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Association between antibiotic-immunotherapy exposure ratio and outcome in metastatic non small cell lung cancer

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ABSTRACT

Objectives: Immunotherapy (IO) is effective in metastatic Non Small Cell Lung Cancer (NSCLC). Gut microbiota has an impact on immunity and its imbalance due to antibiotics may impair the efficacy of IO. We investigated this topic in a case series of NSCLC patients treated with IO.

Materials and Methods: Data about all metastatic NSCLC patients treated with IO between 04/2013 and 01/2018 were collected. Patients were stratified according to antibiotic use during the Early IO Period (EIOP), and according to the Antibiotic-Immunotherapy Exposure Ratio (AIER) defined as "days of antibiotic/days of IO" during the Whole IO Period (WIOP). Survival was estimated using the Kaplan-Meier method. Log-rank test was used to compare the curves. Multivariate analyses were performed with the Cox model.

Results: We analyzed 157 patients. Forty-six patients received antibiotics during the WIOP, 27 patients during the EIOP. No differences in either Progression-Free Survival (PFS) or Overall Survival (OS) were observed according to antibiotic use during the EIOP ($p = 0.1772$ and $p = 0.2492$, respectively). Considering the WIOP, median AIER was 4.2%. The patients with a higher AIER had worse PFS ($p < 0.0001$) and OS ($p = 0.0004$) than the others. Results were significant also after correction for the IO line ($p = 0.0018$ for PFS) and performance status ($p < 0.0001$ for PFS, $p = 0.0052$ for OS).

Conclusion: Although no difference in outcome were observed with antibiotic use in the EIOP, a detrimental effect became evident for patients with a higher AIER in the WIOP. If its relevance is confirmed, AIER may become an innovative variable for estimating the impact of antibiotics on IO efficacy.

1. Introduction

Metastatic Non Small Cell Lung Cancer (NSCLC) is the leading cause of cancer-related deaths worldwide [1].

Only a limited subgroup of patients harbors genetic alterations amenable of targeted treatments (*i.e. EGFR*, *ALK*, *ROS*-1, *MET*). For the remaining ones, platinum-based chemotherapy was the only available option until a few years ago [2].

In the last years, different clinical trials showed superiority of immunotherapy (IO) over standard chemotherapy in terms both of efficacy and toxicity, in metastatic NSCLC [3–8]. This has led first to the approval of anti-PD1 and anti-PD-L1 agents in second and more advanced lines of therapy [3–7], then in first line setting [8]. Many other compounds with similar or slightly different mechanism of action are currently under investigation, with promising results [9–15]. These studies will probably lead to an expansion of the indications for IO, likely also extending to non-metastatic stages of disease and to combinations with other immunotherapeutic or cytotoxic agents [9–15]. Despite such promising premises, only 25–30% of patients appear to derive a durable benefit from IO and the event of primary resistance, up to its worst expression arising as hyper-progression, is well known [16]. This variability strengthens the importance to identify predictive factors of response to IO [17–22].

Emerging evidence suggests that not directly tumor-associated factors may contribute to the response to cancer therapy. Bacteria inhabiting the gut, collectively named as gut microbiota, maintain host

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physiology and health by exerting fundamental functions, spanning from metabolic to immunomodulatory properties [23].

