1). Responders and stable patients had significantly higher eosinophils than progressive patients at t2 ($p < .05$ for AEC and $p < .01$ for REC for

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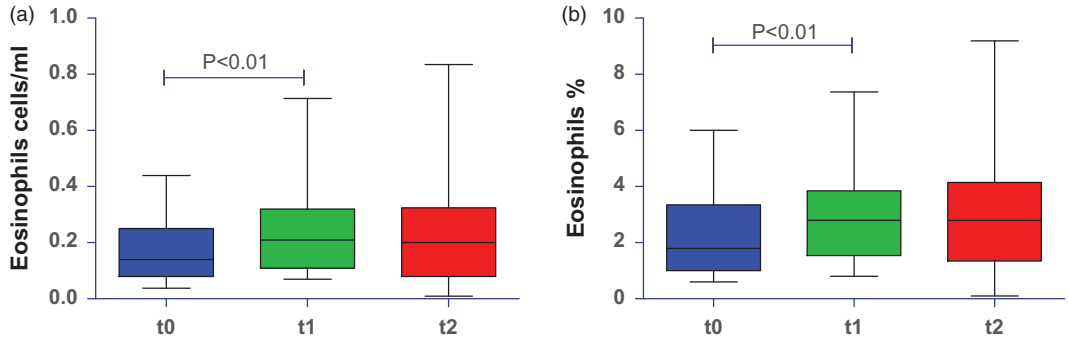

Figure 1. Blood eosinophil levels of the entire study cohort. Results expressed as median ± IQR, confidence interval 10-90%.

Table 1. Blood eosinophil levels according to the type of response.

	Responders		Stable		Progressive	
Eosinophils	AEC	REC	AEC	REC	AEC	REC
t0	0.16 (0.12−0.29)	2 (1.4−3.4)	0.14 (0.08−0.27)	2 (0.95−3.6)	0.11 (0.06−0.2)	1.4 (0.75−2.4)
t1	0.2 (0.1−0.29)	2.7 (1.6−3.8)	0.23** (0.17−0.35)	3.3** (2.05−4.3)	0.16 (0.1−0.32)	1.8 (1.3−3.15)
t2	0.23*# (0.14−0.33)	3.6*## (2.1−5.2)	0.21* # (0.12−0.35)	2.8*# (1.75−4.05)	0.08 (0.04−0.21)	1.4 (0.4−3.4)

AEC: absolute eosinophil count; expressed as number of cells/mL; REC: relative eosinophil count; expressed as percentage of the total white blood cell count. Responders ($n = 27$), stable ($n = 61$) and progressive ($n = 29$) patients: according to the RECIST criteria (see materials and methods). Inter-group analysis: Kruskal-Wallis test followed, if positive, by Dunn's test; *p*-value *vs.* progressive: $^{\#\#}p < .01$; $^{\#}p < .05$. Intra-group analysis: Wilcoxon's paired test; *p*-value: $^{**}p < .01$, $^{*}p < .05$.

responders and $p < .05$ for both AEC and REC for stable patients). Stable patients showed an early (t1) and persistent (t2) significant rise in eosinophils ($p < .01$ for AEC and REC at t1 and $p < .05$ for AEC and REC at t2) (Table 1). Performing univariate analysis (two-way factorial ANOVA) we did not find any impact of histology, type of anti-PD-1 agent, smoking status (current versus former smoker) or PD-L1 status on those results.

Discussion

Response evaluation of patients treated with PD-1 inhibitors remains a challenge. In routine clinical practice RECIST criteria remain the core element for the evaluation of response. However, atypical response patterns have been described following anti-PD-1 use showing the limitations of these criteria. We hypothesised that blood eosinophil kinetics might be an early indicator of response to ICI.

Considering the whole study population, we found a significant and early rise in blood eosinophils, i.e. after two to three months of PD-1 inhibition, compared to baseline values. In a large series ($n = 909$) of patients treated with anti-PD-1 antibodies for various types of cancers the rise in AEC was seen from 3 months after the start of the treatment and peaked at a median of 6.4 months [6]. The significant increase at t1 in our cohort is in keeping with this previous study although we found no further significant rise between t1 and t2, possibly due to the lower number of patients.

Baseline eosinophil counts did not differ between responders, stable or progressive patients. This contrasts with retrospective data from one melanoma series treated with anti-PD-1 agent pembrolizumab where baseline REC > 1.5% was associated with an improved overall survival and more objective responses according to the RECIST criteria, although this positive prognostic and predictive value of eosinophils was only noted for the REC, not for the AEC, and in combination with the relative lymphocyte count [7]. The predictive role of a composite blood biomarker was retrospectively investigated in one series of NSCLC patients and showed a longer progression-free survival, defined as the time between the start of a PD-1 inhibitor and radiological progression, in patients showing the following baseline characteristics: an absolute lymphocyte count >1 cells/mL, an absolute eosinophil count ≥0.15 cells/mL and an absolute neutrophil count <7.5 cells/mL) [8].

Our main results indicate a clear association between blood eosinophils kinetics and the type of response. Indeed, eosinophils were significantly higher in responders and in stable patients than in patients with progressive disease at the time of second evaluation. Also, stable patients showed an early and persistent significant increase in eosinophils. This was not the case for responders, possibly due to a lower number of patients (27 responders *vs.* 61 stable patients). To the best of our knowledge no data exist to date regarding the evolution of blood eosinophil levels and the type of response to ICI in NSCLC.

The exact role of eosinophils in (lung) cancer remains uncertain at present [9]. Indeed, some preclinical studies have shown a lower incidence of squamous cell carcinoma in eosinophil-deficient mice. Most studies, however, highlight their multiple anti-tumour effects: maturation of dendritic cells, polarisation of macrophages to an M1 phenotype, inhibition and normalisation of tumour vasculature, recruitment and activation of T lymphocytes and NK-cells, direct cytotoxic effects on tumour cells [9,10]. Even though we cannot state whether raised blood eosinophils are a consequence or a driver of enhanced activity of PD-1 antibodies our results indicate that they might be indicators of response

to anti-PD-1 drugs for NSCLC. Although we acknowledge the need for a validation study with a greater and ideally prospective cohort, we believe that the highly significant differences between eosinophils of responders and stable patients versus non-responders in our study warrant reporting.

In conclusion, our retrospective cohort suggests a role of blood eosinophils in the early response to PD-1 inhibitors in NSCLC patients.

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LUNG CANCER

White Blood Cells in Patients Treated with Programmed Cell Death-1 Inhibitors for Non-small Cell Lung Cancer

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Abstract

Purpose To investigate whether eosinophils and other white blood cell subtypes could be used as response and prognostic markers to anti-Programmed cell Death-1 or anti-PD-Ligand-1 treatments in non-small cell lung cancer patients.

Methods We retrospectively analyzed data from the NSCLC patients consecutively treated at our hospital with a PD-1/PD-L1 inhibitor in monotherapy for advanced disease. A total of 191 patients were evaluated at three time-points to investigate any relation between tumor response and WBC counts.

Results Baseline WBC and subtypes did not differ according to the type of response seen under treatment. A higher relative eosinophil count (REC) correlated with more objective responses ($p=0.019$ at t1 and $p=0.014$ at t2; OR for progression = 0.54 and 0.53, respectively) independently of the smoking status, PD-L1 status, and immune-related toxicity (IRT). Higher REC was also associated with a longer duration of treatment ($p=0.0096$). Baseline absolute neutrophil count was prognostic ($p=0.049$). At t1 relative lymphocytes, absolute and relative neutrophils, and neutrophil-to-lymphocyte ratio were prognostic ($p=0.044$, $p=0.014$, $p=0.0033$, and $p=0.029$, respectively).

Conclusion Our results show that in NSCLC patients anti-PD-1/PD-L1 therapy induces an early increase only in blood eosinophils, more prominent in responding patients and independent of the smoking status, PD-L1 status, and IRT. Eosinophils are also associated with a longer duration of treatment. Furthermore, our data support a prognostic role of neutrophils, lymphocytes, and their ratio for NSCLC patients with advanced disease treated with PD(L)-1 blockade.

Keywords White blood cells · PD-1 inhibitors · Non-small cell lung cancer · Prognostic marker · Predictive marker · Checkpoint inhibitors

Introduction

The use of immune checkpoint inhibitors (ICI) for non-small-cell lung cancer (NSCLC) is increasing. Currently validated indications include advanced and locally advanced disease [1]. One of the challenges regarding ICI lies in the evaluation of objective response to these drugs. Classically, response evaluation relies on radiological criteria based on the Response Evaluation Criteria In Solid Tumors (RECIST) [2]. However, in the setting of ICI, these criteria seem imperfect. Indeed, several atypical response patterns like pseudoprogression have been observed that make radiological evaluation less clear than it is with chemotherapy [3]. In the search for additional evaluation tools, white blood cell (WBC) count has been investigated, among others, in melanoma and in NSCLC patients treated with Programmed cell Death (PD) Ligand (L)-1 inhibitors [4–7]. Some reports also mention a potential prognostic role of WBC subtypes and/or their ratio for various malignancies among which NSCLC [5, 7–9]. We previously reported a retrospective study investigating peripheral blood eosinophil counts as a parameter in the evaluation of response in NSCLC patients receiving PD-1 blockers [10]. In the present study we first aimed to investigate whether the results obtained in our former cohort could be confirmed. Then, we compared the

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potential predictive value of different subtypes of WBC and investigated the prognostic value of baseline WBC subtypes.

Material and Methods

Patients

All consecutive cases of advanced stage NSCLC were collected from our internal cancer registry from August 1st, 2015 to September 30th, 2019. A computer-based search was performed with the following inclusion criteria: (1) use of an anti-PD-1 or anti-PD-L1 agent (pembrolizumab at 2 mg/kg/3 weeks during the early access program (EAP) and then at 200 mg/3 weeks; nivolumab at 3 mg/kg/2 weeks during the EAP and then at 240 mg/2 weeks; atezolizumab at 1200 mg/3 weeks; durvalumab at 10 mg/kg/2 weeks) or (2) a pathological diagnosis of NSCLC for which the patient was registered in the electronic treatment prescription system. For the 388 patients identified the following exclusion criteria were applied: histology other than NSCLC ($n=27$), missing laboratory values ($n=15$), loss of follow-up ($n=22$); early treatment discontinuation, i.e., before the second evaluation ($n=106$) due to death, toxicity, and progressive disease without death or patient's will; ongoing treatment ($n=7$) or chemotherapy combined with anti-PD-1 ($n=20$). Based on this, 191 patients were included in the present analysis.

Data Collection

We collected the following data: (i) *patient characteristics*: age at the start of immunotherapy, gender, smoking status, concomitant obstructive airway disease, use of inhaled or oral corticoids and the reason for it (underlying respiratory condition, immune-related toxicity (IRT), other), date of death, and baseline Eastern Cooperative Oncology Group (ECOG)-Performance Status (PS); (ii) *lung cancer characteristics*: histology, stage of disease, line of treatment of the anti-PD (L)-1, PD-L1 expression level, based on immunohistochemistry (monoclonal antibody clone 22C3 with Automated Stainer, Dako), and presence or absence of a mutation based on next-generation sequencing analysis and ALK immunohistochemistry; (iii) *treatment characteristics*: dates of the start of treatment [t0], first evaluation (t1) and second evaluation (t2), immunotherapeutic agent, response at t1 and t2 using the RECIST criteria v1.1, immune-related toxicity (IRT), and duration of treatment; (iv) *biological variables*: total WBC counts and differential WBC counts (neutrophils, lymphocytes, eosinophils; absolute and relative) at t0, t1, and t2.

Response Evaluation

A total of 191 patients were assessed for tumor response based on the RECIST criteria v 1.1 at two time-points (t1, t2; 8 to 12 weeks interval in between) and compared with baseline data. We describe patients as responders (R; for complete or partial response), stable (S), or progressive (P). We focused on the first two radiological evaluations as the majority of objective responses occur in the first two months of treatment (t1) with PD-1 blockers in monotherapy for NSCLC [11, 12]. We extended the evaluation period to the second radiological evaluation (t2) in order to include the patients showing a non-significant response at t1 further evolving toward progression or response.

Duration of Treatment

Duration of treatment with anti-PD (L)-1 drugs was calculated from the time of first administration until the last recorded dose administration (data cut-off December 5th, 2019) and expressed in weeks.

Overall Survival

Overall survival (OS) was defined as the time between the first dose of PD (L)-1 blocker and the date of death from any cause and expressed in months. If still alive at data cut-off (December 5th, 2019) the patient was censored.

Statistical Analyses

Biological variables were studied as continuous variables and are described as medians and interquartile ranges. Qualitative data are described using frequencies and percentages. For the analyses on biological variables logarithmic analyses were performed (translated logarithm log $(.+1)$ for the relative eosinophil count (REC) in percentage and log $(.+0.01)$ for the absolute eosinophil count (AEC) in 10^3cells/mm^3). Univariate logistic regression analyses were performed with determination of the Odds ratio (OR), with confidence interval (CI) at 95% and p-values. Survival was calculated, expressed in months, and reported with Kaplan–Meier curves, and Cox regression models were used to analyze the impact of the different variables on the survival and reported as Hazard Ratio (HR), with CI at 95% and p-values. Results were considered significant with an uncertainty level of 5% ($p<0.05$). Calculations were made with the help of SAS software (version 9.4) and graphs with R software (version 3.6.2).

Results

Patient Characteristics

A total of 191 patients were included in the study (Table 1). Approximately two-thirds of the patients were male with a large majority (94.8%) of (former) smokers and in good performance status (PS; 92.7% PS 0–1). Slightly more than half of the patients presented with a chronic obstructive airway disease at the time of PD(L)-1 blocker initiation but only 10.5% used inhaled corticoids and none used oral corticoids during the study period. The predominant histology was adenocarcinoma (55.5%). The majority of patients (69.7%) had stage IV disease at the time of treatment with PD(L)-1 blockade. Most of the patients (67.7%) received an anti-PD(L)-1 antibody in second or later line of treatment.

White Blood Cell Counts Over Time Under PD(L)-1 Blockade

Baseline WBC and subtypes did not differ between responding, stable, and progressive patients. Among the studied biological variables only eosinophils rose under PD(L)-1 inhibition between the start of treatment and the time of first or second evaluation ($p < 0.0001$) (Table 2).

Response

At the time of first evaluation 51 (26.7%) of the 191 patients were responders (R), 103 (53.9%) stable (S), and 37 (19.4%) progressive patients (P). At t2, we found 64 R (33.5%), 67 S (35.1%), and 60 P (31.4%). We found 3 patients (4.7%) showing progression at t1 but response at t2, so-called pseudoprogression. Five R (8.3%) became P at t2.

Higher response rates were noted for high PD-L1 expression (i.e., >50%; $p = 0.0001$ at t1 and $p = 0.0031$ at t2), pembrolizumab use ($p < 0.0001$ at t1 and $p = 0.0096$ at t2), and former smokers ($p = 0.024$; OR = 2.88).

Regarding biological variables none of the baseline values predicted the response at t1 or at t2. Responders had a significantly higher REC than progressive patients at t1 ($p = 0.019$ with OR = 0.54) and at t2 ($p = 0.014$ with OR = 0.53). By univariate analysis (two-way factorial ANOVA) PD-L1 status ($p = 0.18$ for REC and $p = 0.067$ for AEC), smoking status ($p = 0.43$ for REC and $p = 0.13$ for AEC) and immune-related toxicity (IRT) ($p = 0.87$ for REC and $p = 0.93$ for AEC) had no influence on eosinophil levels. No biological variable other than eosinophils was predictive of the response at t1 or at t2 (Table 3).

Table 1 Patient's characteristics

Characteristic	Total ($n = 191$)	Number (%)
Age-years		
Median		66
Range		42–85
Gender	191	
Male		122 (63.9)
Female		69 (36.1)
Smoking status	191	
Non-smoker		10 (5.2)
Former smoker		117 (61.3)
Current smoker		64 (33.5)
Obstructive airway disease	191	
None		83 (43.5)
COPD		88 (46.1)
Asthma		20 (10.4)
Inhaled corticosteroids	191	
No		171 (89.5)
Yes		20 (10.5)
ECOG-PS	191	
0		26 (13.6)
1		151 (79.1)
2+		14 (7.3)
Histology	191	
Adenocarcinoma		106 (55.5)
NOS		7 (3.7)
Squamous cell carcinoma		72 (37.7)
LCNE carcinoma		6 (3.1)
Oncogenic driver	119	
None		77 (64.7)
EGFR		3 (2.5)
ALK		0 (0)
Other		19 (16)
Unknown		20 (16.8)
Disease stage	191	
II		2 (1.0)
III		56 (29.3)
IV		133 (69.7)
PDL-1 category	191	
1		64 (33.5)
2		26 (13.6)
3		29 (15.2)
4		72 (37.7)
IT line stage IV	133	
1L		43 (32.3)
2L+		90 (67.3)
IT Agent	191	
Nivolumab		100 (52.3)
Pembrolizumab		58 (30.4)
Durvalumab		22 (11.5)
Atezolizumab		11 (5.8)

Table 1 (continued)

Smoking status: as registered at the start of PD(L)-1 blockade. Obstructive airway disease: *COPD* Chronic obstructive pulmonary disease, *ECOG-PS* Eastern Cooperative Oncology Group-Performance Status. Histology: *NOS* not otherwise specified. Oncogenic driver: *EGFR* Epidermal growth factor receptor (Tumor Hotspot Mastr kit, Illumina MiSeq), *ALK* Anaplastic lymphoma kinase (monoclonal antibody with Automated Stainer Benchmark, Roche), *other* BRAF, KRAS, and PIK3CA mutations (Tumor Hotspot Mastr kit, Illumina MiSeq), *unknown* No NGS or EGFR/ALK testing done, *no* At least no EGFR mutation/ALK rearrangement identified. Disease stage: according to the TNM 7th classification. PD-L1 category: *1* ≥ 50%, *2* 1–49%, *3* < 1%, *4* Unknown. IT line stage IV: line of treatment for the PD(L)-1 blockade: *1L* First line and *2L* + Second or later line

Table 2 Kinetics of white blood cell counts over time

	t0	t1	t2	*p*-value
WBC 10^3cell/mm^3	8.47 ± 3.70	8.09 ± 3.16	8.56 ± 4.94	0.70
Eosinophils %	2.34 ± 2.00^{ab}	3.38 ± 2.79^{a}	3.29 ± 2.83^{b}	<0.0001
10^3cell/mm^3	0.19 ± 0.20^{ab}	0.27 ± 0.27^{a}	0.29 ± 0.40^{b}	<0.0001
Lymphocytes %	20.16 ± 9.67	20.66 ± 8.54	20.41 ± 9.50	0.43
10^3cell/mm^3	1.56 ± 0.75	1.55 ± 0.62	1.58 ± 0.70	0.43
Neutrophils %	67.13 ± 11.93	65.68 ± 9.81	65.65 ± 13.32	0.20
10^3cell/mm^3	5.90 ± 3.28	5.47 ± 2.85	5.99 ± 4.72	0.15
NLR	4.64 ± 3.83	4.38 ± 5.26		0.20

t0 Pre-treatment, *t1* First evaluation, *t2* Second evaluation. Comparisons made with Scheffé's test between t0–t1 ($p^a < 0.0001$) and t0–t2 ($p^b < 0.0001$). WBC: white blood cells. *NLR* neutrophils-to-lymphocytes ratio

Toxicity

The overall rate for IRT was 24.1%. Most IRT was of low intensity, requiring no immunosuppressive therapy. Indeed, only 12 out of 191 patients (6.3%) required oral corticoids (OCS) for the control of their IRT. Skin (22/191 patients, 11.6%), thyroid (9 patients, 4.7%), joints (5 patients, 2.6%), and lungs (5 patients, 2.6%) were the most frequently involved. For the durvalumab subgroup we identified significantly more IRT (40.9% reported at t1 or t2 vs. 21.9% for non-durvalumab drugs, $p = 0.017$), a higher use of OCS (18.1% vs. 4.7%, $p = 0.0057$), and higher pulmonary and thyroid toxicity (13.6% for both in the durvalumab group, compared to 1.2% and 3.6% for patients receiving non-durvalumab drugs, respectively; $p = 0.0037$). There was no correlation between baseline WBC subtypes and toxicity and no correlation between toxicity and response.

Duration of Treatment

At the time of first evaluation a higher REC and a lower ANC were associated with a longer duration of treatment ($p = 0.0096$ and $p = 0.021$, respectively) (Fig. 1). At t2 all biological variables were predictive for the duration of treatment (data not shown).

Overall Survival

The median OS was 18.8 months with 98 patients (51.3%) alive at data cut-off. No clinico-pathological feature was prognostic in this cohort. The OS was longer in patients responding at t1 ($p < 0.0001$) with medians of OS of 30.4 months for responders, 19.9 months for stable, and 12.8 months for progressive patients. A lower baseline absolute neutrophil count (ANC) correlated with longer OS ($p = 0.049$) while at t1, the relative lymphocyte count (RLC), relative neutrophil count (RNC), ANC, and neutrophil-to-lymphocyte ratio (NLR) were correlated with OS ($p = 0.044$, $p = 0.014$, $p = 0.0033$, and $p = 0.029$, respectively) (Table 4).

Discussion

In this cohort of advanced stage NSCLC patients treated with PD(L)-1 blockade, PD-L1 expression levels and smoking history were associated with response, confirming earlier data [13, 14]. Pembrolizumab was associated with more responses, as it was the only drug used in patients with high PD-L1 expression levels. Regarding biological data we noted an early rise only in eosinophils. Moreover, a higher proportion of eosinophils was associated with an early response and with a longer duration of treatment. Neutrophils, lymphocytes, and their ratio, either at baseline or early in the course of treatment, appeared to be prognostic.

The role of eosinophils in tumors is still a matter of debate. In various tumor types in vitro data and preclinical models show direct and indirect anti-tumor effects [15, 16] but also pro-tumorigenic effects [17–20]. Neutrophils can, like eosinophils, have both anti- and pro-tumor functions [21, 22].

