I.Genotype, stage and the innate immune response in non-smallcelllungcancer (K109008)

1.NK lymphocyte infiltration of resected pulmonary adenocarcinoma tissues

We examinedtheinfluence of cancergenotypeoroncologicstageon tumor infiltrationby NK and Treglymphocytes. Progression-free and overall survival of 121 stage I-IIIA, curativelyresectedlungadenocarcinomapatientswascomparedbetweenpatientshavingabove and lowerthanmedian NK orTreginfiltrated tumor. Immunecellinfiltraton of tumor tissueswasevaluatedbyimmunohistochemistry and repeatedcounting of cells in onehundredlowmagnificationmicroscopicfieldsof eachresecateperformedbytwoindependentinvestigatorswhowereunawareaboutanyclinicaldata. The genotype of theKras (exons 12,13) and p53 genes (exon 6-7-8) wasanalysedbysequencingof DNA (GBS). Figure 1 shows thatabovemedianinfiltrationby NK cellsprognosticatedlongerprogression-free and overall survivalasevaluated during theupcoming 5 and 10 years. Out of the 121 samples 25 provedtocarrymutantKras. Figure 2 and 3 depictthat NK infiltrationwas more intense in KraswildthanKrasmutantpatientsand in womenthan men.Oncologicstagehad no influenceon NK infiltrationwhichfindingcontradictstheparadigmthatearly-stage (e.g. stage IA)lungcancerwould be more immunogenicthanlater (IIIA) stagedisease. Toourbestknowledgethesearethefirstobservationsabouttherelationshipbetweentheintensitiy of NK responsetolungadenocarcinoma and Krasgenotypeorgender.

2. Treglymphocyteinfiltration

Unexpectedly, abovemedianinfiltrationbyTregcellswasalsoprognosticatinglonger overall survival (Figure 4). Thisobservationwascontradictingearlierfindings and needsfurtheranalysis. In contrastwith NK cells, Treginfiltrationwasnotinfluencedbygender, Krasgenotype. Similarlyto NK cells, infiltrationbyTreg-s wasalsoindependentfromoncologicstage.

3. p53 mutation

In the 121 tumor tissuesaltogether 64 mutationwereidentified (Table 1 and 2). Detected p53 mutations were compared to OMIM database ([i]) searching for overlaps.Out of theseonly 40 mutationsresulted in aminoacidchangewithinthe p53 protein molecule(Table 1). Aminoacidchangeinducingmutations of the p53 moleculeareclassifiedasdisruptiveor non-disruptive (Poeta et al. NEJM 2007). Disruptivemutations of p53 have more severeconsequencesontheclinicalcourse of cancerdiseasethat non-disruptivemutations. Withinour 121 samplestherewere 27 disruptive and 13 non-disruptivechanges of aminoacidswithinthe 53 protein molecule (Table 1).

Point mutations of the p53 gene were analyzed with genotyping by sequencing (GBS) in exons 6-7-8. Identified mutations were ranked according to their codon number. Than codons were translated to their coded amino acids to see if the point-mutation resulted in any change of the code (Table 1). The meaning of different colours was explained in the index table below Table 1. Disruptive mutations were those, which resulted in a STOP sequence, or any DNA sequence alteration which occurs within codons 236-251 and replaces an amino acid from one polarity/charge category with an amino acid from another category (Poeta et al. NEJM 2007).

Table 1. p53 mutations resulting in change of amino-acid in 121 surgically resected lung adenocarcinoma tissues

position	poit mutation	wild type AA	mutant AA
	p53/Exon-8		
303	AGC->TGC	Serine (Ser)	Cysteine (Cys)
302	GGG->GAG	Glycine (Gly)	Glutamate (Glu)
301	CCA->CTA	Proline (Pro)	Leucine (Leu)
301	CCA->TCA	Proline (Pro)	Serine (Ser)
300	CCC->CTC	Proline (Pro)	Leucine (Leu)
298	GAG->TAG	Glutamate (Glu)	stop
297	CAC->CCC,	Histidine (His)	Proline (Pro)
295	CCT->TCT,	Proline (Pro)	Serine (Ser)
294	GAG->TAG	Glutamate (Glu)	stop
293	GGG->AGG	Glycine (Gly)	Arginine (Arg)
290	CGC->CAC,	Arginine (Arg)	Histidine (His)
286	GAA->CAA	Glycine (Gly)	Glutamate (Glu)
285	GAG->AAG	Glutamate (Glu)	Lysine (Lys)
284	ACA->ATA	Threonin (Thr)	Isoleucine (Ile)
283	CGC->CAC	Arginine (Arg)	Histidine (His)
282	CGG->TGG	Arginine (Arg)	Tryptophane (Trp)
282	CGG->CCG	Arginine (Arg)	Proline (Pro)
282	CGG->CGC	Arginine (Arg)	Arginine (Arg)
278	CCT->TTT	Proline (Pro)	Phenylalanin (Phe)
278	CCT->CGT	Proline (Pro)	Arginine (Arg)
277	TGT->TAT,	Cysteine (Cys)	Tyrosine (Tyr)
275	TGT->TAT	Cysteine (Cys)	Tyrosine (Tyr)
275	TGT->TTT	Cysteine (Cys)	Phenylalanine (Phe)
274	GTT->TTT	Valine (Val)	Phenylalanin (Phe)
273	CGT->CTT	Arginine (Arg)	Leucine (Leu)
273	CGT->AGT	Arginine (Arg)	Serine (Ser)

	p53/Exon-7		
258	GAA->TAA	Glutamate (Glu)	stop
256	ACA->ATA	Threonin (Thr)	Isoleucine (Ile)
252	CTC->TTC	Leucine (Leu)	Phenylalanine (Phe)
248	CGG->TGG	Arginine (Arg)	Tryptophane (Trp)
248	CGG->CTG	Arginine (Arg)	Leucine (Leu)
247	AAC->CAC	Asparagine (Asn)	Histidine (His)
246	ATG->ATC	Methionine (Met)	Isoleucine (Ile)
245	GGC->TGC	Glycine (Gly)	Cysteine (Cys)
244	GGC->TGC	Glycine (Gly)	Cysteine (Cys)
	p53/Exon-6		
208	CAG->AAC	Glutamine (Gln)	Asparagine (Asn)
207	GAT->GGT	Aspartate (Asp)	Glycine (Gly)

Meaning of colours:

nonpolar
polar
basic
acidic
inactivating
stop

Mutations not resulting in any amino-acid change were excluded from further analysis (Table 2).

Table 2. Mutations not resulting in any amino-acid change in 121 surgically resected lung adenocarcinoma tissues

position	poit mutation	wild type AA	mutant AA
p53/Exon-8			
302	GGG->GGA	Glycine (Gly)	Glycine (Gly)

300	CCC->CCT	Proline (Pro)	Proline (Pro)
293	GGG->GGA	Glycine (Gly)	Glycine (Gly)
291	AAG->AAA,	Lysine (Lys)	Lysine (Lys)
282	CGG->AGA	Arginine (Arg)	Arginine (Arg)
282	CGG->CGA,	Arginine (Arg)	Arginine (Arg)
278	CCT->CCA	Proline (Pro)	Proline (Pro)
276	GCC->GCT	Alanine (Ala)	Alanine (Ala)
274	GTT->GTG	Valine (Val)	Valine (Val)
p53/Exon-7			
260	TCC->TCT	Serine (Ser)	Serine (Ser)
259	GAC->GAT	Aspartate (Asp)	Aspartate (Asp)
257	CTG->TTG	Leucine (Leu)	Leucine (Leu)
253	ACC->ACT	Threonin (Thr)	Threonin (Thr)
252	CTC->CTT	Leucine (Leu)	Leucine (Leu)
251	ATC->ATT	Isoleucine (Ile)	Isoleucine (Ile)
250	CCC->CCT	Proline (Pro)	Proline (Pro)
248	CGG->AGG	Arginine (Arg)	Arginine (Arg)
247	AAC->AAT	Asparagine (Asn)	Asparagine (Asn)
244	GGC->GGT	Glycine (Gly)	Glycine (Gly)
242	TGC->TGT	Cysteine (Cys)	Cysteine (Cys)
p53/Exon-6			
224	GAG->GAA	Glutamate (Glu)	Glutamate (Glu)
204	GAG->GAA	Glutamate (Glu)	Glutamate (Glu)
197	GTG->GTA	Valine (Val)	Valine (Val)

Our research work is currently at this stage. We are analyzing relationships between the presence of disruptive or non-disruptive mutations of p53 on NK and Treg infiltration of lung adenocarcinoma tissues and survival of operated patients. The pathological and clinical significance of combined

presence or absence of Kras and p53 mutations will be clarified. Within the next 6 months one manuscript containing the above new findings will be submitted for publication to a peer-reviewed research periodical.