It was recently demonstrated that gut microbiota constitutes one of the environmental factors affecting response to chemotherapeutic and immunotherapeutic drugs through its ability to regulate the immune response. Indeed, a clear cause-effect relationship between the composition of gut microbiota and the efficacy of both chemotherapy and IO has been showed in mouse models [24–27]. Accordingly, antibiotic-induced dysbiosis in tumor-bearing mice has been associated with the failure of IO containing anti-CTLA4 and anti-PD1 antibodies [26–28]. Moreover, fecal microbiota transplantation of mice with stool of responders and non-responders to IO transfers the capability to respond or not to IO [26,28,29]. In addition to these experimental findings, analyses in human cohorts of metastatic patients affected by different malignancies showed that the use of broad-spectrum antibiotics which are known to severely reduce the bacterial diversity and the function of intestinal flora, around the beginning of IO, has a detrimental effect on patient response and Progression-Free Survival (PFS) [28,29]. In particular, Derosa et al. in a retrospective cohort of patients with metastatic renal cell carcinoma and NSCLC treated with IO, showed that the administration of antibiotics within one month before the beginning of treatment has a detrimental effect on Response Rate (RR) and PFS [29]. Routy et al. confirmed this evidence in a large cohort of patients with different malignancies (NSCLC, renal and urothelial carcinomas), showing that cases receiving antibiotics between 2 months before and 1 month after the first IO administration had worse PFS and OS than their non-treated counterparts. The same researchers performed a molecular characterization of microbiota through shotgun sequencing of stool DNA, finding that the clinical response to Immune Checkpoint Inhibitors (ICIs) is correlated to the abundance of *Akkermansia muciniphila*. These data were subsequently confirmed in mouse models, in which the transplant of a fecal microbiota rich in *Akkermansia* and *Alistipes* induced enhanced response to ICIs potentiating T-mediated response [28]. Another work by Gopalakrishnan et al. prospectively studied patients with metastatic melanoma treated with IO. The patients were classified as responders if they achieved at least disease stability for 6 months, or as non-responders, provided that they showed progressive disease within the first 6 months. The researchers identified significant differences in the composition of bacterial flora between the two subgroups, with a predominance of *Clostridiales, Fecalibacterium* and *Ruminococcaceae* in the stool specimens obtained from responders, and a prevalence of *Bacteroidales*, *Escherichia* and *Anaerotruncus* in the stool specimens obtained from non-responders. Again, these results were confirmed and replicated in germ-free mice, which achieved a better response to IO after fecal transplants with stool specimens of responding patients [30]. A fourth work by Matson et al. analyzed a similar population of melanoma patients treated with IO reporting that cases showing an objective tumor response had basal stool samples enriched in *Bifidobacterium longum, Collinsella aerofaciens* and *Enterococcus faecium.* Germ-free mice transplanted with these specimens showed improved response to anti-PD-L1, with an increase in T-cell infiltrates in tumor masses [31].

We aimed to investigate the association between the antibiotics use and response to IO in patients with metastatic NSCLC treated with IO at our Institution. The impact of antibiotics on outcome was analyzed considering not only the simple antibiotic use in the Early IO Period (EIOP), but also the cumulative exposure to antibiotics in the Whole IO Period (WIOP). For this purpose, we coined a new variable called Antibiotic-Immunotherapy Exposure Ratio (AIER), defined as “days of antibiotic/days of IO” during the WIOP.

2. Materials and methods

2.1. Patients

We analyzed data about all consecutive patients with metastatic NSCLC treated with ICIs at Istituto Nazionale dei Tumori, Milan, Italy, between April 2013 and January 2018.

All the patients signed a written informed consent declaring their agreement to the use of personal data for research purposes at some time of their disease history.

Clinical data were retrospectively collected from institutional database and included age, gender, performance status (PS) according to Eastern Cooperative Oncology Group (ECOG) [32], smoking habits, histology, tumor molecular characterization whenever performed, line of IO, ICI prescribed, response to IO according to Response Evaluation Criteria for Solid Tumors (RECIST) [33], number of metastatic sites at the beginning of IO, and details of antibiotic use (including specific drug, route of administration, dosage, and duration).

For the purpose of the analysis concerning the EIOP, antibiotic use was considered relevant when it happened between 1 month before and 3 months after starting IO. Patients receiving antibiotics outside this time frame were considered as non-antibiotic treated patients. The AIER was defined as the rate “days of antibiotic therapy/days of IO” during the WIOP. First, the AIER was considered as a continuous variable for statistical analyses. Then, the median value of AIER was used as cut-off and patients with an AIER lower than the cut-off were included in the control group as those never receiving antibiotics.

All ICIs were considered, irrespective of mechanism of action. Cases who received only the first dose of drug and then discontinued for toxicity, worsening of general conditions or death were excluded from the analysis. Patients receiving at least two doses of ICI and then discontinued treatment for unequivocal clinical progression were included in the analyses and accounted as patients with progressive disease at first evaluation.

2.2. Statistical analysis

Descriptive statistics were used to analyze and report clinical variables.

All patients were followed until death, loss at follow-up or time of data lock, which was set on the 1st March 2018. PFS was calculated as the time from the beginning of IO to the time of disease progression, or death for any cause. Disease response was assessed using RECIST, at the last version applicable at the time of IO administration (ranging from 2013 to 2018). Overall survival (OS) was calculated as the time from the beginning of IO to death for any cause. Alive patients were right-censored at the time of last contact. PFS and OS were estimated using the Kaplan-Meier method. Duration of follow-up was calculated using the reverse Kaplan-Meier method. Differences between survival curves were analyzed with the log-rank test.