The prognostic and predictive value of blood biomarkers and more specifically WBC and their subtypes in patients treated with ICI have been reported in several tumor types, e.g., colorectal cancer [23], breast cancer [24, 25], prostate cancer [26], melanoma [4, 27–29], and NSCLC [7]. However, these studies lack homogeneity: absolute vs. relative WBC counts, single vs. composite markers, and continuous vs. categorized variables. Weide

Table 3 Biological variables according to the type of response at t2

	Responders		Stable		Progressive	
WBC						
t0	8.53 (5.92–10.61)		7.76 (6.22–18.49)		7.68 (6.04–10.36)	
t1	6.82 (5.74–8.98)		7.79 (5.94–8.60)		7.66 (6.25–9.42)	
t2	6.63 (5.66–8.89)		7.58 (6.54–9.13)		8.52 (6.55–10.61)	
Eosinophils	*AEC*	*REC*	*AEC*	*REC*	*AEC*	*REC*
t0	0.14 (0.08–0.28)	1.85 (0.90–3.40)	0.13 (0.09–0.23)	1.90 (1.00–3.10)	0.12 (0.06–0.21)	1.65 (0.80–8.70)
t1	0.22 (0.14–0.35)	3.1 (2.05–4.75)*	0.2 (0.11–0.30)	2.9 (1.50–4.00)*	0.19 (0.10–0.34)	2.6 (1.30–3.60)
t2	0.24 (0.15–0.39)	3.55 (1.85–5.50)	0.22 (0.13–0.30)	2.50 (1.80–3.70)	0.13 (0.06–0.31)	1.90 (0.80–3.80)
Neutrophils	*ANC*	*RNC*	*ANC*	*RNC*	*ANC*	*RNC*
t0	5.51 (3.75–7.70)	68.75 (60.40–74.55)	5.06 (3.96–6.87)	66.60 (60.20–74.20)	5.31 (3.88–7.59)	68.95 (62.65–74.00)
t1	4.54 (3.67–5.77)	64.45 (56.75–69.70)	5.27 (3.76–5.98)	66.90 (62.00–72.10)	5.30 (4.03–6.63)	67.15 (60.85–74.65)
t2	4.14 (3.19–5.77)	63.90 (52.70–68.50)	5.07 (4.23–6.31)	67.60 (59.50–74.00)	5.95 (4.26–7.77)	69.35 (62.90–80.15)
Lymphocytes	*ALC*	*RLC*	*ALC*	*RLC*	*ALC*	*RLC*
t0	1.62 (1.13–2.02)	19.80 (14.25–25.15)	1.33 (1.04–1.85)	18.20 (13.60–24.40)	1.51 (1.09–1.89)	17.55 (14.40–25.10)
t1	1.55 (1.10–1.86)	21.25 (15.95–26.40)	1.45 (1.10–1.82)	19.90 (15.00–24.40)	1.48 (1.01–1.85)	19.95 (12.85–24.65)
t2	1.67 (1.17–2.06)	22.20 (16.75–29.30)	1.46 (1.15–1.84)	18.50 (13.80–24.90)	1.29 (1.03–1.89)	16.80 (10.75–24.95)

WBC White blood cells (.10^3cell/mm^3), *AEC* Absolute eosinophil count (.10^3cell/mm^3), *REC* Relative eosinophil count (%), *ANC* Absolute neutrophil count (.10^3cell/mm^3), *RNC* Relative neutrophil count (%), *ALC* Absolute lymphocyte count (.10^3cell/mm^3), *RLC* Relative lymphocyte count (%). Responders ($n=64$), stable ($n=67$), progressive ($n=60$) patients: according to the RECIST criteria (see materials and methods). Results expressed as medians and interquartile ranges. Logistic regression analysis; *p*-value vs. progressive. $^*p<.05$

and colleagues proposed a prognostic model based on categorized serum lactate dehydrogenase (LDH), WBC count, and clinical characteristics [4]. The risk of death was 2.4-fold ($p=0.003$) and 2.2-fold ($p<0.001$) for patients with pre-treatment RLC < 17.5% and REC < 1.5%, respectively. In part based on these results Tanizaki and colleagues studied the prognostic and predictive value of peripheral blood biomarkers in a population of NSCLC patients treated with nivolumab for advanced disease ($n=137$) [7]. They found a strong association between baseline low (<7.5 cells/mL) ANC, high (>1.0 cells/mL) absolute lymphocyte count (ALC) and high (>0.15 cells/mL) AEC and higher response rates, progression-free survival (PFS), and OS. In those two studies, authors used categorized variables, i.e., AEC > or < 0.15 cells/mL and REC > or < 1.5%. We, however, considered the variables as continuous. Keeping this in mind, in our cohort a higher proportion in eosinophils at the time of the first evaluation (t1) were associated with a higher chance of objective response to treatment at t1 and t2. In our series, this was independent of the smoking history, PD-L1 status, and immune-related toxicity (IRT). We could, however, not identify a cut-off value for REC at t1 with satisfying sensitivity for discriminating responders from stable and from progressive patients at t2 (32.8% sensitivity and 81.9% specificity for a cut-off of 5.3% REC, *p*-value = 0.0137). Our study also emphasizes the association between blood eosinophils and the durability of clinical benefit for NSCLC patients, as expressed by the duration of treatment. A series of melanoma patients comfort these findings with response to ipilimumab correlated with an early rise in eosinophils [27].

In contrast to other series, toxicity in our cohort was not correlated with a higher probability of response and also not with raised eosinophils. Several retrospective studies [30–32] and one prospective report [33] showed an association between early IRT for advanced NSCLC and outcome. Although these studies are small sized and mostly lack pathological correlation, there is some rationale to explain this link: similarity between tumor antigens and self-antigens leading to cross-reactivity of T cells that are reactivated by the ICI [34], pre-existing autoimmunity with reactivation of T cells primarily directed at self-antigens [35] or B-cell reactivation through PD-1 blockade [36]. The fact that we did not find a correlation with response may be due to the retrospective nature of the study with incomplete data collection during patients' follow-up. On the other hand, a correlation between eosinophilia (i.e., AEC > 0.5 cells/mL) and immune-related toxicity ($p=0.0042$) has been demonstrated in a retrospective series including 146 patients with various solid tumor types treated with anti-PD(L)-1 [37]. As a correlation between eosinophils and response to ICI and between eosinophils and toxicity under ICI were shown, it is tempting to think that both clinical results (response and toxicity) are two sides of one phenomenon: immune (re-)activation. This, however, remains to be formally proven.

Some authors found a prognostic value of baseline eosinophils [4, 7]. This was not the case in our series. However, we found a clear association between eosinophils and

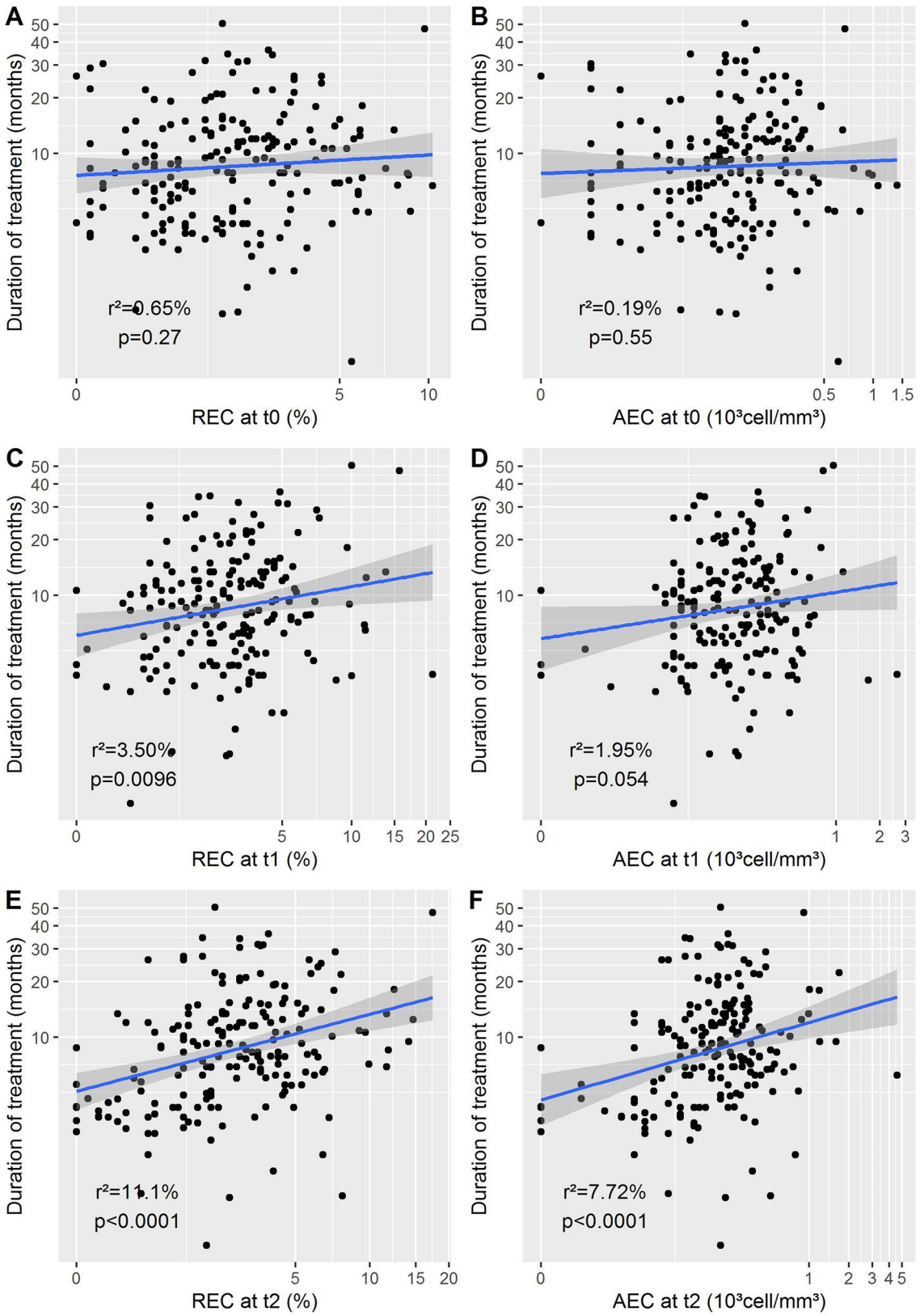

Fig. 1 Eosinophils and duration of treatment. Logarithmic scale representation for relative eosinophil counts (REC) and absolute eosinophil counts (AEC). t0: before treatment; t1: at time of first evaluation; t2: at time of second evaluation

Table 4 Risk of death in the 191 patients according to biological variables at t1

Variable	t1 values	*p*-values	HR	95% CI
White blood cells (10^3cells/mm^3)				
Alive	7.28 (5.74–8.90)	0.094	1.78	0.91–3.51
Dead	7.80 (6.32–8.97)			
Eosinophils (%)				
Alive	2.90 (1.90–4.00)	0.081	0.70	0.47–1.04
Dead	2.80 (1.30–4.10)			
Eosinophils (10^3cells/mm^3)				
Alive	0.21 (0.12–0.33)	0.22	0.84	0.64–1.10
Dead	0.20 (0.11–0.33)			
Lymphocytes (%)				
Alive	20.85 (15.90–25.50)	0.044*	0.64	0.42–0.99
Dead	19.10 (13.80–23.00)			
Lymphocytes (10^3cells/mm^3)				
Alive	1.56 (1.10–1.88)	0.39	0.81	0.50–1.31
Dead	1.47 (1.09–1.81)			
Neutrophils (%)				
Alive	64.10 (59.40–69.20)	0.014*	1.03	1.00–1.05
Dead	68.90 (60.80–73.60			
Neutrophils (10^3cells/mm^3)				
Alive	4.62 (3.54–5.87)	0.0033*	1.11	1.04–1.20
Dead	5.21 (4.11–6.32)			
NLR				
Alive	2.96 (2.38–4.27)	0.029*	1.46	1.04–2.06
Dead	3.56 (2.67–5.35)			

Results expressed as medians and interquartile ranges. Alive ($n=98$)/dead ($n=93$): as recorded at data cut-off (see Materials and methods). *HR* Hazard ratio for death. *CI* Confidence interval. *NLR* Neutrophils to lymphocytes ratio. *significant *p*-value (<0.05)

response to treatment and between response and OS. The lack of prognostic value of REC at t0 may be due to small sample size when compared to the work of Weide and colleagues. Moreover, the prognostic value of baseline neutrophils, lymphocytes, and neutrophils/lymphocytes ratio (NLR) demonstrated in our work supports the findings of several authors [5, 8, 38]. Illustratively, the prognostic value of the iSEND model (immunotherapy, Sex, ECOG-PS, NLR, and Delta NLR) is being investigated in a prospective manner after it showed its value as a predictive tool for patients with advanced NSCLC treated with nivolumab [8]. In earlier stages of disease a study on operated NSCLC specimen revealed an inverse correlation between neutrophils and CD8 + cytotoxic T cells [39].

An additional interesting finding of the present study is that blood eosinophils are the only WBC subtype displaying a rise during the first six months of anti-PD (L)-1 therapy for NSCLC, data that are in keeping with results from a large French cohort and from our previously published data [10, 40]. Further studies will have to explore why this rise is transient and whether raised eosinophils in responders are a consequence of or a trigger for immune anti-tumor activation.

Conclusion

In this study patients receiving PD(L)-1 blockade for advanced NSCLC and showing a raised proportion of eosinophils at the time of first evaluation were more likely to show an objective response according to the RECIST criteria at the time of second evaluation, regardless of smoking history, PD-L1 status, and IRT. A higher REC also correlated with a longer duration of treatment. We could, however, not identify a clear cut-off value to propose eosinophils as a predictive biomarker. It seems necessary to identify the underlying mechanism(s) leading to a rise in blood eosinophils in patients deriving clinical benefit from anti-PD(L)-1 drugs. Further results of this cohort support the prognostic role of neutrophils, lymphocytes, and their ratio, either at baseline or early in the course of treatment.

Author Contribution AS collected the data, contributed to the statistical analyses, and wrote the article. MH conducted the statistical analyses and contributed to the redaction of the manuscript. RA collected the data. JLC, RL, and BD reviewed the manuscript and contributed to its final version. All authors read and approved the final manuscript.

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Code Availability Not applicable.

Declarations

Conflict of interest All authors declare to have no conflict of interest with the submitted work.

Ethical Approval This study was approved by the Ethics Committee of the CHU de Liège with reference number 2019/239.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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Les éosinophiles dans le cancer pulmonaire: pertinence en pratique clinique

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Les éosinophiles sont des granulocytes multifonctionnels. Leurs croissance, survie et migration tissulaire dépendent principalement de l'interleukine-5. Les données précliniques démontrent un rôle immunologique des éosinophiles en tant que cellules du système immunitaire inné et adaptatif, en réponse à des agents endo- ou exogènes. Des rôles à la fois pro- et anti-tumoraux ont été décrits en conditions *in vitro* et dans des modèles précliniques. Par ailleurs, plusieurs études cliniques rapportent un lien entre les résultats des patients traités par immunothérapie pour un cancer de stade avancé et l'éosinophilie sanguine. Une majoration des éosinophiles a également été décrite chez des patients souffrant de toxicité dans le cadre de ces traitements. Jusqu'ici, les publications portent sur des séries rétrospectives mais qui donnent des résultats concordants et ce dans plusieurs sous-types de tumeurs solides. En ce qui concerne le cancer pulmonaire, l'éosinophilie tissulaire a été très peu étudiée et montre des données contradictoires, reflétant possiblement des difficultés techniques pour mettre en évidence les éosinophiles. Le microenvironnement tumoral peut en outre expliquer des fonctions a priori contradictoires de ces cellules, dont le comportement pourrait donc, comme pour d'autres cellules telles que les macrophages, varier en fonction des cytokines, chimiokines et autres cellules immunitaires présentes dans ce microenvironnement tumoral. De nombreux défis persistent afin de mieux déterminer la place de ces cellules dans le cancer, et en particulier dans le cancer pulmonaire: techniques d'identification adéquates, données cliniques prospectives, étude de l'éosinophilie tissulaire dans les différents stades, et modèle(s) explicatif(s) passant par l'étude fonctionnelle des éosinophiles.

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➔ Ce qui est déjà connu à ce sujet:

Peu de données sont publiées au sujet des éosinophiles dans le cancer du poumon. Le rôle de ces cellules myéloïdes rares a principalement été étudié au stade préclinique et dans les maladies obstructives telles que l'asthme et la broncho-pneumopathie chronique obstructive. En ce qui concerne les éosinophiles et le cancer, des rôles anti- mais aussi pro-tumoraux ont été décrits.

➔ Ce que l'article apporte de plus:

Cet article apporte une vue d'ensemble sur les connaissances actuelles du ou des rôles des éosinophiles dans le cancer pulmonaire. Il met en perspective des données d'apparence conflictuelle et souligne l'état d'avancement des données cliniques sur le sujet, ouvrant la perspective à des recherches ultérieures.

Introduction

Il y a plus d'un siècle déjà, Paul Ehrlich décrivait les éosinophiles (Eos) et suggérait que leurs granules contenaient des substances sécrétoires (1). Les Eos sont des leucocytes multifonctionnels. Leur rôle dans les pathologies pulmonaires non oncologiques comme l'asthme et la broncho-pneumopathie chronique obstructive (BPCO) a été mis en exergue par le développement de traitements majeurs tels que les corticoïdes inhalés et les antagonistes de l'interleukine-5 (IL-5) (2, 3). Celle-ci est en effet essentielle pour l'expansion, le recrutement et la migration des Eos en conditions physiologiques et pathologiques. En oncologie également, on observe un intérêt grandissant pour les globules blancs (GB) (neutrophiles, lymphocytes, Eos), a fortiori depuis l'avènement des inhibiteurs de points de contrôle immunitaire (IPC) (4). Dans ce contexte, les rôles potentiels pronostiques et prédictifs des GB ont été explorés, notamment dans le cancer bronchique non à petites cellules (CBNPC) (5). L'étude des cellules myéloïdes en oncologie s'inscrit dans la mise en évidence de l'importance du microenvironnement tumoral, constitué de cellules immunitaires, notamment myéloïdes, de cellules non immunitaires, de chimio- et cytokines (6). Dans cet article, nous décrivons, dans le contexte du cancer pulmonaire, les propriétés biologiques des Eos et leurs rôles dans les conditions homéostatiques et pathologiques. Nous discutons les données cliniques publiées et proposons quelques pistes explicatives pour l'éosinophilie sanguine au cours du traitement par IPC des CBNPC. Nous évoquons ensuite quelques pistes potentielles de recherche clinique sur ce sujet.

Biologie des éosinophiles

Les Eos sont des granulocytes dérivant de cellules souches pluripotentes. Leurs expansion, activation et migration vers des sites extra-médullaires dépendent de trois cytokines majeures: le *granulocyte-macrophage colony stimulating factor* (GM-CSF), l'IL-3 et, de manière plus critique, l'IL-5 (7). Une fois stimulés, les Eos sont largués à l'état mature dans la circulation sanguine. Dans les conditions physiologiques, au *steady-state*, ils migrent ensuite vers le tractus gastro-intestinal et, dans une moindre mesure, vers le thymus, l'utérus et les glandes mammaires. En cas d'inflammation, les Eos sont recrutés, par exemple, vers les poumons par des cytokines (IL-4, IL-5, IL-13), des molécules d'adhésion (beta-intégrines), et les éotaxines-1,-2 et -3 (CCL11, CCL24 et CCL26, respectivement).

Morphologiquement, les Eos peuvent être caractérisés par leur contenu intra-cellulaire et par leurs récepteurs de surface. Chez l'homme, les granules contiennent quatre protéines cytotoxiques majeures: la *major basic protein* (MBP)-1, l'*eosinophil peroxidase* (EPX), l'*eosinophil cationic protein* (ECP) et l'*eosinophil-derived neurotoxin* (EDN). Les granules contiennent également des cytokines, chimiokines et facteurs de croissance permettant aux Eos de remplir leur rôle dans le contexte inflammatoire. Les récepteurs de surface des Eos sont nombreux (8). Parmi eux, les *Pathogen Recognition Receptors* (PRR) qui permettent la reconnaissance de signaux d'alarme («alarmines») exogènes [infectieux; *Pathogen-Associated Molecular Patterns* (PAMP)] ou endogènes [tumoraux; *Danger-Associated Molecular Patterns* (DAMP)]. L'IL-33 semble être ainsi une alarmine centrale pour le recrutement et l'activation des Eos dans le contexte tumoral (9). L'activation des PRR entraîne l'expansion, le chimiotactisme, la dégranulation et les interactions des Eos avec d'autres cellules, induisant ainsi une réponse immunitaire.

Les éosinophiles en conditions physiologiques au ***steady-state***

On retrouve les Eos dans les tissus suivants: moelle osseuse, sang, tractus gastro-intestinal, thymus, tissus lymphoïdes secondaires, utérus et tissu adipeux (7). Quatre **fonctions physiologiques** leur ont été reconnues: le développement tissulaire, la régénération tissulaire, le métabolisme et l'homéostasie immunitaire, à la fois sur le plan de l'immunité innée et de l'immunité adaptative, en jouant le rôle de cellules régulatrices (10). À ce propos, l'activation des lymphocytes B et le maintien des plasmocytes au sein de la moelle osseuse ou de la muqueuse intestinale sont en partie promus par des mécanismes Eos-dépendants. Il en va de même pour la production d'IgA, la composition du microbiome intestinal, l'intégrité de la barrière muqueuse intestinale et le développement des plaques de Peyer. Enfin, les Eos sont médiateurs de tolérance T-cellulaire: dans le thymus, ils participent à la destruction des lymphocytes T auto-réactifs par la sécrétion d'indoléamine 2,3-dioxygenase (IDO).

Les éosinophiles dans le cancer

Données précliniques

Ce sont les cellules tumorales et la réaction inflammatoire précoce (nécrose) qu'elles engendrent, qui recrutent les Eos aux sites tumoraux (11). De nombreuses données *in vitro*, tissulaires, montrent leurs multiples

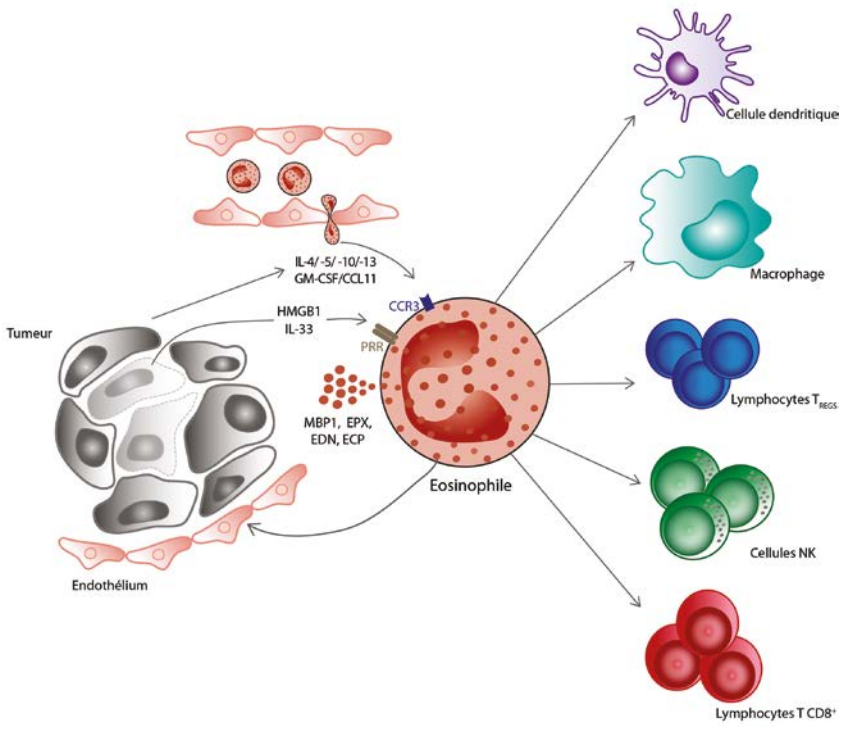
effets anti-tumoraux (**Figure 1**) (9, 12). Mais des effets pro-tumoraux ont également été décrits pour les Eos au niveau préclinique: promotion de la croissance tumorale par l'IL-5 dans un modèle de carcinome spinocellulaire oral, recrutement des lymphocytes T régulateurs (Treg), inhibition des lymphocytes cytotoxiques par la production d'IDO, polarisation des macrophages vers le phénotype M2 immunosuppresseur et effets pro-angiogéniques (11). **Ces effets apparemment contradictoires reflètent l'importance du microenvironnement tumoral**, dont les composants interagissent et influencent leur(s) effet(s) mutuel(s) (6). Il est aussi possible que le rôle de certaines cellules myéloïdes, dont les Eos, diffère selon l'histologie, comme illustré ci-dessus. Enfin, à l'instar des macrophages et des neutrophiles, plusieurs sous-types d'Eos ont été identifiés dans le poumon (13). Leur(s) fonction(s) reste(nt) toutefois à définir dans le contexte tumoral.

Données cliniques-éosinophilie sanguine (Eos-S)

C'est dans une cohorte de 20 patients traités par IL-2 et LAK (*lymphokine-activated killer cells*) pour des tumeurs avancées que la première association entre ces traitements et une Eos-S a été rapportée. Van Haelst-Pisani et al. ont ensuite démontré que c'était l'administration d'IL-2 qui entraînait une sécrétion d'IL-5 et donc une Eos-S (14). Vingt ans plus tard, les IPC immunitaires sont apparus dans l'arsenal thérapeutique oncologique, notamment thoracique. Bien que révolutionnaires au vu du taux et de la durabilité de leur réponse, ces traitements n'entraînent une réponse à long terme que pour une minorité de patients. Cherchant des biomarqueurs d'efficacité et de sélection de patients, plusieurs auteurs ont démontré, de manière rétrospective, une association entre Eos-S, anticorps anti-*cytotoxic T-cell lymphocyte antigen* (CTLA) 4 ou anti-*Programmed Death* (PD) (Ligand-L)-1 et efficacité clinique (4, 5, 15-21). Le travail de recherche réalisé par Voorwerk et al. a permis de démontrer la **spécificité de l'augmentation de l'Eos-S sous IPC** (22). Ce groupe a par ailleurs prouvé que l'amélioration de la survie des souris atteintes de cancer mammaire reposait sur les Eos, puisque leur déplétion résultait en une survie identique à celle de souris non traitées par IPC. Ces résultats ont été confirmés pour plusieurs modèles de cancer, notamment le CBNPC métastatique.

Figure 1:

Multiples effets anti-tumoraux des éosinophiles.