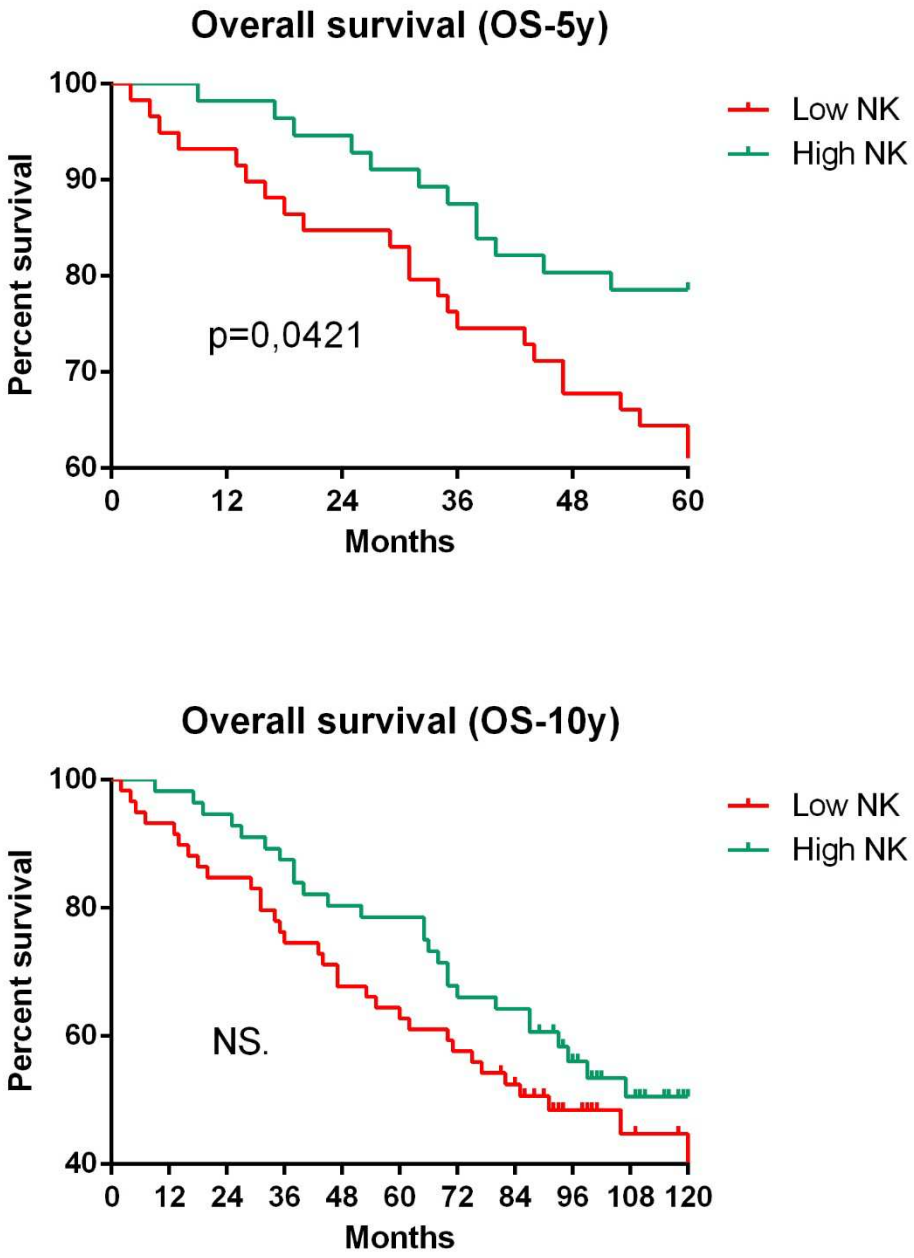

Figure 1. Above median NK infiltration of resected lung adenocarcinoma predicts longer 10 years survival (n=121).

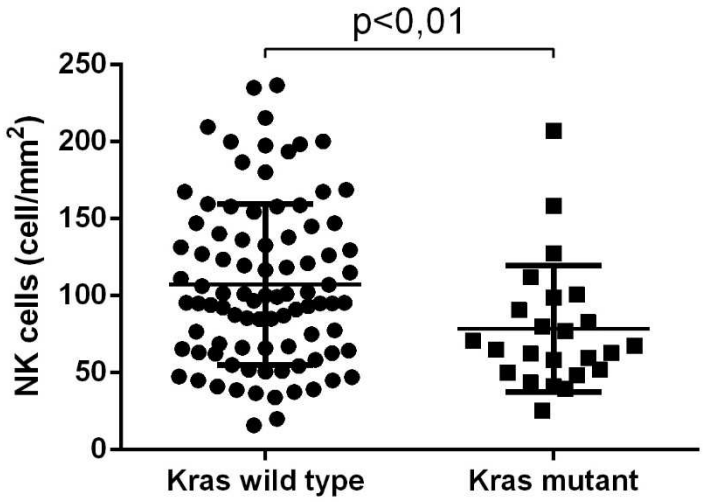

Figure 2. Kras mutatnt lung adenocarcinoma contains less tumor infiltrating NK lymphocytes, tha Kras wild genotype tumors.

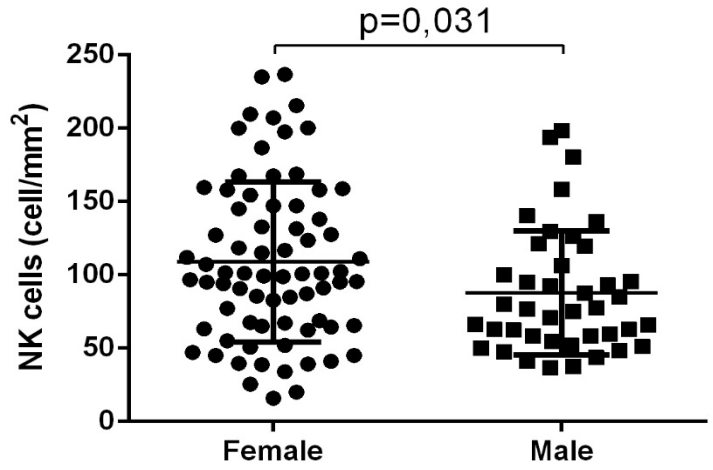

Figure 3. Resected lung adenocarcinoma induces more intense NK infiltration in women than men.

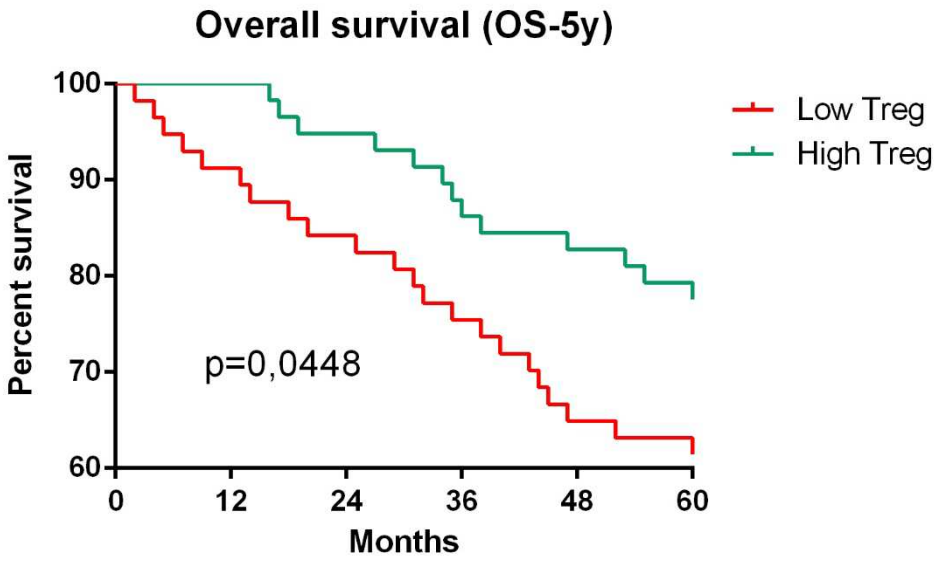

Figure 4. Five year overall survival is longer among those resected patients who had more intense tumor infiltration by Treg lymphocytes.