Fisher’s exact test was used to compare discrete qualitative variables. Differences between continuous ordinal variables were tested using the Mann-Whitney non-parametric U test. Univariate analyses were performed according to sex (male *versus* female), age ($<$ 70 years *versus* $\geq$70 years), smoking status (current or former smoker *versus* never smoker), tumor histology (squamous NSCLC *versus* non-squamous NSCLC), ICI (anti-PD1 *versus* anti-PD-L1 *versus* anti-CTLA4 and combinations), line of IO (first *versus* second *versus* third or more), basal ECOG PS (0 *versus* 1 or 2), and number of metastatic sites at the beginning of IO (0 or 1 *versus* 2 or more). χ square test was used for univariate analyses. Cox proportional hazard model was applied for multivariate analyses, which were calculated for the significant variables at univariate test. All analyses were two-sided and values of $p < 0.05$ were considered statistically significant.

Statistical analyses were performed using SAS (version 9.4, SAS Institute, Cary, NC, USA).

3. Results

3.1. Patients and IO treatment characteristics

A total of 157 patients with NSCLC were identified. The population included 94 men and 63 women. Median age was 66.7 years (range: 30.6–86.5 years). One hundred thirty-seven patients (87.2%) were current or former smokers.

Non-squamous NSCLC accounted for 121 cases (77.1%) and included mostly adenocarcinomas, but also a limited number of sarcomatoid, adeno-squamous, intestinal-like and not otherwise specified lung cancers. PD-L1 expression level was high in 44 patients (28.0%), negative in 46 patients (29.3%), low in 3 patients (1.9%), unknown in 64 patients (40.8%). Only 7 tumors (4.5%) harbored an *EGFR* mutation; *EGFR* was unknown in 27 patients (17.2%). *ALK* rearrangement was found in one patient (0.6%); data were missing in 29 (18.4%) patients. Neither cases of *ROS*-1 rearrangement nor *BRAF* mutations were found.

PS ECOG at the beginning of IO was 0 in 47.8% of cases, 1 in 45.2%, 2 in 7.0%.

Most of the patients (86.0%) had 2 or more metastatic sites at the time of IO start. The most common metastatic sites were lymph nodes (133 cases), lung (78 cases), bones (67 cases), liver and/or adrenal glands (55 cases), brain (32 cases). Patient characteristics are summarized in Table 1.

The majority of patients (98 of 157, 62.4%) received treatment with anti-PD1 (nivolumab in 88 cases, pembrolizumab in 10 cases); 33.1% of patients received an anti-PD-L1 (durvalumab in 31 cases, atezolizumab in 16 cases, avelumab in 4 cases, MSB0011359C in 1 case); 1 patient received an anti-CTLA4 (tremelimumab); 6 patients received a combined IO (durvalumab + tremelimumab).

IO was administered as first line treatment in 25 cases (15.9%), second line treatment in 66 cases (42.0%), third line treatment in 44 cases (28.0%) and as more advanced line of therapy in 22 cases (14.1%).

Stable disease was reported in 52 patients (33.1%), partial response in 34 (21.7%); only 1 complete response was documented (0.6%), while the remaining patients (70, 44.6%) underwent disease progression.

Median time to best response was 1.9 months (range: 0.1–17.5 months).

Eighty-two patients (52.2%) discontinued IO due to disease progression, 30 (19.1%) for physician's decision as a consequence of clinical deterioration, 10 (6.4%) after regular conclusion of the planned treatment program, one (0.6%) for patient's withdrawal of consent. IO was still ongoing at the time of database lock in 34 cases (21.7%).

Median duration of IO was 4.2 months (range: 0.2–91.0 months).

Table 1
Patient characteristics.