Les données cliniques sur l'Eos-S dans le CBNPC sont rares (**Tableau 1**). D'après ces études rétrospectives, il existe une **corrélation entre une majoration des Eos sanguins sous IPC et un meilleur résultat clinique**. L'étude princeps suggère un rôle pronostique et/ou prédictif des Eos chez les patients atteints de CBNPC de stade avancé et traités par nivolumab après échec d'un ou plusieurs traitement(s) antérieur(s) (5). Mesuré avant traitement par nivolumab, un taux d'Eos absolus (AEC) > 0,15cells/mL, de lymphocytes absolus (ALC) > 1,0cells/mL et de neutrophiles absolus (ANC) > 7,5cells/mL était significativement associé à une survie sans progression (SSP) et à une survie globale (SG) meilleures. Ceci a été confirmé dans la catégorie des tumeurs PD-L1 ⩾ 50%, mais pas de manière significative pour les tumeurs PD-L1 < 50%. Pour les patients avec un AEC > 0,15cells/mL, le risque de décès était réduit de 76% et le risque de progression de 47%. Deux autres études s'intéressant aux leucocytes sous IPC sont ensuite venues conforter ces résultats sur un nombre de patients sensiblement plus grand, dans un contexte thérapeutique similaire (majorité de patients pré-traités) (20, 21). Dans notre cohorte, aucune valeur avant traitement ne s'est révélée prédictive ou pronostique (20). Le taux relatif d'Eos (REC) était prédictif d'une réponse objective à la première (8-12 semaines après le premier traitement) et à la seconde (+8-12 semaines) évaluation sous IPC (p = 0,0019, OR = 0,54 et p = 0,0014, OR = 0,53, respectivement). La durée de traitement, reflet indirect du bénéfice clinique, était par ailleurs significativement plus longue pour un ANC plus bas (p = 0,0096) et un REC plus élevé (p = 0,0021) à la première évaluation. Il est à noter qu'aucune

association significative n'a été retrouvée entre toxicité et éosinophilie. Les neutrophiles, lymphocytes et leur ratio se sont révélés pronostiques dans ce contexte de traitement. Okauchi et son groupe ont, eux, uniquement étudié les éosinophiles. Ils ont démontré que l'AEC avant traitement était plus bas chez les patients qui progresseraient sous IPC (p = 0,002); sous traitement, l'AEC et le REC étaient plus bas chez les progresseurs (p = 0,002 et < 0,0001, respectivement) (21). Le temps jusqu'à l'échec du traitement était plus long pour les patients avec une Eos-S plus haute avant initiation de l'IPC (si AEC > 0,15cells/mL et REC > 3%; p = 0,046 et 0,003, respectivement) et sous IPC (si AECmax > 0,3 ou 0,5cells/mL; p < 0,001 pour les deux; et si RECmax > 3% ou 5% (p < 0,001 pour les deux). Cette étude, comme la nôtre, suggère par ailleurs qu'un taux de REC > 5% est prédictif d'un contrôle de la maladie sous IPC, avec une sensibilité et une spécificité toutefois suboptimales (81,9% et 32,8%, respectivement (20); 60,7% et 27,3%, respectivement (21)).

L'Eos-S est également rapportée dans le contexte de toxicité aux IPC au travers de cas cliniques ainsi que d'études, rétrospectives pour la plupart, d'échelle modeste. Les effets secondaires immuno-induits sont spécifiques aux IPC et reflètent un excès d'activation immunitaire. Ils peuvent être corrélés à une meilleure réponse clinique (23). À titre d'exemple, Chu et al. ont mis en évidence un lien entre l'AEC pré-traitement par IPC et, d'une part, la survenue d'une pneumopathie (27,7% pour un AEC ⩾ 0,125cells/mL vs. 9,8% pour un AEC < 0,125cells/mL, p < 0,0001) et, d'autre part, un meilleur résultat clinique (taux de réponse objective 40,9% vs. 28,8%, p = 0,029; SSP 8,9 vs. 5,9 mois, p = 0,038) (24).

Certains auteurs défendent en outre l'idée d'un effet toxique pharmacologique, «non immuno-induit» dans le contexte des IPC (25, 26). Les deux groupes ont rapporté l'existence d'une **Eos-S** (AEC > 0,5cells/mL ou > 1,0cells/mL, selon l'étude) en l'absence d'effet secondaire immuno-induit, bien que la nature rétrospective de leur analyse n'en garantisse pas un enregistrement strict. Ceci dit, la corrélation entre médication et Eos-S est un phénomène bien connu et, en soi, il est concevable que les IPC puissent induire une telle réaction. L'augmentation d'Eos peut alors être la conséquence d'une stimulation de leur production, comme par IL-2, qui induit l'activation d'IL-5. L'éosinophilie peut également résulter d'une réaction allergique de type IVb, comme pour d'autres traitements, caractérisée par une réponse Th2-médiée (27). Au vu du chevauchement de signes cliniques d'une réaction médica-

Tableau 1:

Études sur l'association entre les résultats cliniques de patients atteints de CBNPC traités par IPC et l'éosinophilie sanguine. Ce tableau illustre l'hétérogénéité des objectifs des études et de l'évaluation de l'éosinophilie: variable continue/catégorique; moment d'évaluation; biomarqueur utilisé seul (simple) ou en combinaison avec d'autres facteurs (composite).

Étude	N	Stade clinique	Molécule	Eosinophiles	Paramètres étudiés	Effets	Valeur p
Tanizaki 2017	134	IIIB-IV	nivolumab	AEC t0; catégorique; biomarqueur simple et composite	SG SSP	HR = 0,24 (IC95% 0,09-0,62) HR = 0,53 (IC95% 0,31-0,91) si AECt0 ⩾ 0,15 cells/mL	0,003 0,02
Chu X	300	IIIB-IV	PD-1i ± CT ± AAG	AEC t0; catégorique; simple	TRO SSP	40,9 % vs 28.8 % méd. = 8,93 vs 5,87mo HR = 0,744 (IC95% 0,56–0,99) si AECt0 ⩾ 0,15cells/mL	0,029 0,038
Sibille 2021	191	IIIA-IV	pembrolizumab nivolumab atezolizumab durvalumab	AEC et REC t1; continue	TRO	OR = 0,53 (IC95% 0,32-0,88) si RECt1 > 5.3%	0,014
Okauchi 2021	190	IIIA-IV	nivolumab pembrolizumab atezolizumab ± CT	AEC et REC t0 et q2-3 sem.; RECmax.*; catégorique	TET	OR = 0,39 (IC95% 0,26-0,60) si RECmax. > 5%	< 0,001

IPC: inhibiteur de point de contrôle immunitaire; CBNPC: cancer bronchique non à petites cellules; PD-1i: inhibiteur du *Programmed death*-1; AAG: anti-angiogénique; CT: chimiothérapie (doublet à base de platine); AEC: *absolute eosinophil count*, taux absolu d'éosinophiles; REC: *relative eosinophil count*, taux relatif (pourcentage) d'éosinophiles; catégorique: variable étudiée en valeur catégorisée; continue: variable étudiée en valeur continue; t0: valeur avant traitement par IPC; t1: valeur d'éosinophiles à la première évaluation sous IPC (2-3 mois); q2-3 sem: toutes les 2-3 semaines; *REC max.: valeur maximale d'éosinophiles relatifs relevée sous IPC; SG: survie globale; SSP: survie sans progression; TRO: taux de réponse objective; TET: temps jusqu'à l'échec du traitement, soit le temps sous IPC; HR: *hazard ratio*; OR: *odds ratio*.

menteuse, comme l'éruption cutanée, avec ceux des effets secondaires immuno-induits, l'incidence de l'Eos-S d'origine médicamenteuse pourrait bien être sous-estimée.

Données cliniques-éosinophilie tissulaire (Eos-T):

Peu de données existent sur l'Eos-T pour le CBNPC. Pour le stade précoce, deux études décrivent l'Eos-T et sa potentielle valeur. Ye et al. ont étudié l'expression de l'EPX, une protéine cationique des Eos, sur 30 pièces de résection d'adénocarcinome pulmonaire et sur le tissu normal adjacent (28). Le tissu cancéreux présentait une plus forte expression d'EPX en comparaison au tissu normal ($p < 0,05$). Cette expression était corrélée à un stade pathologique *Tumor Node Metastases* (pTNM) plus élevé ($p = 0,017$) et à une atteinte ganglionnaire ($p = 0,027$). L'analyse multivariée a montré un risque relatif de décès de 3,1 ($p = 0,018$) dans le groupe EPX forte expression. Tataroglu et al. ont quant à eux publié une étude sur la présence de mastocytes, de macrophages et d'Eos, leur association avec la vascularisation tumorale et avec le stade TNM de cas de CBNPC (29). Aucune association significative n'a été notée entre les Eos et le stade ou la vascularisation tumoral(e). Les Eos étaient toutefois évalués par microscopie classique après coloration à l'hématoxyline-éosine. Or, les limites de cette technique d'évaluation des Eos sont bien connues (10). Au-delà de cela, l'Eos-T pourrait aussi varier en fonction du microenvironnement tumoral.

Perspectives et conclusion

Globalement, les données reprises ci-dessus suggèrent fortement (contexte clinique) ou prouvent (contexte préclinique) l'association entre éosinophilie et activation immunitaire, tel qu'illustré par l'efficacité supérieure chez les patients cancéreux (pulmonaires) traités par IPC et par la survenue d'effets secondaires immuno-induits. Voorwerk et al. ont réalisé un travail translationnel fondateur en relation avec le rôle central et spécifique des Eos dans des modèles murins de cancers traités par IPC (22). Dans le CBNPC avancé, le cut-off de 5% de REC en début de traitement par IPC est suggéré comme indicatif d'un bénéfice clinique (20, 21). Bien qu'à ce jour non substituable à une évaluation clinique et radiologique, cette observation peut guider les cliniciens dans leur suivi de patients souffrant de CBNPC et traités par IPC.

Les études discutées ici présentent plusieurs limitations: complétude des données récoltées (notamment concernant les facteurs confondants-expression tumorale du PD-L1- ou influençant l'Eos-S-corticoïdes, atopie, maladies respiratoires obstructives), inhomogénéité des moments et des critères d'évaluation de l'Eos-S (variables catégoriques vs continues), variabilité des Eos sanguins, déjà illustrée dans le contexte des maladies bronchiques obstructives (30). Ces limitations offrent par ailleurs des perspectives de recherche clinique et translationnelle attrayantes: cohortes prospectives, étude de l'éosinophilie sur des matériels biologiques alternatifs, corrélation entre Eos-S et Eos-T, étude de la fonction des Eos, notamment selon le stade tumoral. Gageons que les recherches en cours et futures pourront s'adresser à plusieurs de ces questions.

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LETTER TO THE EDITOR

A prospective, multicenter, noninterventional study of decision factors in the first-line treatment of metastatic non–small cell lung cancer

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The treatment of metastatic non–small cell lung cancer (NSCLC) has evolved rapidly in recent years. For patients with nonsquamous cell carcinoma with oncogene addiction, targeted therapies are the preferred treatment, whereas immunotherapy (IT) has revolutionized treatment options for those without oncogene addiction and those with squamous cell carcinoma. IT treatment options, with or without chemotherapy (CT) are based primarily on expression levels of programmed death ligand 1 (PD-L1) [1]. These rapidly evolving treatment options and the molecular pathology testing required for optimal patient selection can be difficult to implement into daily practice, with technical (i.e., type of diagnostic test, expertise of the pathologist interpreting the results, and turnaround time) and reimbursement issues (i.e., treatment reimbursed without diagnostic test reimbursement) compromising PD-L1 testing. Real-world prescription data can serve as a tool for identifying such barriers to the implementation of optimal treatment. We therefore conducted a cross-sectional study to investigate the relationship between patient, tumor and treatment site characteristics, and systemic treatment choices for patients with untreated, stage IV NSCLC in the public health care system in Belgium with the aim of establishing a better understanding of the characteristics that impact real-life treatment decisions (NCT03959137; VEAP7678).

Consecutive patients with untreated stage IV NSCLC scheduled to receive systemic treatment or best supportive care (BSC) from June 2019 through October 2019 were included. Participants were aged ≥18 years with a histologically or cytologically confirmed diagnosis of stage IV NSCLC. The prospective collection of data started after a maximum of one cycle of treatment, except for patients receiving BSC only. Participants who had previously received systemic treatment for metastatic NSCLC were excluded; however, patients who had received earlier adjuvant or neoadjuvant therapy were eligible. Patients who had received a tyrosine kinase inhibitor, participated in a clinical trial, or received a novel therapy in a medical need program (i.e., patients who received systemic drugs free of charge outside of their usual prescription, based on reimbursement criteria) were also excluded.

A questionnaire was completed by the respiratory oncologist at each participating site regarding treatment site characteristics. Based on the average number of new NSCLC cases per year and on participation in clinical trials, sites were allocated into four categories of: high diagnostic volume (HDV; i.e., more than the median number of patients per year in that hospital) and participating/not participating in clinical trials; low diagnostic volume (LDV, less than the median number of patients) and participating/not participating in clinical trials. Additional site characteristics included

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capability to perform genetic and PD-L1 testing (for both in-house and referral). Treatment and patient characteristics were recorded in an electronic case report form, which included the category of the selected systemic treatment: CT, IT, IT-CT, or BSC. If indicated, palliative radiotherapy was given per standard clinical practice. Variables that positively or negatively impacted the choice of systemic treatment were documented, and physicians were also asked to identify the three most important variables that influenced their selection of treatment. Patient characteristics included in this assessment were demographics, medical history, comorbidities, presence of autoimmune disease, current or recent medications, and prior cancer treatment in earlier stage NSCLC (Supplementary Materials).

For this descriptive study, it was assumed that 200 patients would provide a representative picture of first-line systemic treatment decisions for stage IV NSCLC in Belgium. At most, 20 patients were permitted to be enrolled at a single site and at least 30 patients were required within each hospital category. Because of the issue of quasi-complete separation driven by the factor PD-L1 tumor proportion score (TPS), it was decided to perform subgroup analyses based on the PD-L1 score. Since for patients with high PD-L1 TPS $\geq$50%, the main interest was in the comparison of IT alone with IT-CT, the outcome variable was dichotomized. In both subgroups, simple logistic regression was used to initially identify important variables ($p < .25$) which were then explored through multiple logistic regression. Covariates considered for the simple models were age, sex, weight loss, smoking status, patient treatment preference, metastatic disease status, tumor diameter size (T-size), number of metastatic sites, brain metastases, liver metastases, concomitant malignancies, histology, comorbidities, autoimmune disease, use of corticosteroids/immunosuppressants, antibiotics, prior cancer treatment for local disease, and site type.

Across the 21 participating sites, the median number of newly diagnosed patients during 2018 was 143.7 (standard deviation, 68.5). Based on this median, 10 sites were classified as HDV centers (enrolling 116 patients) and 11 as LDV centers (enrolling 93 patients). Fifteen of the 21 (71.4%) sites were participating in clinical trials. Within the sites that were participating in clinical trials, genetic testing was not performed for 20.7% of patients enrolled at LDV sites compared with 10.6% of those at HDV sites.

A total of 209 patients were included (Supplementary Figure 1). The mean age was 68.2 years; 95.7% of patients were current or former smokers, 65.1% were male, 77% had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, and 65.6% had nonsquamous histology (Supplementary Table 1). In the total population, 33% of patients had PD-L1 TPS <1% and 42.1% had PD-L1 TPS $\geq$50%. Patient characteristics were also generally similar regardless of diagnostic volume and clinical trial participation; however, the proportion of patients with PD-L1 TPS $\geq$50% was higher at HDV sites participating in clinical trials compared with LDV sites or those not participating in clinical trials (51.1% vs. 22.7–37.9%). This may be due to the possibility that patients with lower PD-L1 expression at these centers may have been participating in clinical trials and thus not included in the present study. This logically resulted in the inclusion of a higher proportion of patients with high PD-L1 TPS who were more likely to receive IT alone. LDV sites not participating in clinical trials did not report nonsmokers and had more squamous cell histology (42.9% vs. 18.2–34.5%). The proportion of patients with comorbidities was roughly twice as high at LDV centers compared with HDV centers, although age and smoking habits did not differ significantly. This may be due to physical and/or socioeconomic constraints that prevented these patients attending HDV centers. Overall, there was a relatively low incidence of autoimmune diseases (6.2%) and an even lower incidence of active autoimmune diseases (3.3%).

The six characteristics with the highest rate of impact on treatment decisions were PD-L1 TPS, ECOG PS, metastatic disease status, squamous vs. nonsquamous histology, age, and patient preference (Figure 1). Characteristics with an 'important' impact rate of $\geq$10% for each systemic treatment choice are shown in Supplementary Table 2. For example, 94.1% of physicians indicated that PD-L1 TPS levels assumed a high rate of importance in their decision to prescribe IT. Similarly, PD-L1 TPS was assigned a high rate of importance by 76.8% of physicians prescribing IT-CT and 69.2% of those prescribing CT, whereas only 37.5% considered PD-L1 TPS to have a high rate of importance in the selection of BSC. Overall, PD-L1 expression was considered the most important factor in determining treatment, which seems logical, as it is the only objective factor in Belgium used to guide treatment reimbursement. Other important factors for >50% of physicians were ECOG PS for prescribing IT-CT (62.6%) or BSC (68.8%), the extent of metastatic disease when prescribing IT (54.4%), and patient preference when prescribing BSC (56.3%). Poor ECOG PS tended to guide physicians away from the use of IT-CT which may illustrate a fear of treatment-limiting toxicity in patients with poor ECOG PS and preference for treatment with the highest likelihood of success for fit patients. Poor ECOG PS also was an important factor in selecting BSC alone which again demonstrates the clinical selection of patients deemed fit or unfit for active treatment. Histology was an important factor for 42.4% of physicians in their consideration for IT-CT.

The most common treatment was IT-CT (47.4%), followed by IT alone (32.5%), CT (12.4%), and BSC (7.7%). The high proportion of patients who received IT, either alone or in combination with CT, reflects a high adherence to international treatment guidelines and a similar access to standard-of-care treatment options between hospitals, regardless of their diagnostic volume or participation in clinical trials. Choices of systemic treatment stratified by key patient characteristics are shown in Supplementary Table 3. Most patients with PD-L1 TPS <50% received IT-CT (73.9% in PD-L1 < 1%/73.3% in PD-L1 1–49%), whereas 76.1% of those with PD-L1 TPS $\geq$50% received IT alone. Almost half of all patients with ECOG PS 2 (47.1%) received IT, whereas 63.6% of those with ECOG PS 3/4 received BSC. Never-smokers were rare, but IT was selected for only one of eight (12.5%), compared with 32.5% for the total study population.

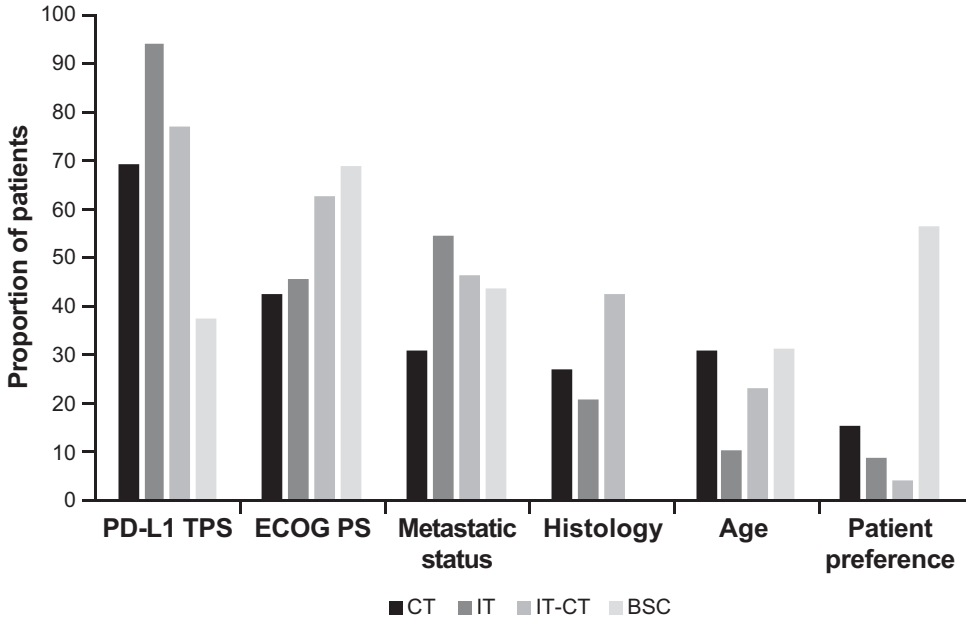

Figure 1. Factors with major impact on treatment choice. BSC: best supportive care; CT: chemotherapy; IT: immunotherapy; IT-CT: immunotherapy + chemotherapy; PD-L1: programmed death ligand 1; ECOG PS: Eastern Cooperative Oncology Group performance status; TPS: tumor proportion score.

Logistic regression was conducted to examine treatment choices in patients with PD-L1 $\geq$ 50% and PD-L1 $<$ 50%. Eight patients were not included because their treatment categories were underrepresented (see Supplementary Materials). Within the group of patients with PD-L1 TPS $<$50%, simple logistic regression identified age, weight loss ($\leq$5% vs. $>$5%), smoking status, ECOG PS, patient preference, and potentially antibiotics as significantly associated with treatment choice which were further assessed using multiple logistic regression (Figure 2). Age (p = .0308) and ECOG PS (p = .0005) had a significant impact on the treatment selection, whereas patient preference was borderline significant (p = .0585). The probability of receiving CT or BSC versus IT-CT increased with age (odds ratio [OR] 1.090; 95% CI 1.005–1.182 and OR 1.148; 95% CI 1.004–1.313). The model also indicated a lower probability of receiving CT or BSC compared with IT-CT in patients with lower ECOG PS (OR 0.084; 95% CI 0.016–0.450 and OR 0.008; 95% CI $<$0.001–0.113). Finally, the probability of receiving BSC was lower if patient preference was not BSC or unknown compared with patients who expressed a preference for BSC (OR 0.010; 95% CI $<$0.001–0.354 and OR 0.029; 95% CI 0.002–0.533).

Within the group of patients with PD-L1 TPS $\geq$50%, age, weight loss, tumor size diameter, comorbidities, and prior cancer treatment were potentially associated with treatment choice; however, there were no variables that significantly impacted treatment selection (age, weight loss, and prior cancer therapy had a borderline significant impact). Ultimately, given the limited sample size in this study, it remains difficult to definitively state which of the factors that were significant according to the univariate analysis are clearly decisive for each PD-L1 category.

This observational study provided detailed information about patient characteristics and factors impacting treatment decision in patients with treatment-naive stage IV NSCLC from Belgium. The strengths of this study are: (1) the national coverage providing a realistic picture of daily oncologic care in Belgium, (2) the well-balanced enrollment between HDV and LDV centers, (3) the data collection period that encompasses the recent changes in treatment guidelines with the availability of IT, and (4) the small proportion of missing data. The limitations of this study include (1) the absence of some treatment options (i.e., IT-CT + bevacizumab and dual IT) because of local reimbursement policies and the exclusion of patients receiving treatment through a medical need program; (2) the limited sample size, which did not permit the optimal representation of specific populations (e.g., patients with autoimmune diseases).

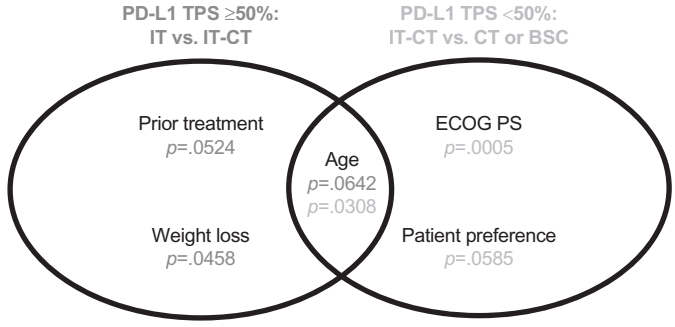

Figure 2. Multiple regression results for patients with PD-L1 TPS $\geq$50% and $<$50%. BSC: best supportive care; CT: chemotherapy; ECOG PS: Eastern Cooperative Oncology Group performance status; IT: immunotherapy; IT-CT: immunotherapy + chemotherapy; PD-L1 TPS: programmed death ligand 1 tumor proportion score.