Reference

[i]https://www.omim.org/allelicVariant/191170

II. Submitted manuscript

Chronic obstructive pulmonary disease prolongs progression-free survival during 1st line treatment in advanced non-small cell lung cancer

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a common comorbidity of stage IIIB-IV non-small cell lung cancer (NSCLC). Progression is accelerated by inflammation and lymphoid dysfunction in NSCLC and similar mechanisms are active in COPD. **Aim:** To find out if COPD influenced tumor induced inflammatory and lymphoid responses and progression-free survival (PFS) during 1st line treatment of advanced NSCLC? **Methods:** Four groups were studied: NSCLC (n=95), NSCLC+COPD (n=80), only COPD (n=54) and controls (n=60). PFS, neutrophil (neu) and lymphocyte (ly) count, the

neu/ly ratio were recorded. IFNγ, TNFα, VEGF and myeloid derived suppressor cells (MDSCs) were measured and systemic T-cell exhaustion was tested by multiplex cytometric bead-based immunoassay and by flow cytometry, respectively. **Results:** Median PFS was increased in NSCLC+COPD to 7.4 months as compared to only NSCLC patients (4.9 months, p<0.01). In multivariate Cox analysis adjusting for age, gender, histology, cancer stage and ECOG performance the adjusted hazard ratio of longer PFS in NSCLC+COPD vs only NSCLC was 1.70 (CI: 1.2-2.4, p<0.002). Neu count, CRP, IFNγ and TNFα concentrations were all reduced in NSCLC+COPD as compared to only NSCLC. NSCLC with concomitant COPD was also associated with reduced IL-10, but more granzyme-B positive CD8 cells versus only NSCLC. NSCLC+COPD patients presented moderation of VEGF and MDSC associated mechanisms of inflammation and lymphopenia. **Conclusion:** Concomitant COPD moderated tumor-induced inflammation and lymphopenia and was an independent positive predictive factor of longer PFS during 1st line therapy of advanced NSCLC.

Introduction

Progression-free survival (PFS) is the length of time during and after the treatment of a disease, such as cancer, that a patient lives without worsening the disease. In a clinical trial, measuring the PFS is one way to see how well a new treatment works (1). In advanced non-small cell lung cancer (NSCLC) standard 1st line treatment is induction and maintanance therapy with cytotoxic and/or biologically targeted agents. Prognosis depends on both tumor- and host-related factors. Stages of primary tumor, lymph node and remote metastasis are major tumor-related, while weight loss and performance state are major host-related factors (2). Performance state, pain, weight loss and functional disability are considered manifestations of tumor-induced inflammation (3), moreover, lower than median neutrophil (neu) count has been demonstrated to be a strong positive predictive biomarker of longer PFS during 1st line treatment in advanced NSCLC (4). On the other hand, T cell exhaustion (5) and lower total lymphocyte (ly) count (6) are signs of negative prognosis in advanced cancer. The neu/ly ratio has been repeatedly shown to be a strong indicator of PFS and overall survival (OS) in advanced NSCLC (7,8). Although advanced NSCLC is the cancer of heavy smokers and the elderly most of whom suffer from comorbidities (9), there has been a lack of information how comorbidities influence PFS during and after 1st line treatment. As an exception, diabetes mellitus has been recently demonstrated to shorten PFS in this state (10). The influence of the 2nd (after cardiovascular disease being the 1st) most frequent comorbidity, chronic obstructive pulmonary disease (COPD (9)) has not been studied, yet. Except a small series including 50 patients (11). This issue is clinically relevant since more than 50% of NSCLC patients suffer from COPD (9,12), which itself is a state of chronic inflammation and lymphoid dysfunction (13).

In advanced NSCLC circulating vascular endothelial growth factor (VEGF) concentration increases (14-16). In addition to angiogenesis VEGF was shown to play role in modulation of myelopoiesis including the formation of myeloid derived suppressor cells (MDSCs) which have been known to contribute to tumor-induced inflammation and immunosuppression (17,18). One manifestation of immunosuppression in cancer is appearance of so-called exhausted T cells (CD4ex and CD8ex) within tumor tissue (19) and the systemic circulation (20,21). VEGF, MDSCs, inflammation, lymphoid dysfunction, the appearance of Tex cells have all been reported to be present also in COPD (22,23), not only cancer.

Therefore, in the present study, we have compared PFS and the myeloid and lymphoid response in peripheral blood together with several pathophysiological factors in the background, like circulating VEGF and MDSCs and exhausted T cells in patients suffering from advanced NSCLC without (NSCLC n=95) or with COPD (NSCLC+COPD, n=80).

Methods

Patients

All patients were recruited and taken care at the Department of Pulmonology, Semmelweis University Medical Center between November 2015 and March 2017. Written informed consent was obtained from every patient and permission was obtained from the Institutional Ethics Committee of Semmelweis University Clinical Center (#238-2/2015). The total number of pathologically confirmed, stage IIIB-IV (advanced) NSCLC patients was 95, while the number of NSCLC patients with concomitant COPD (NSCLC+COPD) was 80. Sixty healthy smokers and 54 COPD patients were included as controls. In addition to general clinical parameters plasma IFNγ, TNFα and interleukin-(IL-) 10, or serum vascular endothelial growth factor (VEGF) and circulating monocytic (M-) and granulocytic (G-) myeloid derived suppressor cells (MDSCs), or subpopulations of CD4 and CD8 T cells positive for IFNγ, granzyme B, PD1 or CTLA4 were quantitated. Since concomitant COPD was observed to markedly alter the relationship between VEGF and MDSCs, as well as neutrophils and lymphocytes, more bevacizumab+paclitaxel+carboplatin treated adenocarcinoma patients (without or with COPD) were included. In some patients PFS and OS could not be have become evaluated, yet. Stage of NSCLC according to Quint (25) and performance state according to Eastern Cooperative Oncology Group (ECOG (26) were determined. Treatment effects on primary tumor, metastatic lymph node (-s) or remote metastasis were evaluated according to RECIST 1.1. Progression-free survival (PFS) was calculated as the time from start of 1st line treatment to disease progression or death. Progression was controlled every 3 week by clinical examination and chest X-ray and if any signs or symptoms have raised suspision of progression, CT, PET-CT and/or MRI were performed. Chest and upper abdominal CT was performed every 3 months minimum. Overall survival (OS) was estimated from start of oncologic treatment until exitus. COPD was diagnosed according to the criteria of the Global Initiative for Chronic Obstructive Lung Disease 2017 (27). COPD patients were in GOLD stage II-III. Lung function results and the bronchoscopic state of large airways of each NSCLC patient were cautiously analysed to exclude those, who had airway obstruction of cancerous or other, not COPD related cause.

None of the included patients and subjects used systemic steroid or antibiotics at least two months before the investigation. All COPD patients (with or without NSCLC) were treated by inhalational long-acting muscarinerg antagonist, or long-acting β2 agonist (LABA), or combination of these, or inhalational corticosteroid combined by LABA. Subjects were excluded if they had a history of another malignancy or other diseases associated with systemic inflammation or immunodeficiency. None of the COPD patients (with or without cancer) suffered from acute exacerbation.

Peripheral venous blood was collected at the time of diagnosis, before the administration of any cytostatic or targeted anticancer drug. Body mass index (BMI) and smoking history was calculated and the plasma concentration of absolute neutrophil (neu) count, monocyte count, lymphocyte (ly) count, platelets and concentration of hemoglobin (Hb) and C-reactive protein (CRP) were measured. Post-bronchodilator forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were tested by Total Body Plethysmograph (Piston, Budapest, Hungary).

Flow cytometry

Peripheral venous blood samples were collected in sodium heparin tubes (Vacutainer, Becton Dickinson, Franklin Lakes, USA). Cell preparations and flow cytometric analyses were performed according to the protocols described earlier (28). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation within 3 hours of sample collection. MDSC measurements were performed right after the PBMC isolation. PBMC samples were frozen and stored in biobank until the exhausted T cell measurements. For cryopreservation, PBMCs were resuspended in freezing medium (10% DMSO and 45% fetal bovine serum in complete RPMI 1640) and stored -80°C until stimulation and flow cytometric measurements. For the CTLA-4 and intracellular IFN-γ measurements defrosted cells were cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate (Sigma), 1% MEM nonessential amino acids and vitamins (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma), 100 µg/ml kanamycin (Gibco) and 10% heat-inactivated FCS (Sigma) in 24 well plates, and stimulated with PMA (25 ng/ml; Sigma) and ionomycin (1 µg/mL; Sigma) for 6 h in the presence of brefeldin A (10 µg/ml; Sigma) for the final 2 h. Cytofix/Cytoperm Fixation and Permeabilization Kit (Beckton Dickinson) were used for cell permeabilization and intracellular staining procedure. Flow cytometry data were acquired on FACSAria (Beckton Dickinson) and Navios (Beckman Coulter, Brea, USA) instruments, and were analyzed with Kaluza software (Beckman Coulter).