	N[1]	%
Total	157	100.0
Gender		
Male	94	59.9
Female	63	40.1
Age, median (range) [years]	66.7 (30.6-86.5)	–
Basal PS[2] (at IO[3])		
0	75	47.8
1	71	45.2
2	11	7.0
Number of metastatic sites (at IO)		
1	22	14.0
≥2	135	86.0
Sites of metastases (at IO)		
Lymph nodes	133	84.7
Lung	78	49.7
Bone	67	42.7
Other viscera	55	35.0
CNS[4]	32	20.4
Smoking history		
Former / current smoker	137	87.2
Never smoker	20	12.8
Tumor histology		
Non-squamous NSCLC[5]	121	77.1
Squamous NSCLC	36	22.9
***EGFR* status**		
Mutated	7	4.5
Wild type	123	78.3
Unknown	27	17.2
***ALK* status**		
Rearranged	1	0.6
Wild type	127	80.9
Unknown	23	18.5
PD-L1 expression		
High	44	28.0
Low	3	1.9
Negative	46	29.3
Unknown	64	40.8

[1] Number.
[2] Performance Status.
[3] Immunotherapy.
[4] Central Nervous System.
[5] Non Small Cell Lung Cancer.

3.2. Antibiotic treatment characteristics

A total number of 46 patients (29.3%) received at least one administration of antibiotic through the WIOP.

Twenty-seven patients (17.2%) received antibiotics during the EIOP. In 7 cases (25.9%), more than one antibiotic was subsequently administered. All patients received single agent antibiotic therapy.

Considering all the 46 patients receiving antibiotics, the prescription was due to a respiratory tract infection in almost all cases. Four patients (8.7%) received an antibiotic for a reactivation of diverticular bowel disease, one for colitis, and one for urinary tract infection; all these cases were also treated with a different antibiotic for pneumonia.

According to the etiology, the most commonly prescribed antibiotic was levofloxacin (30 cases, 65.2%), followed by amoxicillin/clavulanate (10 cases, 21.7%), claritromicin (5 cases, 10.9%), ceftriaxon (4 cases, 8.6%), rifaximin (4 cases, 8.6%), ciprofloxacin (3 cases, 6.5%), and azitromicin (3 cases, 6.5%).

Antibiotics were administered by oral route in 44 cases (95.7%), by intra-muscular route in 3 cases (6.5%), and by intra-venous route in 2 cases (4.4%).

The median duration of single cycle antibiotic was 6 days (range: 2–17 days). Considering the cumulative duration of the antibiotic treatment, thus summing the length of single cycles, the median value was 7.0 days (range: 5.0–33.0 days).

Considering the WIOP, the median AIER was 4.2% (range: 0.6–42.9%). Twenty-three patients (14.7% of the global population) had an AIER higher than the median one. The median duration of antibiotic treatment was significantly different between the 2 subgroups defined by this cutoff (9 *versus* 6 days, p = 0.0087). All other main clinical and pathological variables were balanced between the 2 subgroups (Table 2).

3.3. Outcome results

Median follow-up was 28.6 months.

Antibiotic administration in the EIOP was not associated either with reduced RR (11.1% *versus* 24.6%, p = 0.2018) or with reduced Disease Control Rate (DCR) (51.9% *versus* 56.2%, p = 0.8319).

After stratifying the global population according to the AIER in the WIOP, patients with a higher AIER than the median (4.2%) had a RR of 8.7%, while the RR in the control group was 26.6%. The corresponding DCR was 47.8% and 56.0%, respectively. These differences were not statistically significant (p = 0.1082 and p = 0.5030, respectively).

Median PFS and OS of the global population were 3.0 months (95% confidence interval (CI) 2.3–3.8 months) and 11.3 months (95% CI

Table 2
Clinical and pathologic characteristics according to AIER.

	High AIER[1], N[2] (%)	Low AIER, N (%)
Total	23 (14.7)	134 (85.3)
Gender		
Male	16 (69.6)	78 (58.2)
Female	7 (30.4)	56 (41.8)
	p 0.3624	
Basal PS[3] (at IO[4])		
0	6 (26.1)	69 (51.5)
1	15 (65.2)	56 (41.8)
2	2 (8.7)	9 (6.7)
	p 0.0764	
Number of metastatic sites (at IO)		
1	3 (13.1)	19 (14.2)
≥2	20 (86.9)	115 (85.8)
	p 1.0000	
Smoking history		
Former / current smoker	20 (86.9)	117 (87.3)
Never smoker	3 (13.1)	17 (12.7)
	p 1.0000	
Tumor histology		
Non-squamous NSCLC[5]	17 (73.9)	104 (77.6)
Squamous NSCLC	6 (26.1)	30 (22.4)
	p 0.7886	
PD-L1 expression		
High	4 (17.4)	38 (28.4)
Low	2 (8.7)	1 (0.7)
Negative	6 (26.1)	40 (29.9)
Unknown	11 (47.8)	55 (41.0)
	p 0.8303	
Line of IO		
First	2 (8.7)	23 (17.2)
Second	13 (56.5)	53 (39.6)
Third	6 (26.1)	38 (28.4)
More advanced	2 (8.7)	20 (14.8)
	p 0.4221	

[1] Antibiotic-Immunotherapy Exposure Ratio.
[2] Number.
[3] Performance Status.
[4] Immunotherapy.
[5] Non small Cell Lung Cancer.