In conclusion, our study confirms the adherence of Belgian thoracic oncologists to current guidelines with the large-scale implementation of PD-L1 testing and IT as the first-line treatment for advanced, non–oncogenic driven NSCLC. PD-L1 expression level and ECOG PS were shown to be major determinants in the choice of treatment. Finally, physicians use additional selection criteria, such as age, comorbidities, weight loss, and extent of metastatic disease, when selecting the best treatment options for their patients.

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Data availability statement

Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA (MSD) is committed to providing qualified scientific researchers access to anonymized data and clinical study reports from the company's clinical trials for the purpose of conducting legitimate scientific research. MSD is also obligated to protect the rights and privacy of trial participants and, as such, has a procedure in place for evaluating and fulfilling requests for sharing company clinical trial data with qualified external scientific researchers. The MSD data sharing website (available at: http://engagezone.msd.com/ds_documentation.php) outlines the process and requirements for submitting a data request. Applications will be promptly assessed for completeness and policy compliance. Feasible requests will be reviewed by a committee of MSD subject matter experts to assess the scientific validity of the request and the qualifications of the requestors. In line with data privacy legislation, submitters of approved requests must enter into a standard data-sharing agreement with MSD before data access is granted. Data will be made available for request after product approval in the US and EU or after product development is discontinued. There are circumstances that may prevent MSD from sharing requested data, including country or region-specific regulations. If the request is declined, it will be communicated to the investigator. Access to genetic or exploratory biomarker data requires a detailed, hypothesis-driven statistical analysis plan that is collaboratively developed by the requestor and MSD subject matter experts; after approval of the statistical analysis plan and execution of a data-sharing agreement, MSD will either perform the proposed analyses and share the results with the requestor or will construct biomarker covariates and add them to a file with clinical data that is uploaded to an analysis portal so that the requestor can perform the proposed analyses.

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Traitements ciblés et cancer bronchique non à petites cellules : le point en 2021

Sibille A (1), Corhay JL (1), Vaillant F (1), Paulus A (1), Louis R (1), Duysinx B (1)

Résumé : La majorité des cancers pulmonaires non à petites cellules se présentent à un stade avancé. Une faible proportion des adénocarcinomes, sous-type histologique le plus fréquent, est porteur d'(une) anomalie(s) génétique (s) et moléculaire(s) dont dépend leur survie. Depuis une quinzaine d'années, les anomalies de l'«Epidermal Growth Factor Receptor» (EGFR), «Anaplastic Lymphoma Kinase» (ALK) et ROS1 sont connues et ciblées par des inhibiteurs efficaces. De nouvelles générations permettent actuellement d'augmenter leur efficacité thérapeutique pour une toxicité globalement moindre. De nouvelles anomalies («Kirsten Rat Sarcoma», «Rearranged during Transfection», MET, «NeuroTrophic Receptor tyrosine kinase») sont, elles aussi, à présent ciblées de manière efficace. La recherche des anomalies moléculaires des adénocarcinomes est devenue incontournable car elle modifie fondamentalement la prise en charge thérapeutique et le pronostic d'une proportion grandissante de patients.

Mots-clés : ***Addiction oncogénique - Cancer bronchique non à petites cellules - Inhibiteur de tyrosine kinase - Mutation - Traitement - Traitement ciblé***

Targeted therapies for non-small cell lung cancer : state of the art in 2021

Summary : The majority of non-small cell lung cancers are diagnosed as advanced disease. A subset of the adenocarcinoma subtype presents a molecular aberration leading to tumour survival. Epidermal Growth Factor Receptor (EGFR), Anaplastic Lymphoma Kinase (ALK) and ROS1 have been identified and targeted with good efficacy for fifteen years. Newer inhibitors brought even greater efficacy with a generally better tolerability. Other molecular aberrations (Kirsten Rat Sarcoma, Rearranged during Transfection, MET, NeuroTrophic Receptor yrosine kinase) are targets for newly developed, more selective drugs. As more and more patients will benefit from targeted therapies, the identification of molecular aberration is more than ever crucial for optimal lung cancer patient care.

Keywords : ***Mutation - Non-small cell lung cancer - Oncogenic driver - Treatment - Targeted therapy - Tyrosine kinase inhibitors***

Introduction

En 2018, la Belgique comptait 8815 nouveaux cas de cancers pulmonaires dont une majorité (± 85 %) de cancers dits «non à petites cellules» (CPNPC), avec, pour plus de la moitié, des adénocarcinomes (1). La nature agressive de ces tumeurs et leur présentation généralement à un stade (localement) avancé (70 à 80 %) (1) expliquent leur haute mortalité. L'optique thérapeutique est donc souvent palliative, avec une intention double : allonger la survie globale (SG) du patient tout en maintenant une qualité de vie la meilleure possible (2). La thérapeutique est déterminée par le stade d'extension, par le sous-type histologique de la tumeur, par la présence d'anomalie(s) biomoléculaire(s) et par le choix du patient (3). Par tumeur mutée, on entend un processus néoplasique qui dépend d'une voie d'activation découlant d'une anomalie biomoléculaire spécifique : mutation, gène de fusion… (4). Ces anomalies concernent actuellement quasi exclusivement des adénocarcinomes et, le plus souvent, des patients non ou légers fumeurs (4). Une vaste étude française a démontré l'absence d'aberration moléculaire dans 15 % des adénocarcinomes, la présence d'une mutation de signification indéterminée dans 32 %, une mutation KRAS («Kirsten RAt Sarcoma») dans 32 %, une mutation EGFR («Epidermal Growth Factor Receptor») dans 11 %, un réarrangement ALK («Anaplastic Lymphoma Kinase») dans 5 % et une mutation BRAF dans 2 % (5). A titre comparatif, les mutations EGFR étaient moins fréquentes (7 %) pour les tumeurs interrogées au CHU de Liège en 2019, alors que les chiffres de la BRAF, par exemple, étaient plus élevés (5 %) (Figure 1). L'utilisation systématique de techniques de séquençage d'ADN telles que le NGS («Next Generation Sequencing») permet de cartographier ces différents sous-types de tumeurs, pour lesquels des thérapeutiques spécifiques peuvent être proposées, tant au diagnostic primaire qu'à la progression. La mise en évidence de ces anomalies est d'autant plus bénéfique qu'elle survient tôt dans la prise en charge, permettant de proposer un traitement ciblé dont la tolérance et l'efficacité (survie sans progression (SSP) et SG) sont supérieures à celle de traitements généraux tels que la chimiothérapie (5).

Cet article présente les nouvelles générations de traitements ciblés pour des addictions déjà connues ainsi que les dernières cibles thérapeutiques.

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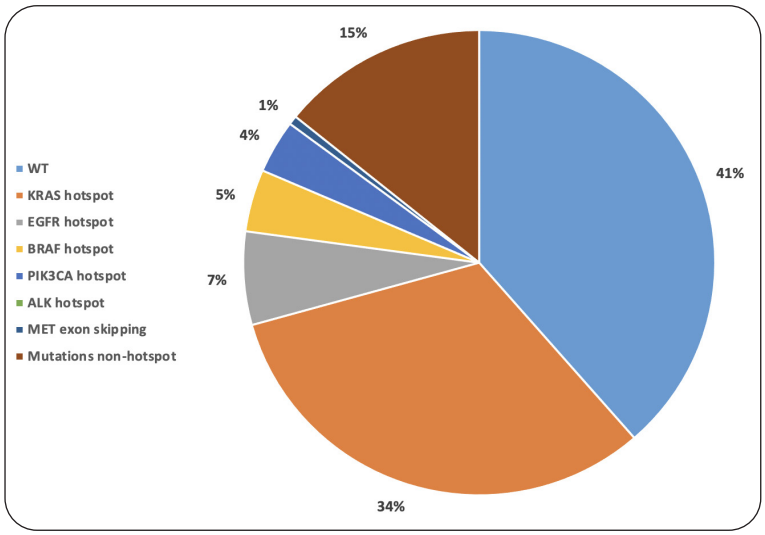

Figure 1. Mutations d'adénocarcinomes pulmonaires-CHU de Liège 2019.

WT : wild type (aucune mutation identifiée); KRAS (Kirsten Rat Sarcoma); MET exon skipping : MET exon 14 skipping; mutations non-hotspot : mutations identifiées, de signification clinique indéterminée; ALK : anaplastic lymphoma kinase; aucune mutation identifiée. Mutation hospot : telle que définie sur le site oncokb https://www.oncokb.org/gene

Les traitements remboursés début 2021

Pour chaque anomalie moléculaire est renseignée la proportion des adénocarcinomes avec mutation, d'abord au niveau mondial puis au niveau du CHU de Liège.

Mutations de l'EGFR : Asie 50-60 %; Europe-USA : 10-15 %; CHU Liège 7 %

Les mutations de l'EGFR concernent les exons 18 à 21 qui codent pour une partie du domaine EGFR, une protéine transmembranaire dont l'activation au niveau des tyrosines kinases entraîne une prolifération et une survie cellulaires (6). On distingue des mutations activatrices communes (80-90 %), sensibles aux inhibiteurs de tyrosine kinase (ITK) de l'EGFR (7-9), et rares (10-20 %), de moindre sensibilité aux ITK de première et de seconde générations (10). Actuellement, trois générations d'ITK sont disponibles (Tableau I). De la première à la dernière génération, on constate une légère amélioration du profil de toxicité, mais surtout de l'efficacité globale (taux de réponse objective (TRO), SSP, SG) et cérébrale. Sur base de ces données, les recommandations actuelles placent l'osimertinib en première ligne de traitement dans les tumeurs «EGFR mutées communes» de stade avancé (3). L'osimertinib est également indiqué en cas de résistance aux ITK de première ou seconde génération présentant une mutation T790M, sur base des résultats de l'étude AURA2 ayant démontré sa supériorité par rapport à la chimiothérapie (11).

L'approche des mutations rares de l'EGFR est moins claire, à cause de leur hétérogénéité et de leur faible prévalence. Les données sont *in vitro*, *in vivo* précliniques ou cliniques, principalement rétrospectives (12). Jusqu'il y a peu, le manque de connaissance concernant ces mutations rares empêchait la prescription de traitements ciblés. Aujourd'hui, les molécules proposées sont : l'afatinib (actif dans les mutations des exons 18 à 21), l'osimertinib (mutations des exons 19 à 21), le poziotinib (mutations de l'exon 20), le mobocertinib (mutations de l'exon 20) et l'amivantamab (mutations de l'exon 20) (12, 13). A noter qu'en Belgique seul l'afatinib bénéficie du remboursement dans le cadre du traitement de ces tumeurs rares; pour les autres molécules, l'inclusion dans des essais cliniques ou des demandes d'usage compassionnel est nécessaire. Outre ces ITK, la chimiothérapie, avec ou sans agents anti-angiogéniques, ou, plus rarement, les inhibiteurs de points de contrôles immunitaires peuvent être envisagés (13).

Gènes de fusion ALK : 3-5 %; CHU Liège 2 %

Depuis l'identification des réarrangements ou gènes de fusion incluant une activation de l'ALK en 2007 (14), plusieurs générations de traitements ciblés ont été développées. L'historique de leurs remboursements en Belgique est illustré en Figure 2. La première molécule à avoir démontré une supériorité par rapport à la chimiothérapie en termes de TRO, de SSP et de qualité de vie a été le crizotinib, un inhibiteur pluripotent ALK/ROS1/MET (15, 16). On notera qu'en Belgique, ce traitement n'est remboursé qu'en seconde ligne, après échec d'une chimiothérapie. Depuis le crizotinib, les secondes générations ont apporté un gain significatif en efficacité globale, mais surtout intracrânienne (Tableau II) (17-19). Parmi les

Tableau I. Inhibiteurs de tyrosine kinase (ITK) de l'«Epidermal Growth Factor Receptor» (EGFR).

Molécule	Génération	Comparateur	SSP	TRO	DR	SG	Toxicité gr3+	Essai Phase III
Gefinitib Iressa®	1	CT	5,7 vs 5,8 (HR 0,74)*	43 *vs* 32,2**	NR	18,6 *vs* 17,3	28,7 *vs* 61	IPASS Mok T 2009
Erlotinib Tarceva®	1	CT	9,7 vs 5,2 (HR 0,54)	58 *vs* 15	NR	19,3 *vs* 19,5	NR	EURTAC Rosell R Lancet Oncol 2012
Afatinib Giotrif®	2	CT	11,1 vs 6,9 (HR 0,58)	56 *vs* 23	11,1 *vs* 5,5	16,6 *vs* 14,2#	49 *vs* 48	LUX-Lung3 Seqvist L JCO 2013
Dacomitinib Vizimpro®	2	1G ITK	14,7 vs 9,2 (HR 0,59)	75 *vs* 72	14,8 *vs* 8,3	NR	63 *vs* 41	ARCHER 1050 Wu YL Lancet Oncol 2017
Osimertinib Tagrisso®	3	1G ITK	18,9 vs 10,2 (HR 0,46)	80 *vs* 76	17,2 *vs* 8,5	38,6 *vs* 31,8##	34 *vs* 45	FLAURA Soria JC NEJM 2018

CT : chimiothérapie; 1G ITK : première génération d'inhibiteur de tyrosine kinase; TRO : taux de réponse objective (%); SSP: survie sans progression (médiane; mois); SG : survie globale (médiane; mois); NR : non rapportée; HR : hazard ratio; * : HR progression/décès pour EGFR mutés 0,48 sous gefitinib; ** : TRO 71,2 *vs* 43,7 mois pour EGFR mutés sous gefitinib *vs* CT; # : p=.60; ## : p=.046. IPASS study : population non sélectionnée sur base de la mutation EGFR, étude de non-infériorité du gefinitib par rapport à la chimiothérapie.

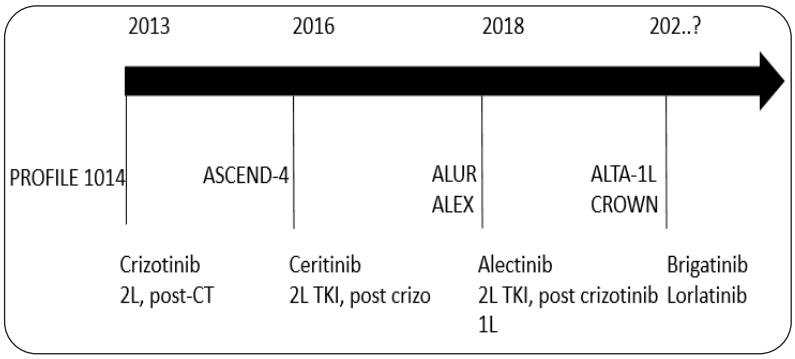

Figure 2. Développement des inhibiteurs ALK.

Ligne du temps représentant le développement des inhibiteurs ALK. PROFIL 1014, ASCEND-4, ALUR, ALEX, ALTAL-1L, CROWN : essais cliniques de phase III ayant mené au remboursement des différentes molécules. 2L : seconde ligne de traitement; CT : chimiothérapie; TKI : tyrosine kinase inhibiteur; 1L : première ligne de traitement. Source : www.inami.fgov.be/fr/programmes-web

Tableau II. Inhibiteurs de «Anaplastic Lymphoma Kinase» (ALK).

Molécule	Génération	Comparateur	SSP	TRO	DR	RIC	SG	Toxicité gr3+	Essai Phase III
Crizotinib Xalkori®	1	CT	10,9 *vs* 7,0 (HR 0,45)	74,0 *vs* 45,0	11,3 *vs* 5,3	NR	NA *vs* NA	NR	PROFILE 1014 Solomon BJ 2014
Ceritinib Zykadia®	2	CT	16,6 *vs* 8,1 (HR 0,55)	72,5 *vs* 26,7	23,9 *vs* 11,1	72,7 *vs* 27,3	immature (HR 0,73)	65 *vs* 40	ASCEND-4 Soria JC 2017
Alectinib Alecensa®	2	crizotinib	34,8 *vs* 10,9 (HR 0,43)	82,9 *vs* 75,5	33,1 *vs* 11,1	81 *vs* 50	immature (HR 0,76)	41 *vs* 50	ALEX Peters S 2017; Camidge RD 2019
Brigatinib Alunbrig®	2	crizotinib	24,0 *vs* 11,0 (HR 0,49)	74 *vs* 62	NA *vs* 13,8	14 *vs* 6	NR*	73 *vs* 61	ALTA-1L Camidge RD 2020
Lorlatinib Lorviqua®	3	crizotinib	12 moPFS 78 *vs* 39**	76 *vs* 58	NR	82 *vs* 23	NR	72 *vs* 56	CROWN Solomon BJ 2020

CT : chimiothérapie; SSP : survie sans progression: médiane (mois); TRO: taux de réponse objective (%); DR : durée de la réponse (mois); RIC : réponse intra-crânienne (%); SG : survie globale: médiane (mois). HR : hazard ratio. NA : non atteinte. NR : non rapportée. * : SG médiane non rapportée, survie à deux ans de 76 *vs* 74 %. ** : SSP exprimée uniquement à 12 mois, analyse intérimaire.

inhibiteurs de seconde génération, le profil de tolérance du ceritinib et du brigatinib est moins bon que celle de l'alectinib. Le lorlatinib, dernier inhibiteur ALK, présente une activité similaire à celle de l'alectinib, avec une tolérance globale comparable, mais un profil de toxicité différent (20). La toxicité spécifique à chaque molécule et le manque d'adhérence au traitement qu'elle peut entraîner chez des patients que l'on traite généralement longtemps doivent aussi guider le choix du traitement. A noter qu'aucune des études publiées jusqu'ici n'a pu démontrer un bénéfice en termes de SG du crizotinib par rapport à la chimiothérapie (imputé à un crosso-

ver du groupe contrôle vers le crizotinib). C'est également le cas si l'on compare les inhibiteurs de seconde versus première génération (possiblement suite à des survies très longues et à un suivi encore trop court).

Gènes de fusion ROS1 : 1-2 %; CHU Liège 0,5 %

Les gènes de fusion impliquant ROS1 présentent une forte similarité biologique avec les réarrangements ALK (21), permettant une utilisation efficace des inhibiteurs ALK tels que le crizotinib et une approche de recherche similaire. Bien que (très) rare, cette anomalie génétique tumorale est facile à rechercher (immunohistochimie) et à cibler d'un point de vue thérapeutique. La première ligne de traitement recommandée reste le crizotinib, apportant un TRO entre 69 et 80 %, une SSP médiane entre 10 et 13 mois et une efficacité cérébrale nette et supérieure à celle de la même molécule dans les réarrangements ALK (3, 22). Outre le crizotinib, une chimiothérapie à base de pémétrexed est également efficace dans ce sous-type tumoral (23).

Les traitements non remboursés début 2021

En l'absence de remboursement actuel, les patients sont soit pris en charge de manière non ciblée, soit de manière ciblée si la firme consent à un usage compassionnel.

Mutation BRAF dont la V600E : BRAF 1-2 % (CHU Liège 3 %; 26 % de V600E)

Ces mutations, dont la fréquence au CHU semble légèrement supérieure à celle de la littérature (Figure 1), se rencontrent typiquement chez des (ex-)fumeurs et sont exclusives d'autres mutations. La combinaison dabrafénib (inhibiteur BRAF) et tramétinib (inhibiteur MEK) a démontré sa supériorité par rapport à la chimiothérapie pour les mutations V600E, en première ou en seconde ligne de traitement (TRO ± 65 %, SSP médiane ± 11 mois, durée de réponse ± 10 mois) (24, 25). Cette combinaison thérapeutique a été approuvée par l'agence européenne des médicaments; on en attend encore le remboursement en Belgique.

Mutation KRAS dont la p.G12C : 25-30 % (CHU Liège 28 %; 44,6 % de G12C)

Les mutations de l'oncogène KRAS, dont la p.G12C, sont fréquemment associées à d'autres mutations (26). Cette hétérogénéité mutationnelle et l'absence de développement d'inhibiteurs directs de KRAS ont longtemps gardé cette mutation loin des cibles thérapeutiques. Récemment, le sotorasib a démontré une toxicité basse (11,6 % de toxicité sévère), un TRO de 32,2 % et un taux de contrôle de la maladie (réponse objective et maladie stable) de 88,1 % chez des patients lourdement prétraités (27). L'essai de phase III est toujours en cours, notamment au CHU de Liège, pour définir sa place dans l'arsenal thérapeutique.

MET exon 14 skipping et amplification MET : CHU Liège 1 %

Les altérations de l'oncogène MET sont de plusieurs types : une amplification, une surexpression, des mutations et des réarrangements (28). Des thérapeutiques ciblées ont été essayées sur le plan clinique pour l'amplification MET et un type de mutation spécifique, appelé METexon14 skipping. Le crizotinib, un inhibiteur pluripotent ALK/ROS1/MET, s'est avéré relativement peu efficace, avec un TRO de 30 à 40 % et une SSP peu modifiée (29, 30). Le tépotinib et le capmatinib ont démontré dans des essais cliniques de phase II une activité intéressante (TRO ≈ 50 %; SSP médiane ≈ 10 mois) pour les METexon14 skipping, y compris en cas de métastases cérébrales (capmatinib) (31, 32). Des résultats complémentaires sont attendus pour le tépotinib (amplification MET) et pour d'autres molécules (savolitinib avec un essai en cours NCT02897479, cabozantinib avec un essai en cours NCT 03911193).

Gène de fusion RET : 0,5-1 % (CHU Liège inconnu)

Le gène «REarranged during Transfection» (RET) code pour un récepteur transmembranaire de type tyrosine kinase, lequel est activé par sa fusion avec d'autres gènes, entraînant une cascade cellulaire activatrice (33). Alors que les premiers inhibiteurs «multikinases» (cabozantinib, lévatinib, vandétanib) se sont avérés peu efficaces et toxiques (34-36), le selpercatinib et le pralsétinib sont nettement supérieurs (TRO 58 à 68 %; durée de réponse non atteinte à 20,3 mois; SSP médiane 18,4 mois) et mieux supportés chez des patients le plus souvent pré-traités (37, 38). Les résultats des études de phase III sont attendus.

Gène de fusion NTRK : 0,5 % (CHU Liège inconnu)

Les gènes de fusion incluant le «neutrotrophic receptor tyrosine kinase rearrangement»

(NTRK) semblent exclusifs d'autres mutations (39) et sans corrélation clinico-pathologique claire. Le larotrectinib et l'entrectinib ont convaincu par leur efficacité dans les essais de phase I/II avec des TRO de 60 à 75 % et des résultats préliminaires de SSP et SG extrêmement encourageants (40-42).

Augmenter la survie sans progression

Les premières études cliniques démontrant l'efficacité de traitements ciblés (TRO, SSP, qualité de vie) ont également démontré leur limitation. En effet, les CPNPC à addiction oncogénique restent des maladies inguérissables. Face à ce constat d'échec partiel, plusieurs stratégies peuvent être envisagées afin d'augmenter la SSP et, idéalement, la SG. Le développement de nouvelles générations de traitements ciblés est un axe activement poursuivi, comme le démontre avec succès l'historique des ITK de l'EGFR et des inhibiteurs ALK. Alternativement, la combinaison de traitements ciblés de première génération avec d'autres agents (anti-angiogéniques, chimiothérapie) a été envisagée par plusieurs groupes (43-45). Enfin, certains auteurs défendent l'idée d'une séquence de traitement, tablant sur un cumul des bénéfices observés pour des molécules utilisées indépendamment les unes des autres, comme par exemple l'utilisation de l'osimertinib après progression avecun ITK de seconde génération (46).

La limite d'efficacité des traitements ciblés illustre le problème de la résistance secondaire. Pour les mutations les plus fréquentes (EGFR, ALK & ROS1), plusieurs de ces mécanismes ont déjà été bien documentés. On distingue des mécanismes «on-target» (directement liés à la cible; mutations ou amplification du gène concerné) et «off-target» (non liés à la cible; activation de mécanismes parallèles ou transformation histologique) (47). Il est recommandé pour les tumeurs EGFR mutées et suggéré pour les remaniements ALK ou ROS1 de rechercher ces mécanismes par le biais d'une nouvelle biopsie, solide ou liquide (3). Une imagerie complète permettra de distinguer une oligoprogression (possibilité d'un traitement local) d'une progression systémique (nécessitant un ajustement de thérapie globale) (3, 47). Toutefois, si la connaissance théorique du mécanisme de résistance permet parfois d'envisager un traitement ciblé de seconde ligne, l'efficacité de ce dernier peut s'avérer décevante (hétérogénéité de la tumeur et de ses clones de résistance, implication clinique variable de mécanismes de résistance identifiés). L'alternative est d'associer au traitement de première ligne un inhibiteur du mécanisme de résistance identifié ou d'abandonner le traitement ciblé pour un traitement systémique général (la chimiothérapie, l'immunothérapie n'étant en général que peu, voire pas, efficace dans cette population de patients généralement non-fumeurs) (48).