The following anti-human antibodies were used for flow cytometry measurements: fluorescein isothiocyanate (FITC)-conjugated anti-CD15 (HI98), phycoerythrin (PE)-conjugated anti-CD33 (WM53) and PE-conjugated anti-IFN-γ (4S.B3), PE/Dazzle 594-conjugated anti-HLA-DR (L234), peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-CD66b (G10F5), PE-indotricarbocyanine (Cy7)-conjugated anti-CD14 (M5E2), allophycocyanin (APC)-conjugated anti-CD11b (ICRF44) and isotype-matched control antibodies conjugated to PE, PE/Dazzle 594 and Alexa Fluor 647 (MOPC-21; all from BioLegend, San Diego, USA); FITC-conjugated anti-CD4 (sk3), PE/Dazzle 594-conjugated anti-CD8 (sk1), PerCP-Cy5.5-conjugated anti-CD3 (UCHT1), PE-Cy7-conjugated anti-CTLA-4 (CD152; L3D10), Alexa Fluor 647-conjugated anti-Granzyme B (GB11), APC-Cy7-conjugated anti-PD-1 (CD279, EH12.2H7; all from Sony Biotechnology, San Jose, USA); isotype-matched control antibodies conjugated to FITC, PerCP-Cy5.5, PE-Cy7, APC and APC-Cy7 (MOPC-21; all from Beckton Dickinson); APC-eFluor 780 anti-human CD19 (HIB19), CD3 (UCHT1), CD56 (CMSSB) and isotype-matched control antibodies conjugated to APC-eFluor 780 (P3.6.2.8.1; all from eBioscience, Affymetrix, Santa Clara, USA). A fixable viability dye (eFluor 780, termed as e-780 on figure; eBioscience) was used for dead cell discrimination.

In order to assess the level of exhaustion in T cell populations the spontaneous cell surface expression of PD-1, and the IFN-γ production as well as the CTLA-4 expression after PMA/ionomycin stimulation both in CD3+CD4+ T helper and CD3+CD8+ T killer cells were measured. The intracellular granzyme B content was also measured in CD3+CD8+ T killer cells (19).

MDSC populations were defined according to Bronte et al (29) as monocytoid-(M-)MDSC: $CD3^{-}CD19^{-}CD56^{-}CD14^{+}HLA\text{-}DR^{-}CD11b^{+}CD33^{high}$ and granulocytoid-(G-)MDSC: $Lin^{-}HLA\text{-}DR^{-}CD11b^{hi}CD15^{hi}CD33^{low}CD66b^{+}$ cells (Figure 1). Lineage markers (Lin) were: CD14, CD3, CD19, CD56. Peripheral blood samples after density gradient centrifugation, especially in cancer patients, were containing low-density granulocytes besides mononuclear cells. Therefore the *bona fide* mononuclear PBMCs (lymphocytes and monocytes) identified by size and complexity (on FSC-SSC plot) were gated, and the M-MDSCs were expressed as percent of the gated PBMCs. G-MDSCs were

expressed as percent of all WBCs in samples after density gradient centrifugation (containing PBMCs and low-density granulocytes).

Analysis of cytokines

Concentrations of IFN, TNF, IL-10 were measured in serum by multiplex cytometric bead-based immunoassays (Bio-Plex system, Bio-Rad, Hercules, CA, USA). Concentration of VEGF in serum samples were measured by commercially available ELISA kit (R&D Systems, Minnesota, USA). The assays were performed according to instructions of manufacturers.

Statistics

Categorical data were compared by Pearson χ^2 test (or Fisher exact test as needed). Continuous data were examined by normality test. Continuous variables with normal distribution were presented as mean ± standard error of the mean (SEM) or as median with {interquartile range} where normal distribution was absent. Continuous data were compared with one-way ANOVA and Pearson test if distribution was normal or with Kruskal-Wallis test and Spearman test if the sample distribution was asymmetrical. We used Tukey post-hoc test for one-way ANOVA. All the p values are two-sided, and $p<0.05$ was considered statistically significant. Differences between groups considering PFS and OS were estimated using Kaplan-Meier method, and log-rank test was used for comparison of outcomes. Independent prognostic variables of PFS were assessed using Cox proportional hazards regression analysis. IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis.

Results

Age, gender ratio and smoking history were similar in patient groups (COPD, NSCLC and NSCLC+COPD). Most NSCLC patients (without or with COPD) were in stage IV (Table I). In both NSCLC groups there were more non-squamous than squamous histologic types, but in the two NSCLC groups the ratio of various histologies was similar. The FEV1/FVC ratio was less than 70% and FEV1 was reduced in the two COPD groups, while BMI was smaller in the two NSCLC groups. Since relatively more non-squamous NSCLC patients were included, more patients received bevacizumab containing chemotherapy. The ratio of the various therapeutic modalities including radiotherapy were similar in the NSCLC and the NSCLC+COPD groups.

The absolute neu count was increased in NSCLC, but less so in case of concomitant COPD. The ly count was reduced in NSCLC vs healthy controls and in NSCLC+COPD vs COPD patients. The neu/ly ratio was increased in NSCLC and relatively reduced in NSCLC+COPD. Monocytosis, increased platelet count and anaemia were also characteristic in NSCLC, but not influenced by concomitant COPD. CRP concentration was also elevated in NSCLC but reduced by concomitant COPD. Based on neu and CRP data inflammation induced by advanced NSCLC became reduced by concomitant COPD (Table I).

Tumor-induced inflammation is a strong, negative prognostic factor of survival in advanced cancers including NSCLC (3,4). In line with these observations of other authors, in the present study PFS was prolonged in NSCLC+COPD patients vs those suffering from NSCLC without COPD. Median PFS was 7.4 months in NSCLC+COPD vs 4.9 months in NSCLC ($p<0.01$). OS was 16.9 and 11.0 months in NSCLC+COPD and NSCLC, respectively ($p>0.05$, Table I). Figure 2 shows the PFS increasing effect of concomitant COPD as confirmed by Kaplan-Meyer analysis ($p=0.0017$). In multivariate Cox proportional analysis adjusting for age, gender, histology, cancer stage and ECOG state the adjusted

hazard ratio of longer PFS in NSCLC+COPD vs only NSCLC was statistically highly significant (HRadj: 1.70, CI: 1.2-2.4, p=0.002, Table II). Thus, concomitant COPD was found to be an independent positive predictive factor of longer PFS during 1st line treatment of advanced NSCLC.

In order to find out some details about the mechanism how concomitant COPD induced moderation of tumor-induced inflammation, different cellular and humoral factors known to mediate this oncopathologic process were analysed in subgroups of each the 4 study groups. The proinflammatory cytokines IFNγ and TNFα were found to be strongly increased in NSCLC but reduced in those patients who had concomitant COPD. IL-10, the cytokine fostering immunologic tolerance of cancer was high in NSCLC, but lower (in the control range) in NSCLC+COPD (Table III).

Synthesis of VEGF in NSCLC is increased which fosters the formation of MDSCs (14-16, 30). Therefore, in the next subgroups VEGF and MDSCs were measured. Serum VEGF as well as M- and G-MDSCs were increased in NSCLC (Table III). When NSCLC was combined with COPD, the M-MDSC fraction remained increased, but the G-MDSC fraction normalized. The relationships between serum concentration of VEGF and counts of various cell populations were analysed. Higher serum VEGF had a direct relationship with M-MDSC-s in NSCLC ($r=0.73$, $p<0.001$, Figure 3a), but not in NSCLC+COPD patients, in whom this relationship became inverse ($r=-0.65$, $p=0.01$, Figure 3b). Thus, concomitant COPD reduced G-MDSCs and moderated or inverted the effect of VEGF on M-MDSCs, which have been known to exert both proinflammatory and immunosuppressive effects in NSCLC (18,24).

There was no relationship between serum VEGF and G-MDSCs in either groups. Higher VEGF was, however, also associated with higher neu count in NSCLC ($r=0.63$, $p=0.006$, Figure 3c), but not in NSCLC+COPD ($r=0.32$, $p=0.269$, Figure 3d). On the other hand, there was no relationship between VEGF and ly count in NSCLC ($r=0.38$, $p=0.135$, Figure 3e), while higher VEGF was accompanied with higher ly in NSCLC+COPD patients ($r=0.62$, $p<0.05$, Figure 3f). Therefore, VEGF-associated mechanisms of both neutrophilia and lymphocytopenia were reduced by concomitant COPD.