8.8–15.2 months), respectively.

At univariate analyses, no impact on PFS was evidenced for smoking status, ICI mechanism of action, gender and age. A significant detrimental effect on PFS was observed for basal PS ≥ 1 and for second or more advanced IO lines. Similarly, no significant differences in median OS were seen when stratifying patients according to smoking status, ICI mechanism of action, gender, age, and IO line. The only variable negatively impacting on OS was the basal PS ($p < 0.0001$) (Table 3).

Regarding the antibiotics effects, the use of one or more anti-microbic agents in the EIOP did not influence either PFS (3.3 months in non-treated patients *versus* 2.2 months in treated patients; $p = 0.1772$) or OS (11.9 months for non-treated patients *versus* 5.9 months for treated patients; $p = 0.2492$), although a numeric trend towards a better prognosis was seen for cases not receiving antibiotics (Table 3 and Fig. 1).

When considering a different time cutoff of 2 instead of 3 months after the first ICI administration, the results did not change (median PFS 3.35 in non-treated patients *versus* 2.2 in treated patients; $p = 0.0992$; median OS 11.9 months in non-treated patients *versus* 5.6 months in treated patients; $p = 0.2147$).

In the WIOP, a higher AIER appeared to have a detrimental effect on PFS and OS when considered as a continuous variable (hazard ratio (HR) 1.053, $p = 0.0029$ for PFS; HR 1.069, $p < 0.0001$ for OS).

Performing the evaluation of the AIER using the cutoff value of 4.2%, a significant difference became evident in terms of both PFS (3.5 months in patients with AIER < 4.2% *versus* 1.9 months in patients with AIER ≥ 4.2%, $p < 0.0001$) and OS (13.2 months in patients with AIER < 4.2% *versus* 5.1 months in patients with AIER ≥ 4.2%, $p = 0.0004$) (Table 3 and Fig. 2).

At multivariate analyses, the impact of AIER on PFS retained significance after correction for the effects of basal PS (HR 1.053, $p = 0.0052$) and IO line (HR 1.059, $p = 0.0018$). Similarly, multivariate analyses confirmed that the detrimental effect of high AIER on OS was independent from that of PS (HR 1.064, $p = 0.0002$) (Table 4).

4. Discussion

The immune system role in maintaining active surveillance against malignancies has been known for decades [34,35]. Pre-clinical and clinical data have demonstrated that immune-compromised hosts have a higher incidence of tumors that often show an aggressive behavior. Moreover, these subjects usually have a poorer response to treatments and a worse prognosis than immune-competent hosts [36].

The concept of immune activation against tumors has dramatically increased its relevance since ICIs introduction in the clinical practice. Indeed, the rationale for the use of these agents relies on the stimulation of systemic immunity against cancer, leading to an immune-mediated killing of malignant cells. In the last years there has been large increase of the therapeutic indications for IO, which is now part of the standard treatment in various malignancies [37].

In lung cancer, different agents have been approved or are in advanced phase of study for locally advanced and metastatic disease. In many trials ICIs have shown a benefit over traditional cytotoxic agents in terms of survival both in first and in subsequent line of therapy [3–14]. Despite the general validity of this observation, it is also well known that only a limited number of patients really benefit from IO [13,14,17,19–22]. While about one-third of cases show disease response and may expect a quite long survival, the remaining two-thirds never respond to ICIs maintaining a dismal prognosis [13,14,17,19–22].

Given the relevance of this topic, many studies have focused on potential variables able to predict the response to IO. Data obtained with different agents and in different settings have supported a role for PD-L1 expression, tumor mutation burden and microsatellite instability [13,14,17,38–40]. Nonetheless, it is likely that the majority of factors accounting for such a variability still remains unknown. In this field, research has recently been developed about gut microbiota and its imbalances due to antibiotics.