Conclusion

La recherche des maladies oncogéniques activables est devenue incontournable avec l'arrivée de nouveaux traitements spécifiques : soit pour des cibles jusqu'ici impossibles à activer (KRAS, BRAF, MET, RET), soit pour des cibles connues (EGFR, ALK, ROS1) avec de nouvelles générations, présentant l'avantage d'une efficacité globale augmentée et/ou d'une meilleure tolérance. Malgré ces traitements (très) performants, le clinicien devra faire face à la résistance tumorale. Rechercher ces mécanismes reste la meilleure garantie pour le patient d'entreprendre un traitement adapté à sa maladie, doté d'une efficacité et d'une tolérance optimales.

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CASE REPORT
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Granulomatosis With Polyangiitis in a Patient on Programmed Death-1 Inhibitor for Advanced Non-small-cell Lung Cancer

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Objectives: To contribute to a precise and thorough knowledge of immune-related adverse events (irAE) induced by immune checkpoint inhibitors (ICI) and to emphasize the importance of this specific form of toxicity in terms of potential predictive value and long-term effects.

Materials and Methods: We report the first case of granulomatosis with polyangiitis (GPA) in a patient treated with an anti-Programmed Death protein-1 (PD-1) antibody for advanced non-small-cell lung cancer (NSCLC).

Results: After a single dose of this drug the patient showed severe myositis associated with a high anti-PR3 anti-neutrophil cytoplasmic antibody titer. Discontinuation of the anti-PD-1 and introduction of corticoids led to a remission of the irAE. Regarding tumor a partial response was noted. A year later a neutrophilic, sterile pleural exudate and cutaneous lesions appeared with the pathological findings of neutrophilic vasculitis. Retreatment with corticoids induced a new remission of symptoms. It remains unclear whether GPA was preexisting and clinically silent but revealed by the use of ICI or primarily induced by this treatment. Conclusions: irAE are rare when anti-PD-1 antibodies are used in monotherapy. They present with a distinct clinical picture and temporal course and require specific treatment. Patients with irAE usually have a favorable oncological outcome.

Keywords: immune checkpoint inhibitor, non-small-cell lung cancer, granulomatosis with polyangiitis, immune-related adverse events, anti-PD-1 antibody

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INTRODUCTION

Immune-checkpoint inhibitors (ICI) have become widely used in advanced non-small-cell lung cancer (NSCLC). Classically, patients with preexisting autoimmune disorder (PAID) have been excluded from clinical trials using ICI. Real-life experience, however, shows that physicians sometimes do prescribe ICI to those patients (1, 2). Although mostly well-tolerated, ICI can cause severe and irreversible immune-related adverse events (irAE), affecting the quality of life and further lines of treatment. Timely recognition of irAE is paramount in order to control them.

We report the case of a patient with advanced NSCLC in whom treatment with pembrolizumab revealed a granulomatosis with polyangiitis (GPA). We then discuss the predictive value of irAE and safety of ICI in patients with PAID.

CASE PRESENTATION

A 64-years old male patient was diagnosed with stage IVB poorly differentiated NSCLC favoring adenocarcinoma of the right upper lobe with several bone lesions (cT4N2M1c). His medical history included a cerebrovascular accident and ischemic heart disease with subacute myocardial infarction in 2003. His chronic medication included acetylsalicylate acid 100 mg once daily (OD) and simvastatin 40 mg 0D, both since 2003. Regarding the tumor no driver mutation was identified by next-generation sequencing analysis. The Programmed Death Ligand-1 (PD-L1) expression level was assessed by immunohistochemistry using a monoclonal antibody to PD-L1 (clone 22C3, Dako) and a Benchmark Ultra (Roche) automated scope with subsequent evaluation by a certified pathologist, revealing 100% staining of a section including at least 100 evaluable tumor cells. Hence, pembrolizumab 200 mg every 3 weeks was started. Ten days after the first dose the patient was admitted to the hospital due to severe myalgia in both lower limbs with severe functional loss. Biochemistry showed creatine kinase (CK) of 1265 IU/L (upper limit of normal (ULN) = 190) and myoglobin of 2361 μg/L (ULN = 72) with normal renal function. Autoimmune serology showed a normal anti-nuclear factor (ANF) titer (1/80) without any characterization (especially for primary immune-mediated myositis with no anti-JO1, PL-7, PL-12, EJ, SRP, Mi-2, MDA-5, HMGCoA reductase) and anti-neutrophil cytoplasmic antibodies (ANCA) with a high titer of anti-PR3 (178 U/mL, ULN = 2); the infectious serology was negative. The statin was taken for several years prior to these symptoms and CK level before the start of the anti-PD-1 was normal. The electroneuromyography before corticoids showed proximal myopathy of moderate intensity without signs of necrosis. The quadriceps biopsy before corticotherapy was normal. He was treated with analgesics, intravenous fluids, and high-dose methylprednisolone (1 mg/kg/day) with favorable evolution. The diagnosis of immune-mediated myositis associated to granulomatosis with polyangiitis (GPA), former Wegener's disease, was established. The anti-PD-1 remained discontinued. Eight months after an initial partial response (PR) to pembrolizumab, progressive disease was noted and second-line doublet chemotherapy was started after antalgic irradiation of a metastatic pelvic mass. Subsequently, PR was noted. A year after the initial presentation of myositis the patient's condition worsened due to dyspnea and arthritis. Evaluation showed a new left-sided pleural effusion and a new lung consolidation. Based on a strong inflammatory syndrome (C-reactive protein (CRP) 116 mg/dL) and a neutrophilic exudate without evidence for empyema the patient was treated with amoxicilline-clavulanate for 14 days. In total, three pleural fluid cultures remained sterile. Due to persistence of the effusion and lack of clinical improvement a pleuroscopy was performed. The fluid appeared unclear and a few non-specific lesions were biopsied on the parietal pleura. They revealed a subacute pleuritis without tumor infiltration, granuloma or vasculitis. The arthritis was symmetrical and located in the wrists, metacarpophalangeal (MCP) joints and knees, without any evidence for infection or crystal-associated disease. A few days later, skin lesions appeared on the MCP and knees (**Figure 1**). Biopsy there showed a neutrophilic vasculitis, as can be seen in cutaneous forms of GPA (3) (**Figure 2**). The new lung consolidation was biopsied and showed only necrosis with no specific features of GPA-related lung involvement. Along with this clinical deterioration the autoimmune serology showed a rise in anti-PR3 titer (352.1 U/mL). The CRP dropped dramatically after initiation of corticoids (methylprednisolone at 1 mg/kg/day) along with clear clinical improvement. Recent clinical and radiological evaluation showed that the patient was in good overall condition with no signs of oncological progression despite discontinuation of the chemotherapy. We noted a progression-free survival (PFS) of 10 months after the second line chemotherapy and an overall survival (OS) of 18 months.

DISCUSSION

With this case we report a unique and rare but severe side effect of pembrolizumab with long-lasting consequences in a NSCLC

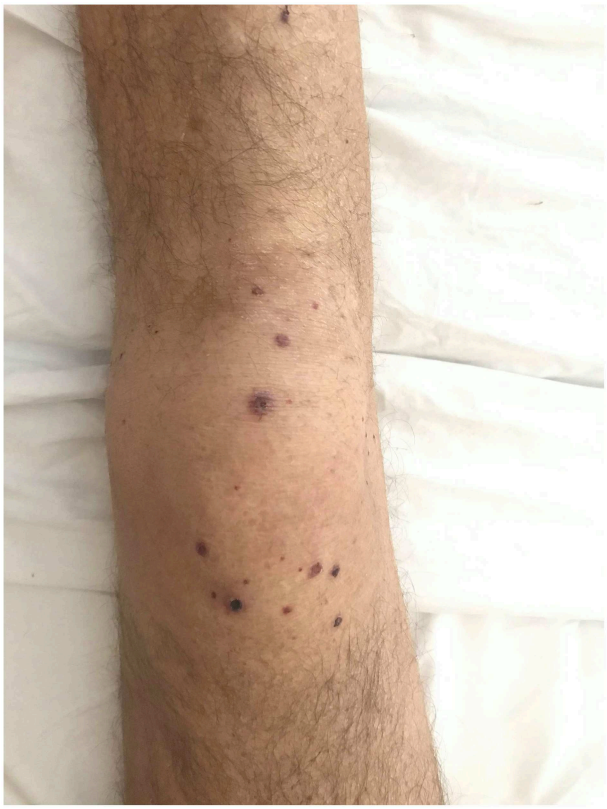

FIGURE 1 | Dark red, necrotic, slightly tender lesions developed symmetrically on MCP joints and knees.

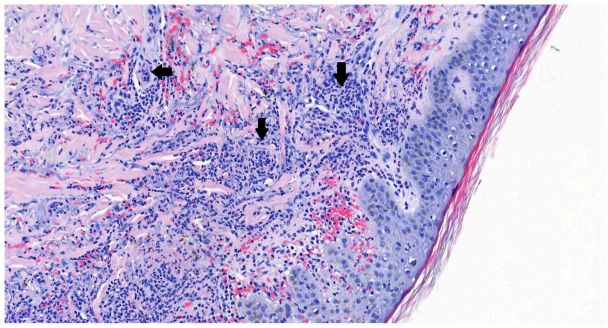

FIGURE 2 | Hematoxylin and eosin (HE) staining shows blood vessels (white areas) with surrounding neutrophilic inflammatory aggregates (arrows), establishing the diagnosis of neutrophilic vasculitis. Picture magnification: 20x; scale bar: 50 μ.

patient. It remains uncertain whether GPA was preexisting and clinically silent but revealed by the use of the anti-PD-1 or primarily induced by this treatment. Indeed, no autoimmune serology was available from before the start of treatment. Retrospectively, the patient mentioned mild myalgia with normal CK values prior to diagnosis and treatment, responding to corticoids. We therefore think they were possibly signs of a low active GPA.

Immune-related adverse events (irAE) are a kind of adverse events specific to immune checkpoint inhibitors (ICI). Virtually all organs can be affected depicting a wide variety of symptoms. They mostly appear early during treatment but can also appear long after drug discontinuation. Relapses are possible, also in the long term, as seen in our patient (4). Severe irAE occur at a low frequency (∼10%) for anti-PD-1/-PD-L1 used in monotherapy (5–9). The general consensus for their management is high-dose corticoids and interruption of the ICI. In the absence of response, adding immunomodulators (mycophenolate mofetyl, anti-TNFα) is recommended (4, 10).

Beside their impact on patients' quality of life and treatment plan, irAE are of specific interest for the clinician. They are considered potential predictors of response to treatment. In NSCLC, several studies showed superior PFS, response rates (RR) and/or OS in patients treated with anti-PD-1/anti-PD-L1 and showing irAE of various types and severity (11–13). The responses were independent of corticoids when needed. These series are, however, small-sized and retrospective. Our patient's tumor showed a PR according to the REsponse Criteria in Solid Tumors (RECIST) version 1.1 with a 50% reduction in the sum of target lesions and a PFS of 8 months after a single dose of pembrolizumab.

Mechanisms of irAE are not yet fully understood. In melanoma, it is argued that there exists a similarity between self-antigens and tumor neoantigens (14). Due to checkpoint inhibition, reactivated and expanding T-cells can interact with both tumor and healthy cells. Preexisting autoimmunity can also explain irAE: preexisting inactivated T-cells directed at self-antigens get reactivated by ICI, clinically translating into irAE (15). ICI might also impact B-cells, either directly via the PD-1 receptor or via the action of T-cells. These effects, however, are not yet fully understood (16).

New onset granulomatous diseases have been described in cancer patients treated with ICI (17, 18). These cases were involving the lungs and/or lymph nodes and pathologically identified as sarcoid-like reactions. To our knowledge, only one case of ICI-induced GPA has been reported in a melanoma patient first treated with ipilimumab, then with pembrolizumab (19). As CD4+ T-cells driven disease, it is understandable that sarcoidosis may be revealed by ICI that will increase the CD4+ T lymphocyte population although the precise mechanisms of this have not been ascertained so far. Wilde et al. suggested the role of the negative coregulatory factor PD-1 in GPA by demonstrating a high level of PD-1 expression in a series of 32 patients as compared to healthy controls and the lack of PD-1 on CD4+ lymphocytes in GPA lesions (20). The IL-17 pathway, known to induce inflammatory and autoimmune phenomena, is also suspected to play a role in GPA as levels of IL-17 producing T cells (Th17) were found to be increased, irrespective of disease activity (21).

Classically, patients known to have an autoimmune disorder have been excluded from clinical trials with ICI. Daily practice, however, shows that this restriction is not always followed. Small-sized studies of such (mainly melanoma) patients treated with an anti-PD-1 are available (1, 2). Reporting on mostly stable PAID, both studies show an increased risk of (new) autoimmune symptoms in ∼40% of patients, mainly with low-grade severity. The permanent discontinuation rate due to irAE was 8% (1) vs. 9% (2) which is similar to patients with no PAID (5–9). The type of PAID might also indicate a higher vs. lower risk of flare on anti-PD-1 treatment, as is the case for rheumatologic disorders, closely linked to the PD-1/PD-L1 axis (1). Danlos et al. found an earlier onset of irAE in patients with PAID compared to patients without PAID (2). Both studies found a similar efficacy of ICI in patients with and without PAID on anti-PD-1 in terms of OS and RR. As a consequence, both authors conclude that ICI treatment in patients with PAID is feasible, albeit requiring adequate follow-up and multidisciplinary management. Systematic autoimmune screening before the start of ICI might reinforce the awareness for irAE.

CONCLUSION

ICI are commonly used in the treatment of advanced NSCLC. They can, although rarely, induce severe irAE that can cause a major morbidity with potentially long-term effects and that can impact further treatment plan. We describe the first case of GPA in a patient treated with an anti-PD-1 antibody for advanced NSCLC. Although stable PAID does not seem an absolute contraindication to the use of ICI, close monitoring for side effects in these patients seems warranted. Patients presenting an irAE seem to have a favorable oncological outcome. The exact mechanisms of irAE remain unclear.

PATIENT INFORMED CONSENT

Written informed consent was obtained from the participant for the publication of this case report and any potentially identifying images/information.

AUTHOR CONTRIBUTIONS

AS reviewed the literature and wrote the paper. RA and MP followed the patient and collected clinical and biological data. OM analyzed the autoimmune serology data and contributed to the redaction of the manuscript. ND gave the pathology input of this case. RL and BD reviewed and contributed to the final version of the paper. All authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

PRISE EN CHARGE DU CANCER BRONCHIQUE NON À PETITES CELLULES

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Résumé : Déjà reconnu comme la première cause de mortalité chez l'homme, le cancer broncho-pulmonaire non à petites cellules (CBNPC) est également devenu l'une des premières causes de décès par cancer chez la femme. Sa prise en charge repose sur un bilan locorégional, médiastinal et extra-thoracique rigoureux permettant une stadification précise qui, non seulement, revêt une signification pronostique, mais conditionne également les options thérapeutiques. Cette revue de la littérature se propose de présenter la stratégie multidisciplinaire actuellement reconnue dans le traitement du CBNPC.
Mots-clés : *Oncologie pulmonaire - Cancer pulmonaire non à petites cellules - Chimiothérapie - Radiothérapie*

Management of non-small cell lung cancer
Summary : Already known as the first cause of mortality in men, non-small cell lung cancer (NSCLC) is nowadays a major cause of cancer-related death in women. Its approach relies on a thorough locoregional and extra-thoracic assessment allowing a precise staging which not only has prognostic value, but also determines the therapeutic options. This review presents the current multidisciplinary strategy agreement on the treatment of NSCLC.
Keywords : *Pulmonary oncology - Non-small cell lung cancer - Chemotherapy - Radiotherapy*

Introduction, incidence et épidémiologie

En 2008, le nombre de nouveaux cas de cancers bronchiques s'élevait, de par le monde, à 1.600.000 (1). En 2011, en Belgique, on a atteint 8.000 nouveaux cas (2). En termes de prévalence et de mortalité, le cancer pulmonaire apparaît en première place chez l'homme et en deuxième chez la femme (3). Il provoque, dans notre pays, quelque 6.000 décès par an. Le nombre de femmes atteintes est en constante augmentation, ce qui reflète la progression de la consommation tabagique dans le sexe féminin. Outre l'âge croissant de la population globale, la prévalence multifactorielle dans les pays en voie de développement explique l'augmentation soutenue de la prévalence globale. Le tabac reste le principal facteur causal, mais d'autres agents étiologiques ont été reconnus comme l'amiante, le nickel, le chrome, l'arsenic, le radon et la pollution intérieure et extérieure (1).

Anatomopathologie

On distingue deux grands types de cancer bronchique : les cancers bronchiques non à petites cellules (CBNPC, 80 à 85 %) et les cancers bronchiques à petites cellules (CBPC, 10 à 15 %).

Les CBNPC comprennent les carcinomes épidermoïdes, les adénocarcinomes, les carcinomes à grandes cellules et les carcinomes dits «NOS» (not otherwise specified). Ceux-ci regroupent les CBNPC ne pouvant être catégorisés, notamment en raison de leur faible différenciation. L'utilisation d'un éventail de tests immunohistochimiques et l'algorithme proposé par le consensus belge ont permis de réduire très nettement le nombre de ces cas (4). L'évolution de la composition des cigarettes (présence de filtre de goudron sur les cigarettes induisant une inhalation plus profonde dans le poumon, ainsi qu'une proportion plus importante de nitrosurée) fait de l'adénocarcinome l'histologie dominante. Son aspect peut être très hétérogène (acinaire, papillaire, lépidique, micropapillaire, solide, mucineux), souvent au sein d'une même tumeur. A noter que l'appellation «bronchiolo-alvéolaire» a été délaissée depuis 2010 pour une classification distinguant des lésions pré-invasives, minimales invasives, invasives ou des variantes, chacune déclinée selon le «pattern» histologique prédominant (5). En 2015, paraîtra une nouvelle classification anatomo-pathologique globale de l'OMS incluant des modifications pour les trois grands sous-types de CBNPC.

Par ailleurs, la recherche moléculaire a permis d'identifier différents profils mutationnels impliqués dans les voies de signalisation intracellulaire qui stimulent la prolifération, l'invasion, la métastatisation de la cellule cancéreuse, l'angiogenèse, ou qui inhibent l'apoptose. En particulier, la voie des tyrosines kinases, activées par le récepteur de l'Epithelial Growth Factor (EGFR), a justifié le recours aux inhibiteurs des tyrosines kinases (TKI), une des thé-

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rapies ciblées les plus utilisées. De nombreuses autres mutations ont été décrites et constituent l'objet de recherches cliniques et précliniques. Il est important de souligner que ces mutations ne sont présentes que dans une petite proportion des CBNPC et presque exclusivement dans les adénocarcinomes. La valeur pronostique de la mutation EGFR et la tolérance nettement meilleure des EGFR TKI justifient la recherche systématique du statut mutationnel EGFR.

Evaluation préthérapeutique

La stratégie thérapeutique dépend, d'une part, de la tumeur (histologie et bilan d'extension) et, d'autre part, du patient (performance status (PS), comorbidités et statut cardio-pulmonaire, préférences).

Le bilan préthérapeutique comportera une biologie générale (hémogramme, fonctions hépatique et rénale, les marqueurs tumoraux n'ayant pas de valeur diagnostique) et des épreuves fonctionnelles respiratoires (éventuellement complétées par une ergospirométrie) (6). L'évaluation histologique de la tumeur constitue évidemment un élément-clé du plan thérapeutique, tout comme le bilan d'extension. Celui-ci comportera une tomodensitométrie (CT) thoraco-abdominale avec contraste incluant les surrénales. En raison de sa meilleure sensibilité, une résonance magnétique nucléaire (IRM) cérébrale sera préférée au scanner avec contraste en cas de symptômes neurologiques ou en l'absence de lésion à distance. La tomographie à émission de positons (TEP) apporte principalement une plus-value dans l'évaluation loco-régionale (7).

L'exploration aussi exhaustive que possible du médiastin représente une étape cruciale qui requiert la plus grande minutie par l'intégration des données morphologiques (CT), métaboliques (TEP) et cyto-histologiques (ponction sous échographie endo-bronchique (EBUS), endo-oesophagienne (EUS) et médiatinoscopie). Un algorithme très récemment publié par l'European Society of Thoracic Surgeons (ESTS) indique la nécessité d'investigation cyto-histologique du médiastin en fonction de la tumeur et des caractéristiques morphologiques (petit axe) et métaboliques (hyperfixation en TEP) des adénopathies (8). La mise en évidence d'un épanchement pleural ou péricardique imposera une investigation (thoracocentèse, pleuroscopie) de manière à ne pas récuser un patient potentiellement curable à une chirurgie d'exérèse.

Ce bilan permet une stadification précise selon les suggestions de l'Union Internationale Contre le Cancer (9). Elle comporte trois volets : la tumeur primitive (T) avec sa taille et ses rapports aux structures adjacentes, l'atteinte ganglionnaire (N, pour «node») par rapport à la tumeur et les métastases à distances (M) (tableau I) (9). Cette stadification conditionne le choix du traitement, mais également le pronostic direct du patient. En effet, plus le stade est élevé, plus brève est la survie (9). Ainsi, la survie à 5 ans pour un stade IA est de l'ordre de 60-65 % tandis qu'elle tourne autour de 1 % pour un stade IV. La majorité des patients se présentant à un stade avancé, on comprend aisément le pronostic sombre du cancer du poumon.

Traitement des CBNPC de stade précoce (stades I et II)

Le sevrage tabagique reste le préliminaire à la prise en charge préventive et curative du CBNPC. La chirurgie d'exérèse constitue la pierre angulaire du traitement des CBNPC de stade précoce et potentiellement curables. Elle nécessite un statut fonctionnel suffisant (VEMS > 1.500 ml pour la lobectomie et > 2.000 ml pour la pneumectomie; DLCO > 80 %; VO2max > 15 ml/kg/min). Cette chirurgie inclut une évaluation minutieuse et aussi exhaustive que possible (curage) des aires ganglionnaires, tant hilaires que médiastinales. L'alternative pour les patients médicalement inopérables consiste aujourd'hui en la radiofréquence ou la radiothérapie stéréotaxique, en particulier pour les tumeurs inférieures à 3 cm (10).

De nombreux essais thérapeutiques ont été menés pour évaluer la place des traitements adjuvants * (tableau II) (11-15, 16) ou néo-adjuvants** (tableau III) (17, 18-21). Quatre essais ont montré un bénéfice significatif de la chimiothérapie (CT) adjuvante dans les stades II et IIIA d'emblée réséqués (gain de survie de + 4,1 à 15 % à 5 ans) (22). L'effet favorable de la CT post-opératoire a été démontré avec des doublets à base de platine (cycle de 3 semaines; dose totale > 300 mg/m^2). C'est avec la vinorelbine que ce concept a été le mieux illustré,

* Traitement adjuvant : traitement auxiliaire, appliqué en post-opératoire pour compléter le traitement chirurgical

** Traitement néoadjuvant : traitement auxiliaire administré avant l'intervention dans le but, notamment, de réduire la taille de la tumeur et de faciliter une chirurgie plus efficace.

mais d'autres choix sont possibles. Pour les stades IA complètement réséqués, il n'y a pas de bénéfice d'une CT adjuvante. L'intérêt de cette CT pour les stades IB est incertain, mais probable pour les tumeurs de plus de 4 cm (16). Pour les stades IIA N0, une CT adjuvante peut être proposée, surtout s'il existe un envahissement pleural, une angio- ou lympho-invasion, un index mitotique élevé ou une hyperfixation intense en TEP. Pour les stades IIA N1 et IIB, la CT adjuvante est recommandée. Quant à la radiothérapie (RT) adjuvante, elle est délétère sur la survie dans les stades précoces (23).

Plusieurs essais ont évalué l'intérêt de l'induction par CT par rapport à une chirurgie seule ou avec CT adjuvante dans les stades précoces. La première étude prospective a montré un bénéfice significatif de la CT dans les stades I et II au prix d'une mortalité post-opératoire légèrement majorée, quoique de manière non significative (17).