In order to evaluate the functional state of circulating T cells and the ratio of CD4ex and CD8ex cells, in further subgroups the CD4 and CD8 fractions with intracellular IFNγ and granzyme-B content, as well as those with surface expression of PD1 and CTLA4 immune checkpoint molecules were measured (Table III). These ly subpopulations were of similar size within both CD4 and CD8 cells, which indicated the absence of systematic T cell exhaustion in either cancer groups. Moreover, the abundance of granzyme-B positive CD8 cells (more than 90% of all CD8 cells) was augmented in NSCLC+COPD patients as compared to those suffering from only NSCLC (about 50% of CD8 cells). Thus, NSCLC+COPD patients had reduced IL-10 concentration and circulating G-MDSCs (both normal, see Table III), as well as more effector CD8 cells. Moreover, only NSCLC+COPD patients presented a direct relationship between VEGF and ly count, but an indirect relationship between VEGF and M-MDSCs.

According to earlier findings lower pretreatment VEGF in plasma (14,31) or serum (15,16) predicted longer survival in advanced NSCLC. Based on that and the present finding that concomitant COPD altered the relationships between VEGF and MDSCs, neu and ly counts, it seemed relevant to find out whether the PFS prolonging effect of concomitant COPD was altered when compared between homogenously bevacizumab+chemotherapy treated patients. Therefore, two further, otherwise matching, non-squamous, stage IIIB-IV NSCLC groups (one without and one with COPD) were

recruited. All patients have received bevacizumab combined with carboplatin+paclitaxel. Patients with concomitant COPD (n=35) had again lower neu, higher ly count and lower neu/ly ratio (all $p<0.05$), as well as longer PFS ($p=0.049$) than those NSCLC patients who had no COPD (n=39). Homogenous anti-VEGF treatment did not change the antiinflammatory and PFS prolonging effects of concomitant COPD (supplementary Table I).

Discussion

Since stable COPD is a chronic inflammatory condition (22,23,32), concomitant COPD could synergize with tumor induced inflammation and worsen survival. This has been previously studied in early stage (I-IIIA) NSCLC patients having had undergone surgical resection of the tumor (33,34). In these studies OS was found shorter in those who suffered also from COPD. But mechanisms of interference between COPD and NSCLC may be very different in curatively and only palliatively treatable NSCLC. While after curative treatment of early-stage NSCLC the expected OS streches to several years, after palliative treatment of advanced NSCLC median OS is only about 12 months (2). Furthermore, in addition to the many negative effects of concomitant COPD in cancer (33,34), COPD is also keeping multiple antiinflammatory and antioxydant mechanisms active (35-37) prior to and probably during tumor progression. α1-antitrypsin, α2-macroglobulin, haptoglobin, orosomucoid (35), as well as ferroxidase (coeruloplasmin), surfactant protein–D (36) and glutathione peroxidase (37) become increased in sputum or plasma or both of COPD patients. But CD8 lymphocytes are also more abundant in COPD (32). Moreover, tertiary lymphoid tissues are formed in bronchial walls of COPD patients, which contain mostly B-cells and immunoglobulin-producing, differentiated plasma cells (38). These antiinflammatory and immunologic mechanisms could, at least in theory, moderate tumor-induced inflammation and improve the effect of therapy in advanced NSCLC+COPD patients. The issue has hardly been studied, yet.

In a relatively small (n=50 patients total) retrospective study concomitant COPD was found not to influence patient ratios with partial response, stable or progressive disease along 1st line combined chemotherapy of advanced NSCLC (11). We have shown here in 175 advanced NSCLC patients that concomitant COPD did significantly prolong PFS during and after 1st line therapy. Some possible background mechanisms were revealed explaining this earlier unknown effect of COPD. NSCLC+COPD patients presented with a lower neu/ly ratio, which was known as a sign of moderated tumor-induced inflammation and/or less inhibited antitumor immune response (7,8). Indeed, lower pretreatment neu/ly ratio was shown by Yao et al (7) to increase PFS in advanced NSCLC after 1st line chemotherapy. Teramukai et al (4) made similar observations by demonstrating longer PFS and OS in advanced NSCLC when pretreatment neu count was lower than median. Lee et al (39) documented that early reduction of the neu/ly ratio was a surrogate marker of better OS in advanced adenocarcinoma of the lung treated by gefitinib or standard chemotherapy in the 1st line.

Changes of the neu/ly ratio in peripheral blood were studied together with the enumeration of effector (IFNγ positive) CD4 and CD8 cells, as well as exhausted fractions of these T cells in an other form of cancer, surgically resected intrahepatic cholangiocarcinoma patients (40). The density of tumor-infiltrating CD3 cells (TIL) was also evaluated. Among these 102 patients those who had higher peripheral blood neu/ly ratio also had lower ratio of peripheral blood IFNγ positive, but higher ratio of PD1 positive T cells, as well as less TIL-s. Higher than median neu/ly ratio patients survived

significantly shorter. Authors concluded that an elevated neu/ly ratio was associated with poor antitumor immunity and could be a biomarker of poor prognosis.

T cell exhaustion has traditionally been evaluated inside tumors among TILs. On the other hand, Kramphorts et al (20) were able to follow the pharmacodynamic effect of pembrolizumab (anti-PD1 antibody) by testing the fraction of PD1 positive CD8 cells in the syetemic circulation. Similarly, Huang et al (21) by peripheral blood analysis found evidence for reinvigoration of CD8 cells (increased fraction of IFNγ+CD8 cells) following the administration of pembrolizumab. These findings support that analysis of exhausted and reinvigorated T cells can be evaluated in peripheral blood, which is easier than on TILs. Our present finding of increased granzyme B+CD8 fraction in NSCLC+COPD patients relative to only NSCLC has supported several other laboratory findings which all pointed to less inhibited immune responsiveness in advanced NSCLC if associated by COPD.

In the present study concomitant COPD was observed to be associated with lower neu/ly ratio indicating reduced tumor induced inflammation as well as lymphopenia. COPD has been known to be associated with repeated viral and bacterial infections of the airways which induce persistent innate and adaptive immune responses (32). In COPD antinuclear antibody (ANA) may be produced, reflecting the associated activation of autoimmune mechanisms in this state (38). COPD associated infectious and autoimmune stimuli may be relevant in NSCLC, because the specific activation of the adaptive immune system is known to inhibit the formation of MDSCs (41). In addition, COPD is associated with the release of neutrophil elastase (NE (42), which enzyme has been observed to be taken up by lung adenocarcinoma cells (43) and lung tumor cells containing NE may become more immunogenic (44). But NE has been also shown to cleave VEGF into fragments which could not bind to their specific cellular receptors (45). We found here that concomitant COPD interfered with various myeloid and lymphoid effects of VEGF. Higher VEGF concentrations were found to be statistically significantly associated with more M-MDSC-s and neutrophils in NSCLC, but not in those who had concomitant COPD. Moreover, only among the latter direct relationship was revealed between VEGF and ly count. It is possible therefore, that NSCLC was more immunogenic in those patients who also had COPD. Moderation of both inflammation and immunosuppression by concomitant COPD could have contributed to the observed prolongation of PFS during 1st line treatment of advanced NSCLC.

Several authors have reported that higher pretreatment concentrations of VEGF in plasma (14,31) or serum (15,16) predicted shorter PFS and OS during chemotherapy (+/-bevacizumab) in advanced NSCLC. Therefore, the concomitant COPD-associated changes in the synthesis of VEGF could become clinically relevant considerting clinical outcome of NSCLC+COPD. Of note, in emphysema patients sputum (46), plasma (47) and lung (48) VEGF was demonstrated to become reduced. On the other hand, in the bronchitic phenotype of COPD, sputum VEGF has been reported to be very high (46,47). Further, as mentioned above, NE can cleave and inactivate VEGF (45). All these mechanisms may be simultaneously active in COPD and VEGF synthesis by NSCLC itself (18,24) may be only one out of several mechanisms to count with in patients suffering from both NSCLC and COPD. Further studies seem to be needed to clarify how various phenotypes of concomitant COPD influence prognosis or predict the effect of various anticancer treatments in advanced NSCLC.

In conclusion, the present results indicated that concomitant COPD had a beneficial influence on advanced NSCLC-induced inflammation and PFS during the period of 1st line oncotherapy. The

lymphocyte count was better maintained and T-cells did not become exhausted systematically in NSCLC+COPD patients. Heavy smoking has been shown to powerfully increase the number of tumor-associated mutations and the number of resulting neoepitopes and potential neoantigens (49), thereby the antitumor immune response during therapy with immune checkpoint inhibitors (49,50). In the era of immunotherapy of advanced NSCLC, elucidation how concomitant COPD modifies the efficiency of immune checkpoint inhibitors may be important.

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References

1. Garon EB[1]. Issues surrounding clinical trial endpoints in solid malignancies with a focus on metastatic non-small cell lung cancer. Lung Cancer. 2012 Sep;77(3):475-81. doi: 10.1016/j.lungcan.2012.06.007. Epub 2012 Jul 12.