In this work, we performed a single Institution retrospective analysis of a cohort of patients with metastatic NSCLC treated with IO. At first, we considered the administration of antibiotics in the EIOP, significant for stratification purposes. We did not identify any differences in RR, PFS and OS between antibiotic treated and non-treated patients, although a numerical tendency in favor of non-treated cases was evident. Afterwards, when we evaluated the impact of AIER on PFS and OS in the WIOP, this continuous variable showed to have a significant detrimental effect on outcome. Given this results, we performed a second analysis using the median value of AIER as a cut-off for patient stratification. When we stratified the population according to AIER, a significant advantage emerged for patients with an AIER inferior to the median value of 4.2%.

The AIER is a completely new empiric variable, that we proposed in order to explore whether the length of antibiotic therapy in relation to that of IO could be more influential that the timing of antibiotic administration. To our knowledge, this is the first time that a variable influencing the duration of treatment and not its relation to the beginning of IO shows a correlation to outcome. The potential rationale behind this observation may lay in the physiological capability of intestinal flora to regain homeostasis after an alteration. In other words, each antibiotic administration induces an imbalance in bacterial species populating the intestinal tract. After the end of the antibiotic cycle, the commensal bacteria species progressively return to their primitive composition and the alteration induced by treatment is overcome. If the

Table 3
Univariate analyses.

Variables	Subgroups	PFS[1], months			OS[2], months		
		Median	95% CI[3]	p value	Median	95% CI	p value
Population	–	3.0	2.3–3.8	–	11.3	8.8–15.2	–
Gender	Male	3.2	2.1–4.8	0.5980	11.2	7.6–15.6	0.8453
	Female	2.9	2.2–3.9		12.4	7.8–16.0	
Smoking status	Smoker	3.2	2.2–3.8	0.8733	15.7	5.0–32.5	0.4285
	N/smoker	2.9	1.8–6.8		11.2	8.3–14.5	
Age	< 70	2.8	2.0–3.8	0.6743	11.2	73.8–15.7	0.4464
	≥70	3.3	2.3–5.1		11.9	7.4–24.2	
Basal PS[4]	0	4.3	2.9–6.8	0.0002	18.6	13.2–28.6	< 0.0001
	≥1	2.1	1.9–3.0		6.6	5.2–9.9	
ICI[5]	PD1	3.0	2.2–3.9	0.5595	11.2	7.6–15.9	0.6644
	PD-L1/other	3.0	1.9–5.5		13.2	8.3–17.8	
Line of IO[6]	First	6.7	2.7–11.4	0.0479	11.3	6.6–NR[7]	0.5214
	≥Second	2.8	2.1–3.7		11.2	8.6–14.5	
Atb[8] use (EIOP[9])	Yes	2.2	1.8–3.2	0.1772	11.9	9.2–15.6	0.2492
	No	3.3	2.6–4.8		5.9	4.5–22.5	
AIER[10] (WIOP[11])	< 4.2%	3.5	2.6–5.0	< 0.0001	13.2	9.9–5.9	0.0004
	≥4.2%	1.9	1.3–3.0		5.1	3.8–5.9	

1 Progression Free Survival.
2 Overall Survival.
3 Confidence Interval.
4 Performance Status.
5 Immune Checkpoint Inhibitor.
6 Immunotherapy.
7 Not Reached.
8 Antibiotic.
9 Early Immunotherapy Period.
10 Antibiotic-Immunotherapy Exposure Ratio.
11 Whole Immunotherapy Period.

process is limited in time, in particular, if IO is carried on meanwhile, it is likely that the effects of antibiotics on intestinal flora and, consequently, on anti-tumor immune response, is negligible. On the contrary, if a patient receives multiple or prolonged antibiotic cycles, the repeated hits on gut microbiota may interfere with bacterial reconstitution, leading to a deeper impact on systemic immunity. The significant relation between AIER and outcome, irrespective of the effects of PS and IO line, and in absence of a clear role for the simple antibiotic use at the beginning of IO, supports the potential role of AIER as a new independent determinant.