Les autres études ont été prématurément interrompues lorsque le bénéfice de la CT adjuvante a été rapporté, la poursuite d'un bras chirurgie exclusive n'étant plus éthique. Néanmoins, trois méta-analyses ont montré que la CT préopératoire améliorait significativement la survie avec un gain comparable à celui obtenu après la CT adjuvante (20, 21, 24, 25).

Le choix entre une CT néoadjuvante ou adjuvante suscite toujours de nombreux débats. La mise en œuvre plus précoce du traitement, le taux de réponse et, surtout, la meilleure compliance plaident pour la CT néoadjuvante. La stadification chirurgicale plus précise et l'étude de marqueurs biologiques sur pièce d'éxérèse dans la perspective d'un traitement personnalisé en cas de rechute plaident, par contre, pour la CT post-opératoire. Aucun essai n'a, en outre, démontré une différence significative dans la survie globale entre la néo-adjuvance et l'adjuvance (26).

Dans plus de 10 % des cas, le bilan médiastinal sera révisé sur base des données opératoires (stade pIIIA «unforeseen» N2). Chez ces patients, une CT adjuvante est recommandée.

Tableau I. Classification TNM selon l'IALSC 2009

T et M	N0	N1	N2	N3
T1 : Absence d'invasion de la plèvre viscérale et d'un tronc souche bronchique T1a: Tumeur ≤ 2cm (grand diamètre) T1b:Tumeur > 2 et ≤ 5 cm	IA	IIA	IIIA	IIIB
T2 : Invasion d'un tronc souche bronchique mais ≥ 2cm de la carène ou de la plèvre viscérale ou associée à une atélectasie consolidation lobaire T2a: Tumeur > 3 et ≤ 5 cm T2b: Tumeur > 5 et ≤ 7 cm	IB	IIB	IIA	IIIB
T3 : Invasion de la plèvre pariétale, de la paroi thoracique, du diaphragme, du nerf phrénique, de la plèvre médiastinale, du péricarde invasion d'un tronc souche bronchique < 2 cm de la carène ou de la plèvre viscérale ou associée à une atélectasie consolidation pulmonaire Plusieurs nodules dans le même lobe Tumeur > 7 cm	IIB	IIIA	IIIA	IIIB
T4 : Invasion du médiastin, cœur, vaisseaux, trachée, nerf récurrent laryngé, de l'œsophage, le corps vertébral, la carène. Plusieurs nodules dans des lobes différents homolatéraux	IIIA	IIIA	IIIB	IIIB
M1a : Effusion pleurale ou péricardique Nodules dans le poumon controlatéral M1b : Métastase extrathoracique	IV	IV	IV	IV
N0 : aucune atteinte ganglionnaire; N1: atteinte ganglionnaire hilaire; N2: atteinte ganglionnaire médiastinale homolatérale; N3: atteinte ganglionnaire hétérolatérale ou supra-claviculaire				

Tableau II. Essais récents de CT adjuvante

Essai	Année	N=	Stade	Protocole	MS Bras Contrôle	MS Bras Expér	Survie 5 ans Contrôle (%)	Survie 5 ans Expér (%)	p
ALPI	2003	1209	I-IIA	MVP**	55,2	48	n.c.	n.c.	NS
BLT	2004	381	I,II,IIIA	Doublet Platine**	32,6	33,9	51 (2 ans)	53 (2 ans)	NS
IALT	2004	1867	I,II,IIIA	VincaP ou P-EP**	44,4	50,8	50,8	44,5	0.03
UFT	2004	999	I	UFT ou P	n.c.	n.c.	85	88	0.04
JBR10	2005	482	IB, II	Vnr P	73	94	54	69	0.009
ANITA	2006	840	IB,II, IIIA	Vnr P**	43,7	65,7	42,6	51,2	0.017
CALGB 9633	2008	344	IB	Carbo-Pac	78	95	95	95	NS

CT : chimiothérapie; n: nombre; MS : moyenne de survie (mois); Contr : contrôle; Expér: expérimental; NS : non significatif; nc : non connu;
MVP : mitomycin, vindesine, cisplatine; ** RT adjuvante (optionnelle; systématique dans ALPI);
VincaP : vincaloïd (vindesine-vinblastine-vinorelbine)-cisplatine; EP : etoposide; UFT : tegafur-uracil; Vnr : vinorelbine; P : cisplatine;
Carbo-Pac : carboplatine-paclitaxel.

Tableau III. Essais récents de CT Néo-adjuvante

Essai	Année	N=	Stade	Protocole	Question posée	MS Contrôle	MS Expér	Survie 5 ans Contrôle (%)	Survie 5 ans Expér(%)	p
MIP91	2002	355	IB,II, IIIA	MIP	CT pré-op ?	36	37	35,3 (4a)	43,9 (4a)	NS
MRCLU 22	2007	519	I-III	Doublet Platine	CT pré-op ?	55	54	45	44	NS
Van Meer-beeck	2007	579	IIIA-N2	Cis-EP	Chirurgie ?	17,5	16,4	14	15,7	NS
Felip	2010	624	IA-II	Carbo-Pac	CT pré vs post-op ?	nc	nc	44	Pré-op: 46,6 % Post-op: 45,5 %	NS

CT : chimiothérapie; n : nombre; MS : moyenne de survie (mois); Contr : contrôle; Expér : expérimental; Pré : pré-opératoire; péri : péri-opératoire; post : post-opératoire;
nc : non connue; MIP: mitomycine, ifosfamide, cisplatine; Cis-EP : cisplatine- etoposide; Carbo-Pac : carboplatine-paclitaxel;

La RT médiastinale post-opératoire est optionnelle.

Traitement des CBNPC de stade loco-régional avancé (stades IIIA et IIIB)

Les CBNPC de stade III constituent un groupe très hétérogène qui reste, de nos jours, le sujet de nombreuses controverses. La chirurgie demeurant le meilleur vecteur de curabilité en oncologie pulmonaire, la première étape consiste à distinguer, lors du bilan préthérapeutique, les tumeurs potentiellement résécables des tumeurs d'emblée inopérables (tableau IV) (27). Pour cela, un bilan médiastinal préthérapeutique minutieux et l'évaluation par un chirurgien expérimenté sont indispensables (résécabilité de la tumeur).

Dans tous les cas, la chimiothérapie est un élément clé du traitement des stades localement avancés. Pour les stades T3N1 et pour les «N2 résécables», la chirurgie sera complétée par une CT périopératoire (néoadjuvante ou adjuvante)

à base de platine. En présence d'une résection incomplète ou d'une atteinte ganglionnaire médiastinale ou pariétale résiduelle, une RT postopératoire doit être proposée. Celle-ci peut augmenter le contrôle local, sans toutefois améliorer la survie globale (28). Dans notre centre du CHU liégeois, l'option d'une induction par CT est le plus souvent privilégiée pour ces stades IIIA résécables; cette attitude repose sur les essais cliniques en faveur de la CT néo-adjuvante (tableau III) (21). L'utilisation de la chimiothérapie permet d'intégrer, dans la décision thérapeutique, la notion de «downstaging» médiastinal (réduction préopératoire du stade anatomopathologique d'une néoplasie) qui est un facteur pronostique important (29) et un traitement des micrométastases potentielles. Par ailleurs, ce traitement néo-adjuvant nous semble permettre de mieux sélectionner les patients susceptibles de bénéficier d'une chirurgie secondaire. La «non-réponse» à la CT d'induction oriente vers un traitement associant radio- et chimiothérapie.

La question du type d'induction (CT *vs* RT-CT concomitante) reste sujet à débat. Actuellement, aucun gain de survie n'a pu être observé en faveur du protocole associatif qui demeure toutefois plus toxique qu'une induction chimiothérapeutique seule (30). Enfin, deux études randomisées ont mis en doute la nécessité d'une chirurgie systématique, en particulier pour la pneumectomie droite post induction en montrant, dans les 2 bras RT-CT *vs* RT-CT suivie de chirurgie, une survie médiane (respectivement 17,5 *vs* 16.4 mois et 22,4 *vs* 23,6; NS) et une survie à 5 ans (14 *vs* 15,7 mois et 27 *vs* 20 mois; NS) équivalentes (19, 31), tout en suggérant un bénéfice en cas de lobectomie.

En cas de CBNPC locorégionalement avancé inopérable (stades IIIA N2 non résécables et IIIB), une association de CT et de RT constitue le standard de traitement. Malgré les progrès thérapeutiques de ces dernières années, leur pronostic reste mauvais avec des taux de survie globale entre 35 et 40 % à 2 ans et autour de 15 % à 5 ans. L'association d'une CT à une RT tant séquentielle que concomitante est supérieure à la RT seule (32). Plusieurs études ont montré un bénéfice significatif sur la survie globale en faveur du schéma concomitant (tableau V) (33-35), mais au prix d'une toxicité plus importante. Une méta-analyse des essais comparant CT-RT séquentielle et concomitante confirme la supériorité, en termes de survie globale, de l'association concomitante, de l'ordre de 4,5 % à 5 ans (p = 0,004) (36). La RT-CT concomitante réduit le taux de rechutes locales de 6 % à 5 ans par rapport au schéma séquentiel (p = 0,01), au prix d'une augmentation très nette de la toxicité œsophagienne aiguë pour une toxicité pulmonaire pratiquement identique. Le gain en survie globale par rapport à l'approche séquentielle semble se faire par un meilleur contrôle local. L'association concomitante de

Tableau IV. Evaluation de la résécabilité tumorale des stades III selon la classification cTNM

Stade	cTNM		Option thérapeutique
IIIA	T3, N1		Tumeur résécable
	T4, N0-1		Tumeur non résécable (excepté de rares exérèses élargies dans des centres sélectionnés d'expertise)
	T1-3, N2	IIIA1 : N2 sur pièce de résection (post-opératoire)	Résécable par définition
		IIIA2 : N2 à la thoracotomie (per-opératoire)	Continuer l'exérèse si la résection est réalisable
		IIIA3 : «non IIIA4» au bilan préopératoire :	Résécabilité discutée au cas par cas
		IIIA4 : au bilan préopératoire : adénopathies multizones, bulky en TDM, fixées ou rupture capsulaire à la médiastinoscopie	Tumeur non résécable
IIIB	T4, N2		Tumeur non résécable
	Tous T, N3		Tumeur non résécable
cTNM: clinical Tumor Node Metastasis			

Tableau V. CBPNPC irrésécable IIIA (N2) et IIIB
Résultats des études randomisées de phase III chimio-radiothérapie séquentielle vs concomitante

Essais	N	Dose RT (Grays)	Médiane de survie globale (mois)	Survie à 2 ans (%)	Survie à 5 ans (%)	Oesophagite grade 3-4 (%)
Radio-chimiothérapie séquentielle						
Furuse K	158	56	13,3	27,4	8,9	3
RTOG 94-10	199	63	14,6	32	12	4
GLOT-GFPC NPC 95-01	101	66	14,4	26	8,8	3
Radio-chimiothérapie concomitante						
Furuse K	156	56	16,5	34,6	15,8	4
RTOG 94-monofractionné	200	63	17	35	21	25
RTOG 94-10 bifractionné	193	69,6	15,2	34	17	44
GLOT-GFPC NPC 95-01	100	66	16,1	39	18,5	32
N: nombre de patients; RT: radiothérapie						

CT-RT est, dès lors, aujourd'hui la meilleure stratégie thérapeutique face aux CBNPC localement avancés non résécables chez les patients en bon état général. La chimiothérapie consiste en un doublet associant le platine à la vinorelbine, l'étoposide, la gemcitabine ou aux taxanes (19, 31); (37-40). Notre groupe privilégie le schéma de Vokes (cisplatine-vinorelbine en 4 cycles; RT monofractionnée jusque 66 Gy en 33 fractions) (41). En cas d'impossibilité de réaliser un schéma concomitant, une administration séquentielle peut être proposée.

Traitement des CBNPC de stade métastatique (stade IV)

La plupart des patients se présentent à un stade avancé de la maladie. Longtemps, une attitude défaitiste a été adoptée face au sombre pronostic de l'affection (médiane sans traitement de 4 à 5 mois) (42). L'introduction des sels de platine a apporté un premier souffle d'espoir en doublant la médiane de survie (43, 44). Depuis une dizaine d'années, la ligne de conduite dans le traitement du CBNPC de stade avancé est celle du traitement individualisé. Cette personnalisation thérapeutique peut se faire par la modulation de la chimiothérapie ou par l'introduction d'un traitement agissant sur les «drivers» oncogéniques tumoraux (EGFR TKI, inhibiteurs de l'ALK ou de ROS1). Outre cette approche du cancer centrée sur la tumeur, une nouvelle approche se dessine, appelée immunothérapie, qui vise à restaurer une réponse immunitaire adéquate et, par là, à contrôler la maladie tumorale.

Première ligne de traitement

La décision du traitement de première ligne du CBNPC métastatique repose sur l'intégration de paramètres liés au patient et liés à la tumeur (type histologique prédominant, présence éventuelle d'une mutation génétique).

Le traitement de première ligne des carcinomes non épidermoïdes dépend de la mise en évidence ou non d'une mutation activatrice de l'EGFR. Plusieurs mutations oncogéniques ont été mises en évidence, les plus connues étant celles de l'EGFR et le réarrangement EML4-ALK. Même si elles sont rares (< 10 % des patients), ces mutations doivent être recherchées de manière systématique au vu de la supériorité des TKI sur la CT standard en termes de taux de réponse (response rate, RR), de survie sans progression (progression free survival, PFS), de moindre toxicité et de qualité de vie (45-51). Jusqu'il y a peu, aucune différence en termes de survie globale (overall survival, OS) n'avait été démontrée, probablement par l'usage de TKI chez les patients initialement traités par CT (cross-over). Mais, une analyse groupée très récente de deux études cliniques comparables démontre un gain de survie globale de 12 à 13

mois pour les délétions de l'exon 19 chez les patients sous afatinib comparativement à la CT standard (52). Le gefitinib, l'afatinib et l'erlotinib sont donc recommandés en première ligne pour ces CBNPC non épidermoïdes mutés pour l'EGFR (53). Les EGFR TKI peuvent aussi, vu leur moindre toxicité, être donnés chez les patients en mauvais état général (PS 3). En présence d'un réarrangement ALK (~3 % des patients), le crizotinib pourra être proposé en seconde ligne et, prochainement peut-être, en première ligne. D'autres thérapies ciblées sont en cours d'investigation clinique.

La grande majorité des carcinomes non épidermoïdes sont de statut non muté. Leur traitement consiste en 4 à 6 cycles d'un doublet associant un platine à un cytostatique de 3ème génération (vinorelbine, gemcitabine, taxanes, pemetrexed). Le cisplatine apparaît préférable au carboplatine (54), mais est plus toxique. Cette combinaison a démontré une augmentation de la survie et une amélioration de la qualité de vie et du contrôle des symptômes chez les patients qui ont un bon état général (PS 0-1) (14). L'association platine-pemetrexed est préférée pour les carcinomes non épidermoïdes au doublet platine-gemcitabine en raison d'un rapport efficacité-tolérance très favorable et d'un bénéfice en survie (+ 2,6 mois), démontré dans une analyse de sous-groupes d'une large étude randomisée de phase 3 (55). Néanmoins, aucune autre analyse prospective n'a confirmé cette donnée. Il n'existe, par ailleurs, aucune étude comparant le doublet platine-pemetrexed avec les autres doublets disponibles. Un doublet sans sel de platine peut être envisagé s'il existe une contre-indication aux platines, notamment en présence d'une altération de l'état général (PS 2) ou chez le patient âgé, et ce, avec un taux de survie semblable bien que pour un taux de réponse inférieur (56). L'adjonction de bevacizumab, anticorps monoclonal anti-angiogénique, a montré une augmentation du RR et de la PFS en association avec un doublet à base de platine (57, 58). Il n'est toutefois pas remboursé en Belgique.

Le traitement de première ligne des carcinomes épidermoïdes repose également sur une association platine-cytostatique de 3ème génération, sans que l'un de ces derniers n'émerge en termes de supériorité. Ici, le bevacizumab est clairement contre-indiqué (57, 58). En présence d'un PS 2, le choix se portera soit sur une monothérapie, soit sur une bithérapie fondée sur le carboplatine associé à l'un des cytostatiques précités, la toxicité du cisplatine étant rédhibitoire. Les patients ayant un PS 3 ou 4 bénéficieront d'un traitement symptomatique de confort seul.

Traitement de maintenance

La maintenance est la poursuite de l'administration d'un traitement après un nombre défini de cycles de CT (4 à 6) lorsque la pathologie est stable ou présente une réponse. Le traitement peut être continué jusqu'à la progression de la maladie ou l'apparition d'une toxicité inacceptable. On distingue la maintenance de continuation (maintien d'une des drogues du traitement d'induction) et la «switch maintenance» (utilisation d'une autre drogue que dans le traitement initial).

Le bénéfice d'une thérapie de maintenance en termes de PFS et d'OS par rapport au bras placebo a été rapporté pour le pemetrexed (59, 60) et l'erlotinib (61), et ce, au prix d'une toxicité majorée, mais acceptable, et sans détérioration de la qualité de vie. La maintenance peut dès lors être proposée par pemetrexed chez les carcinomes non épidermoïdes ou par erlotinib dans tous les types histologiques pour les maladies stables ou ayant répondu à la chimiothérapie d'induction.

Seconde ligne de traitement

La chimiothérapie de deuxième ligne du CBNPC améliore les symptômes et la survie chez les patients avec un PS de 0 à 2. Trois traitements sont validés dans cette indication.

Le docetaxel a montré, comparativement aux soins de confort, un bénéfice de survie significatif malgré un RR objectif faible (62). Dans les carcinomes non épidermoïdes, le pemetrexed présente une activité similaire au docetaxel en termes de réponse et de survie, mais avec un profil de tolérance plus favorable (63). Une bithérapie en deuxième ligne a démontré une meilleure réponse ainsi qu'une PFS améliorée, mais n'influence pas l'OS en comparaison à une monothérapie (64).

Quel que soit le statut mutationnel EGFR, l'erlotinib a montré un bénéfice en termes d'OS, de RR et de PFS par rapport au placebo et une efficacité équivalente au docetaxel ou au pemetrexed, mais avec une meilleure tolérance (65, 66).

Enfin, l'inclusion dans des protocoles d'études cliniques reste fondamental non seulement au bénéfice direct du patient, mais également pour le développement de nouvelles voies de traitement.

Cas particuliers des patients oligométastatiques

En cas de maladie oligométastatique, une approche combinée par CT, RT et exérèse à la fois de la métastase unique et de la tumeur primitive peut se concevoir selon une visée curative, en particulier pour certains sites métastatiques (pulmonaire, surrénalien, cérébral) (53). Dans cette perspective, la chirurgie de la métastase unique ne s'envisage que si la tumeur primitive demeure résécable. Dans un second temps, une prise en charge locorégionale de la tumeur pulmonaire primitive sera envisagée : chirurgie d'exérèse pour les patients en bon état général ou RT ablative avec ou sans CT. De même, devant une récidive sous la forme d'une métastase unique dans les sites précités, une prise en charge curative du site métastatique doit être discutée.

Conclusion

Le CBNPC reste grevé d'une importante mortalité. Tout en revêtant une signification pronostique, la stadification, reposant sur un bilan préthérapeutique rigoureux, en précise les options thérapeutiques. La chirurgie d'exérèse reste le traitement optimal des stades I. Les stades II et IIIA résécables bénéficieront également d'une exérèse combinée à une chimiothérapie périopératoire : la stratégie adjuvante est recommandée pour les stades II tandis qu'une induction néoadjuvante peut être préférée pour les stades IIIA. Un protocole associant, de préférence de manière concomitante, une radiothérapie à une chimiothérapie, est le plus adéquat pour les stades III d'emblée considérés comme non résécables. Enfin, les stades IV seront redevables d'un traitement systémique, soit par CT seule, soit par thérapie ciblée.

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EXPERT REVIEWS

Maintenance therapy for advanced non-small-cell lung cancer: ready for clinical practice?

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The treatment paradigm for advanced non-small-cell lung cancer has changed in recent years with the importance of histological subtyping for the choice of chemotherapy, and the use of molecular markers to select patients for targeted therapy. Maintenance therapy (MT) is another focus of interest. The potential benefit of MT for the patient is that it prolongs tumor control reached with first-line chemotherapy in order to improve overall survival with little added toxicity. Historical studies have never reached this goal, as the agents used for MT were too poorly tolerated. We review the data of the two types of recent MT studies, 'continuation' and 'switch' or 'consolidation' MT. We comment on how the benefits demonstrated in these studies may change clinical practice and reflect on factors that may identify subgroups of patients who derive the greatest benefit from MT in general, as this will help in a rational use of MT.

Keywords: chemotherapy • maintenance treatment • non-small-cell lung cancer • review • targeted agents

Advanced non-small-cell lung cancer

Approximately 85% of all lung cancer patients have non-small-cell lung cancer (NSCLC). A treatment with radical intent can be offered to patients with nonmetastatic stages, but the majority of patients present with advanced disease at the time of diagnosis. Over the last few decades, several steps have brought modest improvement in the average survival prospects of the latter, along with better symptom control and quality of life (QoL).

The median overall survival (OS) of untreated patients with advanced NSCLC was approximately 4–6 months, with <10% of patients alive after 1 year [1]. Platinum-based doublet chemotherapy was proven to be beneficial in terms of symptom control as well as in survival [2]. The standard of care for patients with advanced NSCLC and a good performance status (PS) became four to six cycles of platinum-doublet chemotherapy [3]. The doublet consisted of a platinum compound (cisplatin or carboplatin) in combination with a 'third generation' compound (gemcitabine, vinorelbine, paclitaxel or docetaxel). This 'any platinum doublet fits all' strategy resulted in a median OS of 8–10 months and a 1-year survival rate of 33%.

Different strategies to prolong survival and improve QoL have been developed over the last decade. The first important change was customization of chemotherapy according to the histological subtypes of NSCLC. This occured when superior efficacy of pemetrexed over gemcitabine was shown for nonsquamous tumors, especially adenocarcinoma, while the reverse was true for squamous-cell carcinoma [4]. Second, whenever possible, treatment is now guided by the tumor's genetic profile. Targeted therapies such as EGF receptor (EGFR) tyrosine kinase inhibitors (TKIs) in patients harboring an activating *EGFR* mutation [5] and *ALK* inhibitors in those having the *EML4–ALK* fusion gene [6] have brought major progress in molecularly selected subsets of advanced NSCLC. Third, the chemotherapy doublet can also be optimized in subsets of patients by adding a monoclonal antibody (mAb) such as bevacizumab [7,8] or cetuximab [9,10]. However, as long as lung cancer genetics and targeted therapies remain limited to small

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subsets of patients, all others will still have to rely on cytotoxic chemotherapy as the basis of their treatment.

Given the observation that most advanced NSCLC patients experience disease progression 2–3 months after the end of first-line therapy, over the past years attention was focused on the possible effect of prolongation of therapy immediately after first-line chemotherapy, so-called maintenance therapy (MT).

The concept of MT

Before the true concept of MT was considered, different studies had examined the optimal number of cycles of first-line doublet chemotherapy [11–13]. No study showed a difference in OS between six cycles or less, one study showed a benefit in time to progression (TTP) with six cycles instead of four [13], and one reported similar symptom control but a decrease in global QoL for patients receiving six cycles compared with three [12]. This led to the current recommendation that four cycles of chemotherapy appear sufficient in most NSCLC patients, but six cycles may be considered depending on response and toxicity [14].

The principle of true MT studies is to try to prolong disease control obtained with first-line chemotherapy (Figure 1). In the classical approach, patients have a careful follow-up period without any therapy after their first-line chemotherapy. Second-line therapy is given when disease progression is detected or clinically apparent (Figure 1A). In the maintenance approach, some type of therapy is continued with the intent of prolonged disease control, and thus longer TTP and progression-free survival (PFS) (Figure 1B). The ultimate aim for the patient is that this longer PFS is translated into longer OS.

MT consists of either prolongation of agent(s) already used in the initial treatment regimen (so-called 'continuation' MT) or introduction of another non-cross-resistant agent ('consolidation' or 'switch' MT).