2.Gospodarowicz MK, O'Sullivan B, Sobibn LH, eds. Lung Cancer. In: Prognostic Factors in Cancer. 3rd Ed. New York: Wiley-Liss, 2006: Chapter 19.

3. Trajkovic-Vidakovic M, de Graeff A, Voest EE, Teunissen SC. Symptoms tell it all: a systematic review of the value of symptom assessment to predict survival in advanced cancer patients. Crit Rev Oncol Hematol 2012; 84: 130-148.

4. Teramukai S[1], Kitano T, Kishida Y, Kawahara M, Kubota K, Komuta K, Minato K, Mio T, Fujita Y, Yonei T, Nakano K, Tsuboi M, Shibata K, Furuse K, Fukushima M. Pretreatment neutrophil count as an independent prognostic factor in advanced non-small-cell lung cancer: an analysis of Japan Multinational Trial Organisation LC00-03. Eur J Cancer. 2009 Jul;45(11):1950-8. doi: 10.1016/j.ejca.2009.01.023. Epub 2009 Feb 21.

5.Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol 2015; 15: 486-499.

6. Ray-Coquard I[1], Cropet C, Van Glabbeke M, Sebban C, Le Cesne A, Judson I, Tredan O, Verweij J, Biron P, Labidi I, Guastalla JP, Bachelot T, Perol D, Chabaud S, Hogendoorn PC, Cassier P, Dufresne A, Blay JY; European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group. Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. Cancer Res. 2009 Jul 1;69(13):5383-91.

7.Yao Y, Yuan D, Liu H, Gu X, Song Y. Pretreatment neutrophil to lymphocyte ratio is associated with response to therapy and prognosis of advanced non-small cell lung cancer patients treated with first-line platinum-based chemotherapy. Cancer Immunol Immunother. 2013 Mar;62(3):471-9. doi: 10.1007/s00262-012-1347-9. Epub 2012 Sep 18.

8. Berardi R[1], Rinaldi S[1], Santoni M[1], Newsom-Davis T[2], Tiberi M[3], Morgese F[1], Caramanti M[1], Savini A[1], Ferrini C[1], Torniai M[1], Fiordoliva I[1], Bower M[2], Cascinu S[1,4]. Prognostic models to predict survival in

patients with advanced non-small cell lung cancer treated with first-line chemo- or targeted therapy. Oncotarget. 2016 May 3;7(18):26916-24. doi: 10.18632/oncotarget.8309.

9. Kocher F, Hilbe W, Seeber A, Pircher A, Schmid T, Greil R, Auberger J, Nevinny-Stickel M, Sterlacci W, Tzankov A, Jamnig H, Kohler K, Zabernigg A, Frötscher J, Oberaigner W, Fiegl M. Longitudinal analysis of 2293 NSCLC patients: a comprehensive study from the TYROL registry. Lung Cancer. 2015 Feb;87(2):193-200. doi: 10.1016/j.lungcan.2014.12.006. Epub 2014 Dec 18.

10. Zhu L[1], Cao H, Zhang T, Shen H, Dong W, Wang L, Du J. The Effect of Diabetes Mellitus on Lung Cancer Prognosis: A PRISMA-compliant Meta-analysis of Cohort Studies. Medicine (Baltimore). 2016 Apr;95(17):e3528. doi: 10.1097/MD.0000000000003528.

11. Czyżykowski R, Nowak D, Janiak A, Włodarczyk A, Sarniak A, Krakowska M, Potemski P. A retrospective evaluation of associations between chronic obstructive pulmonary disease, smoking, and efficacy of chemotherapy and selected laboratory parameters in patients with advanced non-small cell lung cancer. Contemp Oncol (Pozn). 2016;20(5):407-413. doi: 10.5114/wo.2016.64605.

12. Young RP[1], Hopkins RJ, Christmas T, Black PN, Metcalf P, Gamble GD. COPD prevalence is increased in lung cancer, independent of age, sex and smoking history. Eur Respir J. 2009 Aug;34(2):380-6.

13. MacNee W, Vestbo J, Agusti A. COPD: Pathogenesis and Natural History. in Murray & Nadel's Textbook of Respiratory Medicine. Sixth Edition, Elsevier Saunders 2016, pp. 751-766.

14. Mok T, Gorbunova V, Juhasz E, Szima B, Burdaeva O, Orlov S, Yu CJ, Archer V, Hilton M, Delmar P, Pallaud C, Reck M. A correlative biomarker analysis of the combination of bevacizumab and carboplatin-based chemotherapy for advanced nonsquamous non-small-cell lung cancer: results of the phase II randomized ABIGAIL study (BO21015). J Thorac Oncol. 2014 Jun;9(6):848-55. doi: 10.1097/JTO.

15. Hu P, Liu W, Wang L, Yang M, Du J. High circulating VEGF level predicts poor overall survival in lung cancer. J Cancer Res Clin Oncol. 2013 Jul;139(7):1157-67. doi: 10.1007/s00432-013-1425-1. Epub 2013 Apr 4.

16.Kaya A, Ciledag A, Gulbay BE, Poyraz BM, Celik G, Sen E, Savas H, Savas I. The prognostic significance of vascular endothelial growth factor levels in sera of non-small cell lung cancer patients. Respir Med. 2004 Jul;98(7):632-6.

17. Johnson B, Osada T, Clay T, Lyerly H, Morse M. Physiology and therapeutics of vascular endothelial growth factor in tumor immunosuppression. Curr Mol Med 2009; 9: 702-707.

18. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. Nature Rev Cancer 2013; 13: 739-752.

19. Zarour HM. Reversing T-cell dysfunction and exhaustion in cancer. Clin Cancer Res 2016; 22: 1856-1864.

20. Kamphorst AO, Pillai RN, Yang S, Nasti TH, Akondy RS, Wieland A, Sica GL, Yu K, Koenig L, Patel NT, Behera M, Wu H, McCausland M, Chen Z, Zhang C, Khuri FR, Owonikoko TK, Ahmed R, Ramalingam SS. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. Proc Natl Acad Sci U S A. 2017 May 9;114(19):4993-4998. doi: 10.1073/pnas.1705327114.

21. Huang AC, Postow MA, Orlowski RJ, Mick R, Bengsch B, Manne S, Xu W, Harmon S, Giles JR, Wenz B, Adamow M, Kuk D, Panageas KS, Carrera C, Wong P, Quagliarello F, Wubbenhorst B, D'Andrea K, Pauken KE, Herati RS, Staupe RP, Schenkel JM, McGettigan S, Kothari S, George SM, Vonderheide RH, Amaravadi RK, Karakousis GC, Schuchter LM, Xu X, Nathanson KL, Wolchok JD, Gangadhar TC, Wherry EJ. T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. Nature. 2017 May 4;545(7652):60-65. doi: 10.1038/nature22079. Epub 2017 Apr 10.

22. Scrimini S, Pons J, Agustí A, Clemente A, Sallán MC, Bauçà JM, Soriano JB, Cosio BG, Lopez M, Crespi C, Sauleda J. Expansion of myeloid-derived suppressor cells in chronic obstructive pulmonary disease and lung cancer: potential link between inflammation and cancer. Cancer Immunol Immunother. 2015 Oct;64(10):1261-70.

23. Kalathil SG[1], Lugade AA, Pradhan V, Miller A, Parameswaran GI, Sethi S, Thanavala Y. T-regulatory cells and programmed death 1+ T cells contribute to effector T-cell dysfunction in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2014 Jul 1;190(1):40-50. doi: 10.1164/rccm.201312-2293OC.

24. Abdullah A, Belal M, Bastawisy AE, Gaafar r. Plasma vascular endothelial growth factor 165 in advanced non-small cell lung cancer. Oncol Lett 201; 7: 2121-2129.

25. Quint LE. Staging of non-small cell lung cancer. Cancer Imaging 2007**;** 7: 148-159.

26. Buccheri G, Ferrigno D, Tamburini M. Karnofsky and ECOG performance status scoring in lung cancer: a prospective, longitudinal study of 536 patients from a single institution. Eur J Cancer. 1996; 32A(7):1135-41.

27. http://www.goldcopd.org/Guidelines/guideline-2010-gold-report.html

28. Komlósi ZI, Kovács N, van de Veen W, Kirsch A, Fahrner HB, Wawrzyniak M, Rebane A, Stanic B, Palomares O, Rückert B, Menz G, Akdis M, Losonczy G, Akdis CA. Human CD40L-expressing type 3 innate lymphoid cells induce IL-10-producing immature transitional regulatory B cells. J Allergy Clin Immunol. 2017 Sep 19. pii: S0091-6749(17)31460-4. doi: 10.1016/j.jaci.2017.07.046.