As regards to the lack of association between antibiotic use in the EIOP and outcome, our results differ from most recent research data showing a significant negative impact of early antibiotics use on PFS. We can be made only hypotheses to explain this discrepancy, maybe due to the population characteristics and to the small sample size. In any case, a numerical trend towards superior PFS and OS in patients not receiving antibiotics was identified, in line with literature data. Nonetheless, it has to be underlined that also a previous retrospective work on 74 NSCLC cases treated with nivolumab did not find differences in RR and PFS according to antibiotic use in the 3 months before the beginning of the ICI treatment [41]. This underlines the lack of definitive evidence in the field of microbiota and IO, in particular for

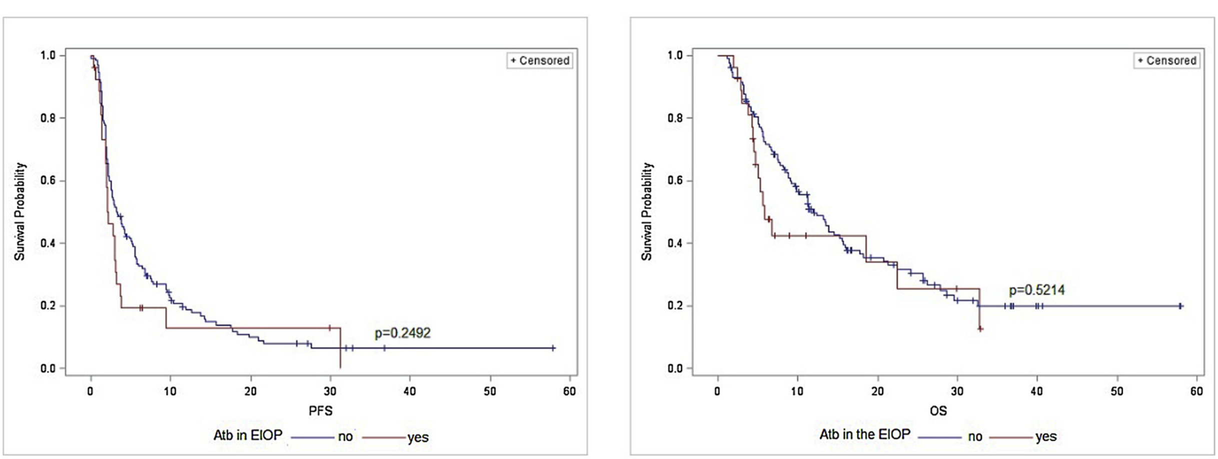

Fig. 1. Kaplan-Meier curves for PFS and OS according to antibiotic use in the EIOP.
PFS = Progression Free Survival.
Atb = Antibiotics.
EIOP = Early Immunotherapy Period.
OS = Overall Survival.

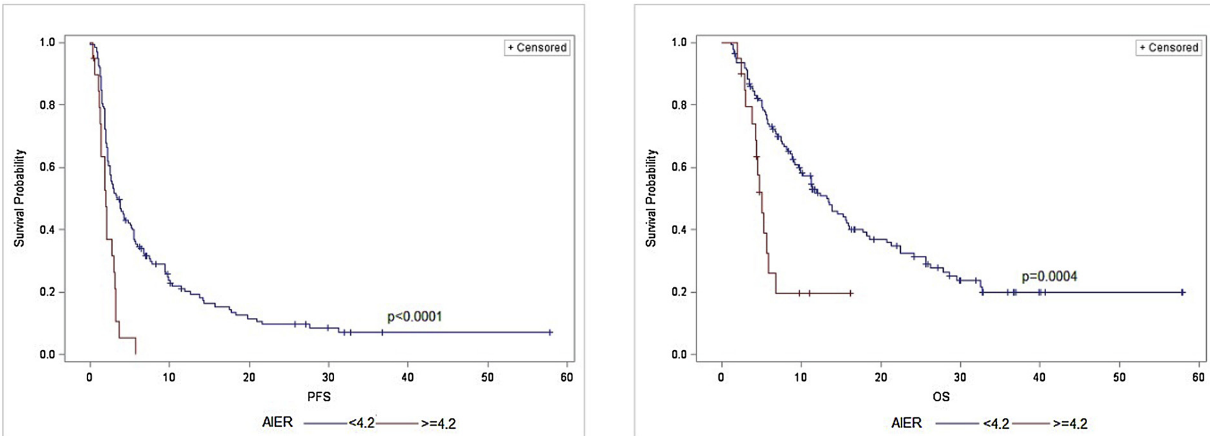

Fig. 2. Kaplan-Meier curves for PFS and OS according to AIER during the WIOP.
PFS = Progression Free Survival.
AIER = Antibiotic/Immunotherapy Exposure Ratio.
OS = Overall Survival.