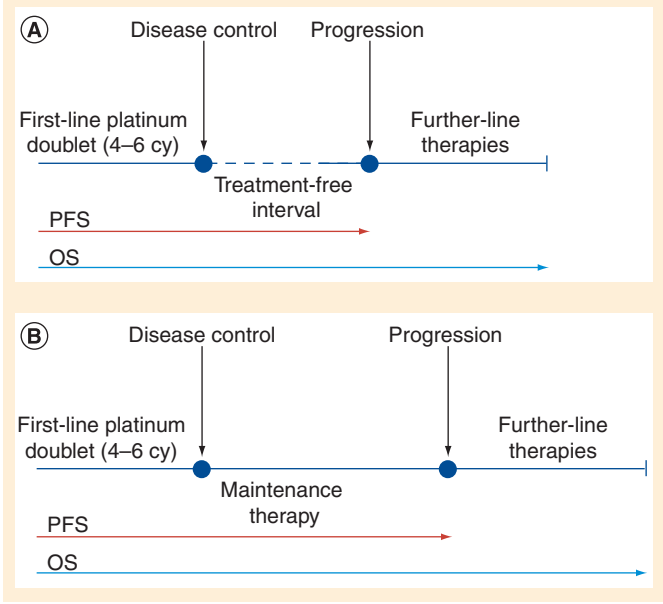

Figure 1. The principle of maintenance treatment.
(A) Classical approach and **(B)** maintenance approach.
OS: Overall survival; PFS: Progression-free survival.

Continuation maintenance studies

In continuation MT (Figure 2, upper section), the first-line regimen, the nonplatinum cytotoxic agent of the first-line, or a targeted agent used with the first-line platinum doublet is prolonged after the standard first-line therapy. Advantages of this approach are the already known tolerance and effectiveness of the therapy that will be prolonged, and other drugs remaining spared for the time of a later relapse. Cumulative toxicity is a potential issue, especially in studies that try to continue the platinum-doublet chemotherapy, while continuation of only the third-generation cytotoxic agent or the targeted agent is expected to be more feasible.

Continuation of the first-line regimen

A small historical study looked at maintenance with the nonplatinum-containing regimen MACC (methotrexate–doxorubicin–cyclophosphamide–lomustine) (Table 1, upper section) [15]. Seventy-four patients with stable disease after three cycles of MACC had either continuation of this regimen or observation. There were no significant differences in TTP or OS.

A Phase III randomized trial compared OS and QoL in patients receiving four cycles of carboplatin–paclitaxel followed by either observation (n = 114) or carboplatin–paclitaxel until disease progression (n = 116) [16]. At progression, both groups received paclitaxel on a weekly basis. No difference was noted in response rate (22% for the four cycles only group vs 24% for the other; p = 0.80) and OS (11.6 vs 12.5 months; p = 0.63). At disease progression, only 65% of the patients received second-line paclitaxel; the most frequent reason for not receiving the planned second-line therapy was residual neuropathy by paclitaxel. QoL, assessed with functional assessment of cancer therapy-lung (FACT-L), decreased over time, similarly between the two treatment arms.

Continuation of single-agent chemotherapy

In a randomized trial with three different drug administration schedules of first-line carboplatin and paclitaxel, nonprogressive patients were randomly assigned after four cycles to either weekly MT with paclitaxel (n = 65) or observation (n = 65) (Table 1, lower section) [17]. The mean PFS was 38 weeks in the paclitaxel MT group versus 29 weeks in the other (hazard ratio [HR]: 0.76; 95% CI: 0.33–1.75). Median OS was 75 versus 60 weeks (HR: 0.85; 95% CI: 0.42–1.73). The primary goal was to examine the feasibility of paclitaxel as MT; the study was not powered to conclude on PFS or OS. The results, however, suggested a trend in favor of MT and prompted other trials.

The Central European Cooperative Oncology Group was the first to investigate gemcitabine as MT. Patients with advanced stage (IIIB/IV) achieving objective response or stable disease after four cycles of cisplatin–gemcitabine were randomly assigned to best supportive care (BSC) and gemcitabine continued until progression (n = 138) or BSC only (n = 68) [18]. The primary end point – a significant difference in TTP – was achieved with a median TTP in the maintenance phase of 3.6 versus 2.0 months (p < 0.001). Some aspects of tumor evaluation limit the interpretation of these results. First, timing of tumor evaluation was not identical across arms (due to treatment delay of more than one out of five gemcitabine

MT patients), which may impact on the comparison of TTP [19]. Second, the Southwest Oncology Group criteria (less stringent than RECIST [20,21]) were used for evaluation, and the imaging methods used were computed tomography and the less precise chest x-ray as well. Median OS was better in the MT arm, but this was not statistically significant (p = 0.172). Exposure to second-line treatment at disease progression was approximately 60% in both groups. There was a significantly higher need for red blood cell transfusion in the gemcitabine arm (20 vs 6.3%; p = 0.018). QoL – assessed by the LCSS – was similar, with a trend toward better symptom control with gemcitabine MT. Subgroup analysis revealed that the benefits were limited to patients with a good PS (Karnofsky score > 80).

In another trial with a very similar design, patients were randomized between gemcitabine MT plus BSC (n = 128) or BSC only (n = 127) after four cycles of carboplatin–gemcitabine [22]. Gemcitabine MT failed to improve PFS or OS in this trial. A possible explanation is the high proportion (64%) of patients with a PS of 2 or more, potentially less fit to receive sufficient therapy. Exposure to second-line therapy at disease progression was also very low (16–17%). Gemcitabine MT was associated with a higher incidence of grade 3/4 hematological toxicity (anemia: 9.4 vs 2.4%; neutropenia: 13.3 vs 1.6%; thrombocytopenia: 9.4 vs 1.4%). No QoL data were provided.

The French Intergroupe Francophone de Cancerologie Thoracique/Groupe Français de Pneumocancérolgie (IFCT-GFPC) conducted a Phase III trial in fit patients achieving disease control after four cycles of cisplatin–gemcitabine for advanced NSCLC [23]. There were three arms: gemcitabine continuation MT (n = 154) versus erlotinib switch MT (n = 155) versus observation (n = 155). All patients had a PS of 0 or 1, and – quite unique – the second-line treatment at the time of progression was predefined as pemetrexed. The primary objective – PFS – was significantly prolonged by gemcitabine MT (HR: 0.56; 95% CI: 0.44–0.72). Preplanned subgroup analysis showed that patients with objective response after first-line treatment benefited the most from MT with gemcitabine. The study was not powered for OS, but the median time of randomization to death was 12.1 months in the gemcitabine arm versus 10.8 months in the observation group (HR: 0.89; 95% CI: 0.69–1.15) [24]. QoL data have not yet been reported.

The largest continuation MT Phase III study was presented at the 2011 annual meeting of the American Society of Clinical Oncology [25]. In the PARAMOUNT study, patients with nonsquamous NSCLC in disease control after four cycles of cisplatin–pemetrexed were randomly assigned to pemetrexed MT or placebo MT. The primary objective – PFS – was met, with a median PFS of 4.1 versus 2.8 months (HR: 0.62; 95% CI: 0.49–0.79). Preplanned subgroup analysis again showed that patients with objective response after first-line therapy benefit more from MT with pemetrexed. OS data are eagerly awaited for 2012. Pemetrexed MT resulted in higher number of grade 3/4 side effects (18.1 vs 5.0%), especially fatigue (4.2 vs 0.6%), anemia (4.5 vs 0.6%) and neutropenia (3.6 vs 0%). This did not result in a significantly higher hospitalization rate (patients with at least one hospitalization 15.4 vs 14%). QoL was assessed with the FACT-L scale [26], and there were no major differences between the arms, except for a significantly longer time to pain for the erlotinib arm in a *post hoc* analysis [27].

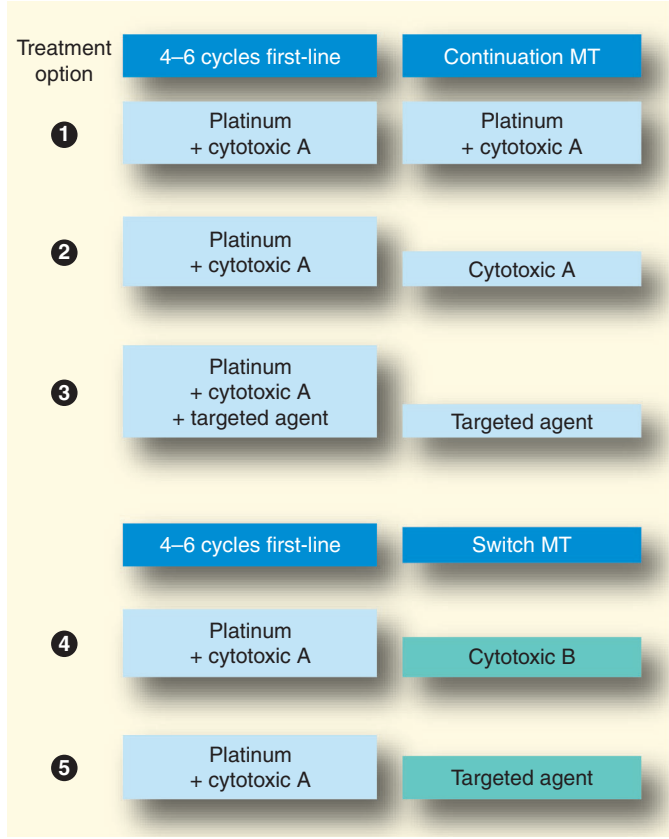

Figure 2. The different types of maintenance treatment.
MT: Maintenance therapy.

Very recently, a randomized study (labeled Phase III, but more of a Phase II size) was presented at the 2011 European Cancer Conference [28]. In this AVAPERL study, patients with nonsquamous NSCLC in disease control after four cycles of cisplatin–pemetrexed plus bevacizumab were randomly assigned to continued pemetrexed and bevacizumab or bevacizumab alone. The primary end point – PFS – was clearly in favor of pemetrexed maintenance: median PFS of 7.4 months with pemetrexed MT versus 3.7 months for the bevacizumab-containing backbone arm (HR: 0.48; 95% CI: 0.35–0.66). These findings thus confirm the PFS benefit seen in PARAMOUNT.

Continuation of a mAb

Large Phase III trials have examined if the addition of a mAb to standard doublet chemotherapy improves the outcome of patients with advanced NSCLC. Cetuximab, a mAb against the EGFR, was studied in all histologies [9,29]; bevacizumab, a mAb against the VEGF, was studied in patients with nonsquamous NSCLC [7,8]. In the experimental arms of all of these studies, the mAb was continued beyond first-line treatment until progression. In the lack of data on patients without continuation of the drug after first-line therapy, the benefit of continuation cannot be assessed in this approach.

Table 1. Chronologic overview of the randomized continuation maintenance therapy studies.

Study (year)	Patients included (n)	Comparison	Randomized (n)	Median PFS HR (95% CI)	Median OS HR (95% CI)	Ref.
Continuation of the first-line regimen						
Buccheri *et al.* (1989)	116	MACC Observation	38 36	5.8 months† 5.3 months† NS	10.4 months† 6.7 months† NS	[15]
Socinski *et al.* (2002)	230	Carboplatin–paclitaxel Observation	116 114	NR NR	12.5 months† 11.6 months† NS	[16]
Continuation of single-agent chemotherapy						
Belani *et al.* (2003)	401	Paclitaxel Observation	65 65	8.7 months† 6.7 months† 0.76 (0.33–1.75)	17.2 months† 13.8 months† 0.85 (0.42–1.73)	[17]
Brodowicz *et al.* (2006) CECOG	352	Gemcitabine Observation	138 68	3.6 months 2.0 months 0.57 (0.42–0.78)	10.2 months 8.1 months 0.84 (0.52–1.38)	[18]
Belani *et al.* (2010)	519	Gemcitabine Observation	128 127	3.9 months 3.8 months 1.09 (0.81–1.45)	8.0 months 9.3 months 0.97 (0.72–1.30)	[22]
Perol *et al.* (2010) IFCT-GFPC	834	Gemcitabine Observation	154 155	3.8 months 1.9 months 0.56 (0.44–0.72)	12.1 months 10.8 months 0.89 (0.69–1.15)	[23]
Paz-Ares *et al.* (2011) PARAMOUNT	939	Pemetrexed Placebo	359 180	4.1 months 2.8 months 0.62 (0.49–0.79)	13.9 months‡ 11.1 months‡ (0.78 [0.61–0.98])‡	[25]
Barlesi *et al.* (2011) AVAPERL	376	Pemetrexed + bevacizumab Bevacizumab	128 125	7.4 months 3.7 months 0.48 (0.35–0.66)	NR NR	[27]

†These studies reported PFS/OS from start of first-line therapy. More recent studies report PFS and OS from the time of randomization into the maintenance phase.
‡Preliminary data based on second OS interim analysis requested by the regulatory authorities [102].
CECOG: Central European Cooperative Oncology Group; HR: Hazard ratio; IFCT-GFPC: Intergroupe Francophone de Cancerologie Thoracique/Groupe Français de Pneumocancérolgie; MACC: Methotrexate–doxorubicin–cyclophosphamide–lomustine; NR: Not reported; NS: Nonsignificant; OS: Overall survival; PFS: Progression-free survival.

Switch or consolidation maintenance studies

In switch MT (Figure 2, lower part), an as yet unused agent is started immediately after disease control is achieved by first-line doublet chemotherapy. Possible advantages are that a potentially non-cross-resistant agent is used when the tumor burden is small, and that all patients are exposed to this agent (otherwise it may be used in only some as second-line treatment). Concerns here are that tolerance and effectiveness of this new drug is not yet known, and the 'loss' of a well-established second-line agent for treatment of a patient without progression.

Switch to single-agent chemotherapy

The first randomized trial investigating switch maintenance chemotherapy investigated vinorelbine [30]. The first-line chemotherapy was mitomycin C–ifosfamide–cisplatin. Patients with objective response were randomized between vinorelbine for a maximum of 6 months (n = 91) or observation (n = 90). The primary outcome – OS – was identical in both arms, with a trend for better PFS with vinorelbine MT (Table 2). The low activity of vinorelbine as a second-line drug [31], the poor tolerance of prolonged vinorelbine (only a quarter of the patients received the planned 6 months of therapy) and the small patient numbers may have played a role in the absence of difference.

Docetaxel, a well-established second-line agent, was chosen for a Phase III study with a particular design [32]. Patients with disease control after four cycles of carboplatin–gemcitabine were randomized and received docetaxel at the time of relapse (standard approach; n = 156) or immediately after the first-line therapy (switch MT approach; n = 153). The primary objective – OS – was better in the immediate docetaxel group, although not statistically significant (12.3 vs 9.7 months; HR: 0.84; 95% CI: 0.65–1.08). PFS was significantly improved in the immediately treated group (5.7 vs 2.7 months; HR: 0.71; 95% CI: 0.55–0.92). There was no major increase in toxicity, and evolution of symptoms, measured with the Lung Cancer Symptom Scale [33], was similar across both arms.

Pemetrexed was investigated as a switch maintenance drug in comparison to placebo in patients without progression after four cycles of platinum-based, non-pemetrexed-containing, doublet chemotherapy [34]. This randomized Phase III trial was planned and run before the change in the pemetrexed label that introduced

Table 2. Chronologic overview of the randomized switch maintenance chemotherapy studies.

Study (year)	Patients included (n)	Comparison	Randomized (n)	Median PFS HR (95% CI)	Median OS HR (95% CI)	Ref.
Westeel *et al.* (2005)	573	Vinorelbine Observation	91 90	5.0 months 3.0 months 0.77 (0.56–1.07)	12.3 months 12.3 months 1.08 (0.79–1.47)	[29]
Fidias *et al.* (2009)	566	Immediate docetaxel Delayed docetaxel	153 156	5.7 months 2.7 months 0.71 (0.55–0.92)	12.3 months 9.7 months 0.84 (0.65–1.08)	[31]
Ciuleanu *et al.* (2009) JMEN	663	Pemetrexed Placebo	441 222	4.3 months 2.6 months 0.60 (0.49–0.73)	13.4 months 10.6 months 0.79 (0.65–0.95)	[33]
Nonsquamous subanalysis			321 160	4.4 months 1.8 months 0.47 (0.37–0.60)	15.3 months 10.3 months 0.70 (0.56–0.88)	
Squamous subanalysis			120 62	2.4 months 2.5 months 1.03 (0.71–1.49)	9.9 months 10.8 months 1.07 (0.77–1.50)	

HR: Hazard ratio; OS: Overall survival; PFS: Progression-free survival.

its first-line use, but restricted the drug to nonsquamous NSCLC in all settings. The primary end point – PFS – was significantly in favor of pemetrexed MT (4.3 vs 2.6 months; HR: 0.60; 95% CI: 0.49–0.73). The same was true for OS (13.4 vs 10.6 months; HR: 0.79; 95% CI: 0.65–0.95). In a subanalysis for histology, the benefit was even more pronounced: the gain in PFS and OS increased to 2.6 and 5.0 months, respectively, while no benefit was seen for squamous cell carcinoma patients. The benefit was mostly in patients with stable disease after first line therapy (OS HR: 0.61), less in those achieving objective response (OS HR: 0.81). The grade 3 and 4 toxicity of pemetrexed MT consisted of fatigue (5% of the patients vs 1% in the control arm), and neutropenia (3 vs 0%). There was a significant delay in time to worsening of pain and hemoptysis as measured with the Lung Cancer Symptom Scale [33] in favor of the pemetrexed arm.

Switch to a targeted agent

Phase III trials with this approach have been reported for the class of EGFR TKIs (Table 3). The ATLAS trial included nonsquamous NSCLC patients without disease progression after four cycles of the carboplatin–paclitaxel–bevacizumab regimen [35]. Patients were randomized between switch MT with erlotinib and continued bevacizumab (n = 370) versus placebo and continued bevacizumab (n = 373). Adding the EGFR TKI to

Table 3. Chronologic overview of the randomized switch maintenance EGF receptor tyrosine kinase inhibitor studies.

Study (year)	Patients included (n)	Comparison	Randomized (n)	Median PFS HR (95% CI)	Median OS HR (95% CI)	Ref.
Miller *et al.* (2009) ATLAS	1160	Erlotinib/bevacizumab Placebo/bevacizumab	370 373	4.8 months 3.7 months 0.72 (0.59–0.88)	15.9 months 13.9 months 0.90 (0.74–1.09)	[34]
Cappuzzo *et al.* (2010) SATURN	1949	Erlotinib Placebo	438 451	2.8 months 2.5 months 0.71 (0.62–0.82)	12.0 months 11.0 months 0.81 (0.70–0.95)	[36]
Perol *et al.* (2010) IFCT-GFPC	834	Erlotinib Observation	155 155	2.9 months 1.9 months 0.69 (0.54–0.88)	11.4 months 10.8 months 0.87 (0.68–1.13)	[23]
Zhang *et al.* (2011) INFORM	NR	Gefitinib Placebo	148 148	4.8 months 2.6 months 0.42 (0.32–0.54)	18.7 months 16.9 months 0.84 (0.62–1.14)	[39]
Gaafar *et al.* (2011) EORTC	173	Gefitinib Placebo	86 87	4.1 months 2.9 months 0.61 (0.45–0.83)	10.9 months 9.4 months 0.83 (0.60–1.15)	[40]

EORTC: European Organisation for Research and Treatment of Cancer: HR: Hazard ratio; IFCT-GFPC: Intergroupe Francophone de Cancerologie Thoracique/Groupe Français de Pneumocancérolgie; NR: Not reported; OS: Overall survival; PFS: Progression-free survival.

bevacizumab brought the primary end point – PFS – from 3.7 to 4.8 months (HR: 0.72; 95% CI: 0.592–0.881). The effect on OS was reported later and not significantly different (HR: 0.90; 95% CI: 0.74–1.09) [36]. Exposure to second-line treatment at disease progression was approximately 50%. The safety profile was consistent with previously reported toxicity profiles for both agents.

The SATURN trial included 1949 patients treated with a first-line non-bevacizumab and non-pemetrexed-containing platinum doublet for four cycles [37]. Patients in response or stable disease were randomized to switch MT with erlotinib (n = 438) or placebo (n = 451). The primary end point – PFS – was significantly better with erlotinib (HR: 0.71; 95% CI: 0.62–0.82), although the absolute difference in median PFS was very modest (2.8 vs 2.5 months). The effect was present both in *EGFR* mutated and nonmutated tumors, although the PFS benefit was much more impressive in the mutated group (PFS HR: 0.10; 95% CI: 0.04–0.25) than in the wild-type tumors (0.78; 95% CI: 0.63–0.96). OS was also significantly better, with a HR of 0.81 (95% CI: 0.70–0.95). Subgroup analysis showed that the benefit in OS was significant only for patients achieving stable disease after first-line chemotherapy, and for adenocarcinoma histology, although neither of these were significant interactions [38]. Erlotinib MT was associated with a higher number of grade 3 or 4 rash (9 vs 0%) and diarrhea (2 vs 0%). QoL was assessed with the FACT-L scale [26], and there were no major differences between the arms, except for a significantly longer time to pain in a *post hoc* analysis. Exposure to second-line treatment at disease progression was fairly high (~70%) in both groups, and quite some patients in the placebo arm (~20%) received erlotinib at the time of progression.

The earlier mentioned IFCT-GFPC three-arm Phase III trial also investigated erlotinib MT compared with observation after initial cisplatin–gemcitabine first-line chemotherapy [23,24]. There was a PFS advantage for erlotinib (median 2.9 vs 1.9 months; HR: 0.83; 95% CI: 0.73–0.94) and a nonsignificant OS advantage (median 11.4 vs 10.8 months; HR: 0.87; 95% CI: 0.68–1.13). Planned second-line pemetrexed was administered in the erlotinib arm in 63%, and in the observation arm in 76% of the patients. On subgroup analysis, patients with response or stable disease after first-line therapy had comparable benefit.

Gefitinib was investigated in a Chinese trial with advanced stage NSCLC patients and WHO PS 0–2, who had no progression or unacceptable toxicity after four cycles of first-line platinum-based doublet chemotherapy [39]. Randomization was between gefitinib MT (n = 148) or placebo (n = 148) until progression. PFS – the primary end point – was superior for gefitinib, with a difference of 4.8 versus 2.6 months (HR: 0.42; 95% CI: 0.32–0.54). OS was slightly better for gefitinib (18.7 vs 16.9 months), albeit not significantly (HR: 0.84; 95% CI: 0.62–1.14). Adverse events were as expected (rash any grade :49.7%; diarrhea any grade: 25.2%). There were no QoL data provided.

A study by the EORTC investigating gefitinib as maintenance drug had to be discontinued early because of poor accrual [40]. The primary end point – improvement of OS – was not reached, but a prolonged PFS was reported for the gefitinib arm (HR: 0.61; 95% CI: 0.45–0.83).

Registration of maintenance therapy

Pemetrexed is registered by the EMA as MT for advanced nonsquamous NSCLC patients with response or stable disease after first-line chemotherapy, irrespective of the type of chemotherapy doublet used [101]. For this registration, an additional (second) interim analysis of the PARAMOUNT study was requested by the authorities. At that point in time, the OS data were in favor of pemetrexed MT with a HR of 0.78 (95% CI: 0.61–0.98) [102]. For erlotinib, the registration is MT for advanced NSCLC patients after four cycles of platinum-based first-line chemotherapy, only in case of stable disease, but irrespective of histology or *EGFR* mutation status [103].

MT with pemetrexed is indicated by the US FDA for nonsquamous advanced NSCLC patients whose disease has not progressed after four cycles of platinum-based first-line chemotherapy [104]. Erlotinib is listed as MT for all patients with advanced NSCLC whose disease has not progressed after four cycles of platinum-based first-line chemotherapy [105].

Expert commentary

Several Phase III trials on MT showed improvements in PFS. A trend for improved OS was present in the gemcitabine continuation MT trials of the Central European Cooperative Oncology Group [18] and IFCT-GFPC [24]. The final OS results of the large PARAMOUNT trial [25] are still awaited, but the HR for PFS of 0.62, as well as the preliminary OS data delivered for registration purposes, are very promising. Thus, gemcitabine and pemetrexed continuation MT seem to be interesting strategies. Statistically significant differences in OS were reported in two recent switch MT studies [34,37]. The absolute difference in median OS in the nonsquamous patients of the switch MT trial with pemetrexed of 5 months is especially remarkable in a population with advanced NSCLC.