29. Bronte V[1], Brandau S[2], Chen SH[3], Colombo MP[4], Frey AB[5], Greten TF[6], Mandruzzato S[7,8], Murray PJ[9], Ochoa A[10], Ostrand-Rosenberg S[11], Rodriguez PC[12], Sica A[13,14], Umansky V[15,16], Vonderheide RH[17], Gabrilovich DI[18]. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun. 2016 Jul 6;7:12150. doi: 10.1038/ncomms12150.

30. Voron T, Marcheteau E, Pertot S, Colussi O, Tartour E, Taieb J, Terme M. Control of the immune response by pro-angiogenic factors. Front Oncol 2014; 4: 1-9.

31. Tamiya M[1], Tamiya A[2], Yasue T[3], Nakao K[2], Omachi N[2], Shiroyama T[4], Tani E[4], Hamaguchi M[4], Morishita N[4], Suzuki H[4], Okamoto N[4], Okishio K[5], Kawaguchi T[6], Atagi S[5], Hirashima T[4]. Vascular Endothelial Growth Factor in Plasma and Pleural Effusion Is a Biomarker for Outcome After Bevacizumab plus Carboplatin-Paclitaxel Treatment for Non-small Cell Lung Cancer with Malignant Pleural Effusion. Anticancer Res. 2016 Jun;36(6):2939-44.

32. Baraldo S, Zanin ME, Zuin R, Saetta M. Lymphocytes. In: Chronic Obstructive Pulmonary Disease. Stockley R, Rennard S, Rabe K, Celli B. (eds.). Blackwell Publishing 2007, pp. 269-274.

33. Zhai R[1], Yu X[1], Shafer A[2], Wain JC[3], Christiani DC[4]. The impact of coexisting COPD on survival of patients with early-stage non-small cell lung cancer undergoing surgical resection. Chest. 2014 Feb;145(2):346-353. doi: 10.1378/chest.13-1176.

34. Qiang G[1], Liang C[1], Xiao F[1], Yu Q[1], Wen H[1], Song Z[1], Tian Y[1], Shi B[1], Guo Y[1], Liu D[1]. Impact of chronic obstructive pulmonary disease on postoperative recurrence in patients with resected non-small-cell lung cancer. Int J Chron Obstruct Pulmon Dis. 2015 Dec 29;11:43-9. doi: 10.2147/COPD.S97411. eCollection 2016.

35. Engström G[1], Segelstorm N, Ekberg-Aronsson M, Nilsson PM, Lindgärde F, Löfdahl CG. Plasma markers of inflammation and incidence of hospitalisations for COPD: results from a population-based cohort study.Thorax. 2009 Mar;64(3):211-5. doi: 10.1136/thx.2008.102079. Epub 2008 Nov 6.

36. Bridges JP[1], Davis HW, Damodarasamy M, Kuroki Y, Howles G, Hui DY, McCormack FX. Pulmonary surfactant proteins A and D are potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury. J Biol Chem. 2000 Dec 8;275(49):38848-55.

37. Ambade VN[1], Sontakke AN[2], Barthwal MS[3], Tyagi R[3], Basannar DR[4]. Diagnostic Utility of Biomarkers in COPD. Respir Care. 2015 Dec;60(12):1729-42. doi: 10.4187/respcare.03753. Epub 2015 Jun 23.

38. Morissette MC, Jobse BN, Thayaparan D, Nikota JK, Shen P, Labiris NR, Kolbeck R, Nair P, Humbles AA, Stämpfli MR. Persistence of pulmonary tertiary lymphoid tissues and anti-nuclear antibodies following cessation of cigarette smoke exposure. Respir Res. 2014 Apr 22;15:49. doi: 10.1186/1465-9921-15-49.

39. Lee Y, Kim SH, Han JY, Kim HT, Yun T, Lee JS. Early neutrophil-to-lymphocyte ratio reduction as a surrogate marker of prognosis in never smokers with advanced lung adenocarcinoma receiving gefitinib or standard chemotherapy as first-line therapy. J Cancer Res Clin Oncol. 2012 Dec;138(12):2009-16. doi: 10.1007/s00432-012-1281-4.

40. Lin G, Liu Y, Li S, Mao Y, Wang J, Shuang Z, Chen J, Li S. Elevated neutrophil-to-lymphocyte ratio is an independent poor prognostic factor in patients with intrahepatic cholangiocarcinoma. Oncotarget. 2016 Aug 9;7(32):50963-50971. doi: 10.18632/oncotarget.7680.

41. Kusmartsev S, Cheng F, Yu B, Nefedova Y, Sotomayor E, Lush R, Gabrilovich D. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. Cancer Res. 2003 Aug 1;63(15):4441-9.

42. Hoenderdos K, Condliffe A. The neutrophil in chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol. 2013 May;48(5):531-9. doi: 10.1165/rcmb.2012-0492TR.

43. Gregory AD[1], Hale P, Perlmutter DH, Houghton AM. Clathrin pit-mediated endocytosis of neutrophil elastase and cathepsin G by cancer cells. J Biol Chem. 2012 Oct 12;287(42):35341-50. doi: 10.1074/jbc.M112.385617. Epub 2012 Aug 22.

44. Mittendorf EA, Alatrash G, Qiao N, Wu Y, Sukhumalchandra P, St John LS, Philips AV, Xiao H, Zhang M, Ruisaard K, Clise-Dwyer K, Lu S, Molldrem JJ. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. Cancer Res. 2012 Jul 1;72(13):3153-62. doi: 10.1158/0008-5472.CAN-11-4135. Epub 2012 May 7.

45. Lee KH[1], Lee CH[2], Jeong J[1], Jang AH[1], Yoo CG[3]. Neutrophil Elastase Differentially Regulates Interleukin 8 (IL-8) and Vascular Endothelial Growth Factor (VEGF) Production by Cigarette Smoke Extract. J Biol Chem. 2015 Nov 20;290(47):28438-45. doi: 10.1074/jbc.M115.663567. Epub 2015 Oct 9.

46. Kanazawa H, Asai K, Hirata K, Yoshikawa J. Possible effects of vascular endothelial growth factor in the pathogenesis of chronic obstructive pulmonary disease. Am J Med. 2003 Apr 1;114(5):354-8.

47. Valipour A[1], Schreder M, Wolzt M, Saliba S, Kapiotis S, Eickhoff P, Burghuber OC. Circulating vascular endothelial growth factor and systemic inflammatory markers in patients with stable and exacerbated chronic obstructive pulmonary disease. Clin Sci (Lond). 2008 Oct;115(7):225-32. doi: 10.1042/CS20070382.

48. Kasahara Y, Tuder RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. Am J Respir Crit Care Med. 2001 Mar;163(3 Pt 1):737-44.

49. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, Hollmann TJ, Bruggeman C, Kannan K, Li Y, Elipenahli C, Liu C, Harbison CT, Wang L, Ribas A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med. 2014 Dec 4;371(23):2189-99. doi: 10.1056/NEJMoa1406498. Epub 2014 Nov 19.

50. Rizvi NA[1], Hellmann MD[2], Snyder A[3], Kvistborg P[4], Makarov V[5], Havel JJ[5], Lee W[6], Yuan J[7], Wong P[7], Ho TS[7], Miller ML[8], Rekhtman N[9], Moreira AL[9], Ibrahim F[10], Bruggeman C[11], Gasmi B[12], Zappasodi R[12], Maeda Y[12], Sander C[8], Garon EB[13], Merghoub T[14], Wolchok JD[15], Schumacher TN[4], Chan TA[16]. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science. 2015 Apr 3;348(6230):124-8. doi: 10.1126/science.aaa1348. Epub 2015 Mar 12.

Figure legends

Figure 1. Flow cytometric identification of myeloid-derived suppressor cells.

Figure 2. Kaplan-Meyer analysis of progression-free survival in NSCLC and NSCLC+COPD patients

Figure 3. Relationships between serum VEGF and M-MDSC (a,b), neutrophil (c,d) and lymphocyte counts (e,f) in NSCLC (a,c,e) and NSCLC+COPD (b,d,f) patients.