Table 4
Multivariate analyses.

	PFS[1], months			OS[2], months		
	HR[3]	95% CI[4]	p value	HR	95% CI	p value
Variables						
Line of IO[5]	0.570	0.336–0.965	0.0365	–	–	–
AIER[6]	1.059	1.022–1.098	0.0018	–	–	–
Basal PS[7]	1.860	1.321–2.620	0.0004	–	–	–
AIER[6]	1.053	1.016–1.092	0.0052	–	–	–
Basal PS	–	–	–	0.405	0.271–0.607	< 0.0001
AIER[6]	–	–	–	1.064	1.030–1.099	0.0002

1 Progression Free Survival.
2 Overall Survival.
3 Hazard Ratio.
4 Confidence Interval.
5 Immunotherapy.
6 Antibiotic.
7 Performance Status.

NSCLC, and the consequent need of further research.

This study has some limitations. First, it is a retrospective analysis of a single Institution population, with a small number of patients. This might limit the possibility of generalizing its results. Second, as this is a retrospective work on an unselected population, we did not perform either biologic analyses on stool samples, or translational correlations on animal models. This prevents from identifying a biologic rationale and confirmation for our observations. Indeed, in absence of a translational correlative, the possibility that patients receiving repeated courses of antibiotics have a worse prognosis because of frequent infections and not for the use of antibiotics itself, cannot be excluded. In the end, AIER is a merely empiric variable, based on a theoretical rationale, which will require additional studies to prove its scientific basis. Moreover, the cutoff chosen for stratification derives from the analysis of the population itself, being defined as the median value of the observed cases. This cutoff may be different when analyzing other case series, and the results may change consequently.

Despite these limitations, the present work presents some interesting points. For the first time it proposes the AIER as a new variable that may condition the impact of antibiotic use on the efficacy of IO. Although the selection of a specific cut-off may be questionable, we conducted our analysis in the attempt of testing the potential applications of this new variable. Indeed, a definite cut-off is essential for any tool aiming to be useful in clinical practice, whereas continuous variables are barely applicable. The AIER has been tested on an unselected, intentionally heterogeneous population, which reflects the characteristics of patients in clinical practice. The variability in treatments, disease extension and patient PS, and the prolonged time span of data collection, instead of being a bias, may constitute a strong point of the analysis, depicting a realistic population of outpatient cases with NSCLC.

In conclusion, this work suggests that the recently emerged concept that antibiotics impair IO efficacy may be considered from a different point of view. In particular, the most relevant factor modulating this effect may be the duration of the antibiotic use in relation with the duration of IO, instead of the prescribed antibiotic type or the time between the beginning of the antibiotic use and the beginning of IO. Given the limited number of patients and the retrospective nature of the analysis, this hypothesis deserves confirmation and further investigation in larger case series. Furthermore, a translational and biological correlation with stool analyses and animal models may contribute to give a strong scientific evidence to our observation, which still remains a hypothesis-generating finding. However, if this relevance is confirmed, the AIER may become a new variable to help predicting the response to IO in NSCLC patients. Finally, it suggests that clinicians should weigh the risk/benefit ratio of prescribing antibiotics carefully, in particular for repeated or long courses, to patients with metastatic NSCLC.

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6. Conflict of interest statement

CP declares travel accommodations and honoraria with MSD International GmbH, BMS, Eli Lilly. DS declares travel accommodations and honoraria with AstraZeneca, MSD International GmbH, BMS. FdB provided consultation, attended advisory boards and/or provided lectures for the following organizations, from whom received honoraria or education grants: Amgen, AstraZeneca, Boehringer-Ingelheim, BMS, Eli Lilly, F. Hoffmann-La Roche, Ignyta, Merck Sharp and Dohme, Merck Serono, Novartis, Pfizer. MCG declares personal financial interests with the following organizations: AstraZeneca, MSD International GmbH, BMS, Boehringer Ingelheim Italia S.p.A, Celgene, Eli Lilly, Ignyta, Incyte, Inivata, MedImmune, Novartis, Pfizer, Roche, Takeda; she also declares Institutional financial interests with the following organizations: Eli Lilly, MSD, Pfizer (MISP), AstraZeneca, MSD International

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