One of the first questions raised by these data is why trials are successful now in a setting where they failed over at least two decades. Both the agents used for MT and trial size may account for this. Even during early trials comparing three to six cycles of chemotherapy, it was not surprising that a fairly toxic regimen such as cisplatin–vinblastine–mitomycin C did not perform better when more cycles were delivered [11]. This aspect of tolerance also affected the results in MT trials trying to continue carboplatin–paclitaxel [16], or even paclitaxel alone [17], given the neurotoxic aspect of longer duration paclitaxel. The same was true in the trial with switch MT with vinorelbine [30]. This clearly changed with better tolerated agents such as gemcitabine, pemetrexed, erlotinib or gefitinib. Furthermore, the size of several trials was too small to evaluate possible OS differences, both for the older trials [15–17,30], as well as for some recent ones with the better tolerated agents [18,23,40].

A second observation is that the efficacy of therapeutic agents in the maintenance setting is dependent on the choice of study design and end points.

Unfortunately, it is impossible to know if MT with the monoclonal antibodies cetuximab [9,29] or bevacizumab [7,8] is of use, as these drugs were administered until progression in all Phase III clinical trials, leaving us without any comparison between stopping these agents at the time of discontinuation of first-line chemotherapy or continuing beyond. This is a disturbing gap in our knowledge, as continued administration can be associated with toxicity and substantial cost. Therefore, these trials were not mentioned in our tables.

Many of the recent Phase III trials had PFS as their primary end point [18,23,34,35,37,39]. While this end point may be appropriate in the clinical trial setting, we feel that in the noncurative setting of advanced NSCLC, the aim of treatment we offer to our patients should be to prolong OS as much as possible with a toxicity as small as possible in order to maintain the best QoL possible. However, most of the studies did not achieve this goal: until now, all except two switch MT studies [34,37] did not show significant OS benefits. Symptom evolution or QoL analysis showed either smaller differences or was reported as 'not negatively affecting QoL' [25,32,34,37]. Finally, PFS is a less solid end point than OS, as it is much more dependent on timing and type of disease assessment [19], which may have been a problem in, for example, the Central European Cooperative Oncology Group (CECOG) trial [18] as previously discussed.

OS benefits have until now only been demonstrated in two trials with switch MT. Both of these trials, however, suffer from one major limitation in interpretation: the MT agent was delivered in only 19% of the control-arm patients in the pemetrexed trial [34] and 21% in the erlotinib trial [35]. Consequently, it remains unclear how much of the OS benefit is attributable to the MT principle *per se* (i.e., in how far the PFS difference generated in the MT arm is translated into OS difference), and how much is related to the strong differences in exposure to a well-established second-line agent across arms. By contrast, in the switch MT arm of the IFCT-GFPC study, all patients were planned to receive a valid second-line option (pemetrexed), and 50% of the control-arm patients received the MT agent at a later point in time [23]. In that approach, there was no remaining significant difference in the OS analysis (HR: 0.87; 95% CI: 0.68–1.13) [24]. Further information on this debate comes from the comparison of 'early' versus 'late' docetaxel [32]. In the intent-to-treat evaluation, there was a significant PFS advantage and a clear trend for improved OS in the patients that received early docetaxel. When OS was compared between patients that received early docetaxel and those who actually received the late docetaxel, the OS outcome was superimposed, with an identical median OS of 12.5 months in both arms. Therefore, how much of the benefit in OS is generated by delivery time of versus exposure to a valid second-line agent remains, in our view, an open question. Nonetheless, there is an OS benefit in the existing trials, and it remains unlikely that close follow-up would allow 'late' delivery of the second-line agent at the time of relapse in all patients, as relapse goes along with the deterioration of general condition in a significant number of patients.

Studies with continuation MT, on the other hand, have a more pure design, as their potential OS benefits are not generated by 'early' use of valid second-line options. In these studies, the potential OS benefit is thought to be generated by the PFS benefit with the MT agent, at least if the latter is large enough, and if not diluted by imbalances in further lines of therapy, which is in general not the case in large Phase III trials. Continuation of gemcitabine resulted in a trend for improved OS in both the CECOG [18] and IFCT-GFPC [24] experience, but without statistically significant difference, maybe also because of the number of patients randomized (206 in the CECOG trial; 309 in IFCT-GFPC). Therefore, the OS results of the PARAMOUNT trial on pemetrexed continuation [25], in which 539 patients were randomized, are eagerly awaited. Especially as this study is powered for both PFS and OS outcome analysis.

Five-year view

Pemetrexed and erlotinib are approved in the MT indication in many regions of the world, albeit with differences in application across countries, based on, for example, the type of first-line regimen used, the outcome of the first-line therapy (response vs stable disease), histological selection for pemetrexed and molecular selection for erlotinib, among others. We expect that based on the currently available data, MT will gradually gain a more important position in the treatment patterns of patients with advanced NSCLC. As the above mentioned dilemma in the interpretation of switch MT studies – is this a benefit of timing or of delivery of a valid second-line drug? – will probably never be solved, the future preference may be continuation MT, at least if the concept is confirmed in the final OS analysis of the PARAMOUNT study. Pemetrexed continuation may then be the MT option for nonsquamous NSCLC. Gemcitabine continuation MT has been studied in smaller trials, and therefore has less strong evidence on its record, but it could be considered as an option for squamous cell cancer.

Nonetheless, MT with the agents used in the two studies with significant OS benefit (pemetrexed and erlotinib) for all patients will probably not remain economically feasible in many healthcare systems. There are no pharmacoeconomic analyses in the original JMEN or SATURN publications. However, with a median OS difference of only 1 month in the SATURN study [37], it is unlikely that an expensive molecularly targeted drug like erlotinib will produce incremental cost per life-year gained results that fall within the benchmark of that parameter in many countries; for example, NICE did not approve for erlotinib MT [41]. On the other hand, NICE – known for its critical appraisal of new drug indications – approved pemetrexed as MT for nonsquamous NSCLC, based on an incremental cost–effectiveness ratio of GBP£47,000 (~€55,000) [42].

So how could clinicians deal with the positive signals in MT studies of the last decade in a rational way, when faced with a patient in disease control after first-line doublet chemotherapy? As mentioned above, advanced NSCLC is a noncurative setting, where the aim is either to prolong OS without negative effects on QoL, or to prolong PFS if associated with clearly documented

Table 4. Hazard ratios for progression-free survival in recent maintenance studies according to the evaluation after first-line chemotherapy.

Study (year)	HR for PFS in responders	HR for PFS in stable disease	Ref.
Perol *et al.* (2010) IFCT-GFPC/gemcitabine	0.44	0.68	[23]
Paz-Ares *et al.* (2011) PARAMOUNT	0.48	0.74	[25]
Ciuleanu *et al.* (2009) JMEN	0.81	0.61	[33]
Capuzzo *et al.* (2010) SATURN	0.94	0.72	[36]
Perol *et al.* (2010) IFCT-GFPC/erlotinib	0.80	0.85	[24]

HR: Hazard ratio; IFCT-GFPC: Intergroupe Francophone de Cancerologie Thoracique/Groupe Français de Pneumocancérolgie; PFS: Progression-free survival.

benefits in symptom control or QoL. Thus, on the one hand, there is the opportunity to give the patient a drug-, toxicity- and cost-free period, but with careful follow-up with adequate and timely use of second-line therapy when needed. On the other hand, there is the argument to use MT to prolong the disease control achieved by first-line therapy, and thereby avoid rapid deterioration of the disease, which may lead to inability to administer second-line therapy when needed.

Thus, information on who are the best candidates for MT is most welcome. Several factors in the available data point at subgroups that may have a balance in favor of MT. A first factor is fitness of the patient: individuals with low PS derived little or no benefit in several trials, probably because they are more vulnerable to the toxicity of MT [18,24]. In one trial with a majority of low PS patients, the MT with gemcitabine resulted in no benefit at all, with HRs close to 1 both for PFS and OS [22]. A second factor is the nature of the tumor: patients with a large tumor burden at the end of first-line chemotherapy are more likely to benefit from switch MT, while those with little remaining tumor and no symptoms at all may be better candidates for continuation MT. Histology matters as well, as gemcitabine or erlotinib can be used for all NSCLC histologies, while pemetrexed is restricted to nonsquamous tumors. Finally, for the choice between 'second-line agent at the time of relapse', or 'use of this agent in a switch MT strategy', some indicators of probability of not receiving a second-line therapy have been described recently: a large amount of tumor initially (expressed as >70 mm sum of lesions according to RECIST), no response after first-line therapy, or low PS (2 or 3) [43]. Treatment of the latter patients is difficult anyway, as they perform poorly in continuation MT studies [22], while low PS patients were not included in recent switch MT trials [34,37].

A possibly emerging trend when looking at the HR for the primary end point PFS in the data is that continuation MT seems to be better for patients with a response to first-line therapy, while switch MT maybe preferred for those with stable disease (with the exception of the erlotinib-treated patients in the IFCT-GFPC study, Table 4). At a first view, there may be some logic in this as it is in line with the simple wisdom 'never change a winning team' and 'switch gears when unsatisfied'. We should, however, be careful with this interpretation. First, while there are differences between HRs for PFS in subanalyses of response versus stable disease, none of these had a statistically significant interaction. Second, response rate is an end point to be used for assessment of activity of a new drug in Phase II trials, but not as a parameter in Phase III trials, nor for clinical decisions in a patient. Finally, the distinction between response and stable disease is not always easy, as the reproducibility of tumor measurements is far from optimal in study settings [44], let alone in the standard clinical practice setting.

Aside from the fact of how we could deal with the data from MT studies of the last decade, in the noncurative context of advanced NSCLC, we also want to emphasize that we should

Key issues

- Maintenance therapy (MT) is a new treatment paradigm that has improved prospects of patients with advanced non-small-cell lung cancer (NSCLC), aside from histological subtyping for the choice of chemotherapy, and the use of molecular markers to select patients for targeted therapy.
- Effective and well-tolerated MT after first-line chemotherapy provides longer disease control, which ideally should be translated into improved overall survival, along with acceptable toxicity for the patient.
- MT can be the continuation of an agent already used in the first-line treatment, or the switch to another non-cross-resistant agent.
- Modern continuation MT studies have convincingly demonstrated good tolerability of MT with agents such as gemcitabine or pemetrexed, with significant and clinically relevant improvements in progression-free survival, and some overall survival benefits, which until now did not reach statistical significance.
- Switch MT studies with agents such as pemetrexed and erlotinib have demonstrated significant and clinically relevant benefits in progression-free survival and overall survival, but the imbalance in the exposure of the tumor to the non-cross-resistant agent in the MT – arm (all) versus control arm (very few) complicates the interpretation.
- In the same way that a 'one size fits all' strategy for first-line chemotherapy in advanced NSCLC is no longer correct, MT is unlikely to benefit all patients. Several factors, such as performance status, histology and response to first-line treatment, may help in a rational use of MT; that is, in selection of patients who will most benefit from either continuation or switch MT.
- As the choice for MT fits in the noncurative setting of advanced NSCLC, its benefits should be weighed by the patient against a drug- and toxicity-free period. Owing to the lack of data on the patients' attitudes towards MT, more research on this aspect is needed.

listen to the opinion of the patient, in an open discussion of the pros and cons of the MT approach. The views of our patients on benefits such as 'delay of disease' or 'longer life' versus more hospital visits and potential toxicity of prolonged treatment do not necessarily coincide with our estimation of PFS or OS end points in clinical trials. Research on this aspect of MT is just emerging [45], and there is a clear need for more data from the patients' perspective.

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Three colleagues with sarcoidosis?

To the Editors:

Bilateral pulmonary nodules are a relatively common finding in thoracic radiology and hence are an important problem that chest physicians often face. Aetiology can be diverse, ranging from neoplastic conditions to infectious lesions. An accurate and prompt diagnosis is needed in order to target the most appropriate and least harmful treatment. We report the case of a 49-yr-old, Caucasian, otherwise healthy male patient with bilateral lung nodules and an unusual diagnosis.

The patient had an unremarkable history and was referred due to asymptomatic bilateral lung nodules. He had stopped smoking 6 months earlier and had a normal chest radiograph a year before referral, taken for follow-up of benign colon polyps. At the time of presentation he was not on any medication.

The patient was an engineer working in a Belgian high-tech facility. His job mainly consisted of office work with occasional visits to production sites where coating of medical material was performed. When visiting these sites, he did not systematically wear a protective mask. The problems occurred after a business trip to the USA where he met with three colleagues. He first visited his general practitioner with a flu-like syndrome. Other symptoms included a productive cough, night sweats and loss of weight (5 kg in 2 weeks). He was treated symptomatically with paracetamol. Two of his colleagues developed the same complaints. One never visited a doctor but the other did and was diagnosed with sarcoidosis based on the finding of non-necrotising granulomatous lesions in transbronchial biopsies. He was put on steroids and his condition improved. Oddly, the third colleague who did not visit the doctor had an existing diagnosis of sarcoidosis but remained asymptomatic.

Due to persistent cough and low-grade fever, our patient was subsequently referred to a pneumologist. Biochemistry revealed an inflammatory syndrome (C-reactive protein 4.6 $mg \cdot dL^{-1}$, sedimentation rate 28 $mm \cdot h^{-1}$) and normal tumour markers, angiotensin-converting enzyme (ACE) levels and anti-nuclear factor (ANF). *Mycoplasma pneumoniae* serology was compatible with a recent infection. Computed tomography (CT) of the chest showed bilateral lung nodules, some with cavitation, in a subpleural and more central distribution. Extensive mediastinal lymphadenopathy was also present. Bronchoscopy revealed a normal bronchial tree. Bronchoalveolar lavage was neutrophilic (60%) with no bacterial pathogens and a normal cytology. Pulmonary function tests were normal (vital capacity 4.9 L, forced expiratory volume in 1 s (FEV_1) 4.4 L, FEV_1/forced vital capacity (FVC) 88%, total lung capacity 9.2 L, diffusion capacity 99%). Atypical pneumonia was diagnosed and the patient was treated with doxycycline 200 mg daily for 10 days.

2 months after initial presentation the symptoms disappeared and biochemistry normalised. The lung nodules persisted but the cavitations and adenopathy regressed. The patient was then referred to our centre for a second opinion. At that time he was completely asymptomatic and the initial weight lost had been regained. Our differential diagnosis included chronic berylliosis, sarcoidosis and, less likely, malignancy and opportunistic infections. A lymphocyte transformation test (LTT) performed for berylliosis was negative. Bronchoscopy with lavage, brushing and biopsies was repeated and showed a normal cell count and differentiation, a CD4/CD8 ratio of 3.8 and no bacterial pathogens on cultures. Bronchial brushing and biopsies were normal. HIV test was negative.

5 months after his business trip the patient remained asymptomatic. No further change of the radiological picture was noted. Exposure to beryllium seemed very unlikely since the LTT was negative. Given the uncertainty of the diagnosis, a lung biopsy was performed. This revealed necrotising granulomas with a positive Grocott methenamine silver (GMS) stain (fig. 1). A serological test for *Histoplasma capsulatum* antibodies confirmed

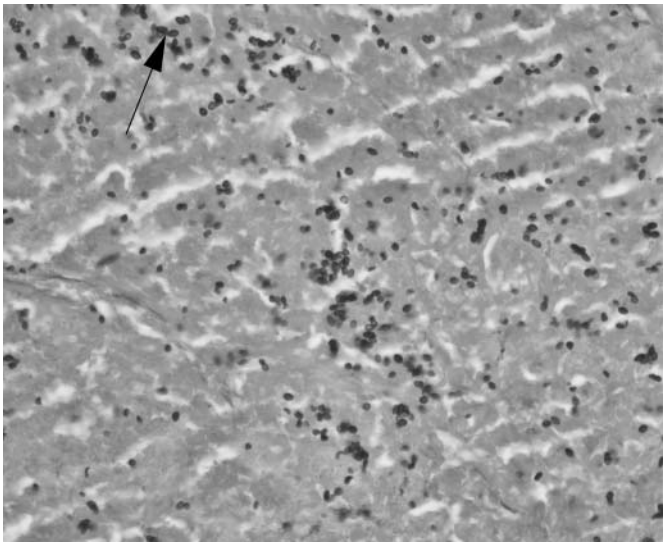

FIGURE 1. Necrotising granuloma from lung biopsy specimen. Small ovoid yeasts, some with narrow-based budding (arrow), representing *Histoplasma capsulatum* in a background of necrosis. Grocott methenamine silver stain. Original magnification ×200.

pulmonary histoplasmosis. Our patient did not receive any specific treatment and remained clinically stable 6 months after initial presentation. Intriguingly, retrospective examination of the histopathological samples showed the presence of the same micro-organism in our patient's colleague who was diagnosed with sarcoidosis and treated with steroids. His clinical improvement seemed unexpected as steroids are known to depress the cellular immunity, which would facilitate the infection with *H. capsulatum* [1]. We did not ask to review the transbronchial biopsies of the third colleague as his diagnosis of sarcoidosis was prior to the business trip and he remained asymptomatic thereafter.

Granulomas are amongst the most common pathological findings in pulmonary medicine; yet diagnosis often remains challenging due to incomplete clinical data and the difficulty of interpreting some histological features. Granulomas are compact aggregates of histiocytes (macrophages). They may also contain necrosis (necrotising granuloma), lymphocytes, plasma cells or multinucleated giant cells [2]. Infections are the first cause of granulomatous lung disease, with mycobacteria and fungi as the two most common infectious agents. Sarcoidosis is a primary non-infectious cause, but other causes include chronic berrylium disease, hypersensitivity pneumonitis and, less frequenly, hot tub lung and Wegener's granulomatosis [2].

H. capsulatum is a dimorphic fungus existing as a mould in the soil and as a yeast at body temperature [1, 3]. Soil containing bat or bird droppings is the reservoir. The fungus is endemic to certain regions such as North and Central America, but also to Africa, Southern Europe and South-Eastern Asia. Exposure in these locations is extremely frequent but symptomatic infection is less common and depends on the balance between cell-mediated immunity and infectious burden. In an immunocompetent host, a large inoculum is needed to cause the disease, while immunocompromised patients are at risk of developing it with a much smaller inoculum. Contamination occurs with inhalation of the microconidia that reach the alveoli and infect the macrophages before spreading *via* the reticuloendothelial system [1, 3].

Clinical pictures can be acute or chronic, local or disseminated and possibly complicated. The acute pulmonary forms are a mostly mild pneumonia mimicking an atypical pneumonia and are thus often treated as such, as in our patient. Severe forms also exist and can lead to acute respiratory distress syndrome (ARDS). Chronic cavitary pulmonary histoplasmosis occurs generally in patients with emphysema, resembling a reactivation of tuberculosis. Complications of these pulmonary forms include granulomatous mediastinitis, fibrosing mediastinitis and pericarditis, but these are less common. Disseminated histoplasmosis can be either acute, leading to a sepsis-like illness, or chronic. Immunocompromised patients develop acute illness. Chronic disseminated histoplasmosis is seen in older, mostly male patients with underlying emphysema but normal immunity [1].

Diagnosis is *via* careful review of patient history and can be confirmed using various tests. The test used depends on the clinical syndrome [1, 2, 4]. For chronic histoplasmosis, serology is the test of choice. Complement fixation and immunodiffusion techniques can both be used. Antibody production takes 4–8 weeks, however, and repeating the test might be necessary in case of strong clinical suspicion and initial negative serology. False-positive serology can be seen in lymphoma, tuberculosis, sarcoidosis and other fungal infections. A persistent low-positive titre can also be noted years after exposure and therefore does not always correspond to active infection [1]. Culture of respiratory specimen is also indicated in chronic pulmonary forms but pathogen growth may take weeks. In acute and particularly disseminated forms, antigen detection is rapid. Urinary testing has the best sensitivity. PCR techniques are not yet reliable. Histopathology can be divided into three main types [2]. The first is an intra-alveolar lymphohistiocytic infiltrate with small granulomas and variable necrosis, mainly observed in acute pulmonary histoplasmosis. The second shows well-formed, necrotising granulomas surrounded by a rim of epithelioid histiocytes and a fibrotic capsule. These lesions, also called ''histoplasmomas'', are often resected in the work-up of a solitary pulmonary nodule [2, 5]. The third histological subtype is mostly observed in disseminated disease and consists of heavily infected histiocytes within the interstitium [2, 6]. *H. capsulatum* are usually not visible in granulomas on haematoxylin–eosin stains but GMS stains enlighten small, uniform, ovoid organisms, sometimes typically narrow-based [1, 4].

Treatment is only proven to be effective in severe pulmonary and disseminated forms. The agent of choice is amphotericin B for the most severe syndromes. It is recommended to use the liposomal form rather than the deoxycholate because of the lower nephrotoxicity. Itraconazole is used as a maintenance drug and in milder cases. Treatment can last from weeks to months, depending on the severity of the infection [1, 7].

In conclusion, this case highlights the importance of pathology and reminds us of the endemically important differential diagnoses for sarcoidosis. It also proves that, in all countries, the first step when analysing lung granulomas should be to rule out infection.

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An unusual presentation of sarcoidosis with tetraplegia and severe osteolytic bone lesions

To the Editors:

Sarcoidosis is a granulomatous multisystem disorder of unknown aetiology. Pathology is characterised by non-necrotising granulomas, which may affect virtually any organ, most commonly the lungs, lymph nodes, skin and eyes. Lytic foci in the phalanges of the hands and feet are common, whereas it is rare to see a widespread effect to the bone.

We present a case of severe diffuse osteolytic bone involvement and tetraplegia presenting as the first manifestation of sarcoidosis.

A 54-yr-old male was admitted to hospital after a bike accident, presenting with total paralysis of his left arm and paresis of his right arm and both legs. Computed tomography (CT) revealed fracture of C2 and widespread osteolytic lesions in the vertebrae, sternum, clavicles, skull, costae and pulmonal nodular infiltrates. The patient had no respiratory complaints.

Positron emission tomography with ^{18}F-fluoro-2-deoxy-D-glucose (FDG-PET)/CT showed areas of pathological uptake that would suggest malignancy, but biopsy from the bone marrow showed non-necrotising granulomas compatible with sarcoidosis. The suspicion of cancer was maintained due to the rareness of osteolytic sarcoidosis, but repeated biopsies from os ileum and os parietale confirmed the diagnosis of sarcoidosis, and prednisolone and methotrexate were initiated. A stabilising operation of the cervical spine was performed; 2 months later, another operation with laminectomy at C1 level was necessary for decompression of the spinal cord.

At the time of diagnosis, pulmonary function tests showed a forced expiratory volume in 1 s (FEV1) of 1.8 L (43% of predicted), rising to 2.9 L (69% pred) 6 months later. FEV1/forced vital capacity (FVC) was normal; total lung capacity (74% pred) and diffusion capacity (76% pred) were both slightly decreased and did not improve with treatment.

Interleukin (IL)-2 receptor was elevated to 1,180 kU·L^{-1} (normal range 223–710 kU·L^{-1}), whereas angiotensine-converting enzyme (ACE) was normal. There was a light hypercalcaemia (1.43 mmol·L^{-1}), slightly elevated C-reactive protein (134 nmol·L^{-1}) and a mild normochrome, normocytic anaemia (7.5 mmol·L^{-1}). These parameters were normalised after a few months of treatment.

Response to treatment was monitored with serum ACE, IL-2 receptor and bone scintigraphy. FDG-PET/CT and bone scintigraphy were performed early in the course of disease, and showed excellent correspondence (figs 1 and 2). Bone scintigraphy was chosen for monitoring.

Remission of the scintigraphic bone manifestations began after more than 1 yr of treatment, and was almost complete after 2 yrs. IL-2 receptor fell to normal levels within 2 months.

Prednisolone was gradually tapered and finally stopped after nearly 2 yrs of treatment. Immunosuppressive treatment was completed with intravenous biphosphonate (zoledronic acid), administered yearly for 3 yrs, and daily supplementation of calcium and vitamin D.

The patient underwent intensive rehabilitation after the operation with very satisfactory results. After 3 months, he was able to walk again, and he resumed work 2 yrs after the diagnosis. At the time of writing, the patient was still taking methotrexate and the disease was well controlled; methotrexate treatment was planned to be ceased during the coming months.

Bone involvement is frequent in sarcoidosis, and is seen in up to 13% of patients [1]. It is usually a late manifestation and is often associated with chronic pulmonary, cutaneous or multivisceral sarcoidosis. The most common manifestations are asymptomatic lytic lesions of the phalanges of the hands and feet [2]. Involvement of the vertebral column, the skull and the