Table I. Clinical data, progression-free and overall survival

Group	Control	COPD	NSCLC	NSCLC+COPD
	n=60	n=54	n=95	n=80
male	15	27	54	50
female	45	27	41	30
age (years)	54.3±0.9	63.2±1.0***	63.3±0.9***	65.3±0.9***
smoking (pack-year)	28 {15-45}	42 {31-50}***	40 {28-48}	40 {30-50}*
oncologic stage				
IIIB	-	-	12	14
IV	-	-	83	66
adenocarcinoma	-	-	73	60
squamous cell cc.	-	-	22	20
ECOG state				
0	-	-	56	44
1	-	-	33	31

2	-	-	4	3
FEV_1 (%pred)	103.1±2.4	46.1±2.4***	78.8±2.3*** $^{###}$	62.0±2.3*** $^{###}$ $^{+++}$
FEV_1/FVC	81.7±1.0	51.1±1.9*** $^{###}$	79.2±1.6$^{###}$	62.5±2.1*** $^{###}$ $^{+++}$
BMI (kg/m^2)	26.9±1.2	28.3±1.1	26.2±0.8$^{#}$	24.2±0.8$^{#}$
treatment				
platinum+gemcitabine	-	-	14	11
platinum+pemetrexed	-	-	15	13
platinum+taxan	-	-	14	9
platinum+taxan+bevac.	-	-	48	40
other$^{¤}$	-	-	4	7
radiotherapy	-	-	32	20
no radiotherapy	-	-	63	60
WBC (G/L)	7.2±0.2	9.0±0.3*	11.9±0.5*** $^{###}$	10.3±0.4*** $^{+}$
neu count (G/L)	4.3±0.2	5.7±0.3	9.2±0.4*** $^{###}$	7.5±0.3*** $^{##}$ $^{++}$
ly count (G/L)	2.3±0.1	2.4±0.1	1.7±0.1*** $^{###}$	1.9±0.1$^{##}$
neu/ly ratio	1.96±0.08	2.72±0.23	7.41±0.76*** $^{###}$	4.54±0.27** $^{+++}$
monocytes (G/L)	0.42±0.02	0.56±0.03	0.62±0.03***	0.67±0.04***
hemoglobin (g/L)	143±2	148±2	133±2** $^{###}$	137±2$^{##}$
platelets (G/L)	240±7	257±10	346±17*** $^{###}$	324±12*** $^{##}$
CRP (mg/L)	3 {2-5}	4 {3-8}	17 {6-59}*** $^{###}$	8 {4-19}$^{+++}$
median PFS (month)	-	-	4.9	7.4^{++}
median OS (month)	-	-	11.0	16.9

BMI: body mass index, WBC: white blood cell count, neu: neutrophil, ly: lymphocyte, CRP: C-reactive protein concentration; unindexed data indicate p>0.05 vs other groups, *: p<0.05, **: p<0.01, ***: p<0.001 vs. control, $^{#}$: p<0.05, $^{##}$: p<0.01, $^{###}$: p<0.001 vs. COPD, $^{+}$: p<0.05, $^{++}$: p<0.01, $^{+++}$: p<0.001 vs. NSCLC; $^{¤}$other treatments: gemcitabine (n=3), docetaxel (n=2), TKI monotherapy (n=2), platinum+vinorelbin (n=2), platinum+etoposid (n=2);

Table II. Cox-regression analysis of factors influencing progression-free survival (n=175)

Variable	n (%)	Progression-free survival			
		Univariate analysis		Multivariate analysis*	
		Median (mo)	p value	HR (95% CI)	p value
age					
<65 years	93 (53)	5.7	NS	-	NS
≥65 years	82 (47)	5.3	-	-	-
gender					
Male	104 (59)	5.3	NS	-	NS
Female	71 (41)	5.8	-	-	-
COPD					
No	95 (54)	4.9	0.0017	1.7 (1.2-2.4)	0.0020
Yes	80 (46)	7.4	-	-	-
ECOG					
0	101 (58)	6.1	NS	-	NS (0.12)
1-2	74 (42)	4.4	-	-	-
histology					
Adenocarcinoma	133 (76)	5.8	NS	-	NS
Squamous cell carcinoma	42 (24)	5.7	-	-	-
stage					
III/B	26 (15)	11.5	0.0422	-	NS (0.09)
IV	149 (85)	5.3	-	-	-
radiotherapy					
Yes	52 (30)	5.3	NS	-	NS
No	123 (70)	5.9	-	-	-

*Cox-regression model also included the type of chemotherapy, which did not influence PFS (not shown). HR=hazard ratio, CI=confidence interval, NS=not significant

Table III. Cytokines, vascular endothelial growth factor, myeloid suppressor cells and T cell subpopulations

Group	Controls	COPD	NSCLC	NSCLC+COPD
cytokines	n=24	n=26	n=29	n=19
IFNγ	10.8±1.9	36.6±5.9	83.0±19.9***$^{\#}$	32.7±8.1^{+}
TNFα	7.6±1.2	21.8±3.7	38.5±6.3***	19.0±5.3^{+}
IL-10	0.85±0.08	1.49±0.17	2.97±0.45***$^{\#\#}$	1.45±0.22^{++}

VEGF, MDSC	n=27	n=17	n=21	n=19
plasma VEGF (pg/mL)	545±43	526±67	1123±159**$^{\#\#}$	1243±158***$^{\#\#\#}$
M-MDSC/CD14^{+} (%)	3.77±0.55	3.71±0.77	9.71±1.44***$^{\#\#\#}$	7.88±0.84**$^{\#}$
G-MDSC/all WBCs (%)	1.35±0.24	2.55±0.58	4.48±0.81***	2.27±0.45^{+}
T cell subpopulations	n=9	n=11	n=14	n=13
CD3^{+}CD4^{+}(x10^{8}cell/mL)	12.49±0.99	10.15±1.61	7.43±1.61	9.73±1.89
IFNγ^{+} (x10^{7}cell/mL)	13.01±2.68	11.53±2.25	9.45±1.98	16.95±4.32
granz-B^{+} (x10^{7}cell/mL)	3.89±0.63	9.79±2.52	6.95±2.84	8.70±5.31
PD1^{+} (x10^{8}cell/mL)	1.38±0.20	1.29±0.24	1.07±2.53	1.51±0.45
CTLA4^{+} (x10^{7}cell/mL)	4.56±0.80	4.21±1.11	3.18±0.64	4.96±1.42
CD3^{+}CD8^{+}(x10^{8}cell/mL)	4.11±0.49	5.34±1.05	3.10±0.66	4.77±0.93
IFNγ^{+} (x10^{8}cell/mL)	1.36±0.34	1.52±0.42	1.24±0.29	2.06±0.50
granz-B^{+}(x10^{8}cell/mL)	1.94±0.32	2.61±0.64	1.48±0.40	4.35±1.12^{+}
PD1^{+} (x10^{7}cell/mL)	8.35±1.07	9.16±2.26	7.15±1.51	12.65±3.37
CTLA4^{+} (x10^{6}cell/mL)	7.19±1.40	5.91±1.30	5.59±1.38	9.01±2.08

VEGF: vascular endothelial growth factor, M-MDSC: monocytic-myeloid derived suppressor cells, G-MDSC: granulocytic myeloid derived suppressor cells, granz: granzyme B; unindexed data indicate p>0.05 vs other groups, *: p<0.05, **: p<0.01, ***: p<0.001 vs. control, $^{\#}$: p<0.05, $^{\#\#}$: p<0.01, $^{\#\#\#}$: p<0.001 vs. COPD, $^{+}$: p<0.05, $^{++}$: p<0.01, $^{+++}$: p<0.001 vs. NSCLC;

Supplementary Table I. Clinical data of stage IIIB-IV, non-squamous NSCLC patients having received bevacizumab with carboplatin+paclitaxel

variable	NSCLC	NSCLC+COPD
	n=39	n=35
Male	20	17

Female	19	18	
Age (years)	62±2	65±1	
Oncologic stage			
IIIB	4	4	
IV	35	31	
ECOG state			
0	27	23	
1	11	11	
2	1	1	-
FEV_1 (%pred)	81±3	$62±4^{+++}$	
FEV_1/FVC	73±1	$58±2^{+++}$	
BMI (kg/m^2)	24.2±0.8	25.3±0.9	
CRP (mg/L)	15 {6-53}	$6 \{3-16\}^{+}$	
WBC (G/L)	12.4±0.7	11.2±0.6	
neu (G/L)	10.1±0.7	$7.8±0.5^{+}$	
ly (G/L)	1.6±0.1	$2.2±0.1^{+}$	
neu/ly ratio	6.7±0.7	$4.5±0.4^{++}$	
monocytes (G/L)	0.6±0.0	0.7±0.1	
hemoglobin (g/L)	131±2	141±3	
platelets (G/L)	367±29	330±16	
median PFS (months)	3.3	6.1^{+}	
median OS (months)	11.0	15.0 ns	

BMI: body mass index, WBC: white blood cell count, neu: neutrophil, ly: lymphocyte, CRP: C-reactive protein concentration; unindexed data indicate $p>0.05$, $^{+}$: $p<0.05$, $^{++}$: $p<0.01$, $^{+++}$: $p<0.001$ vs. NSCLC;

Figure 1.

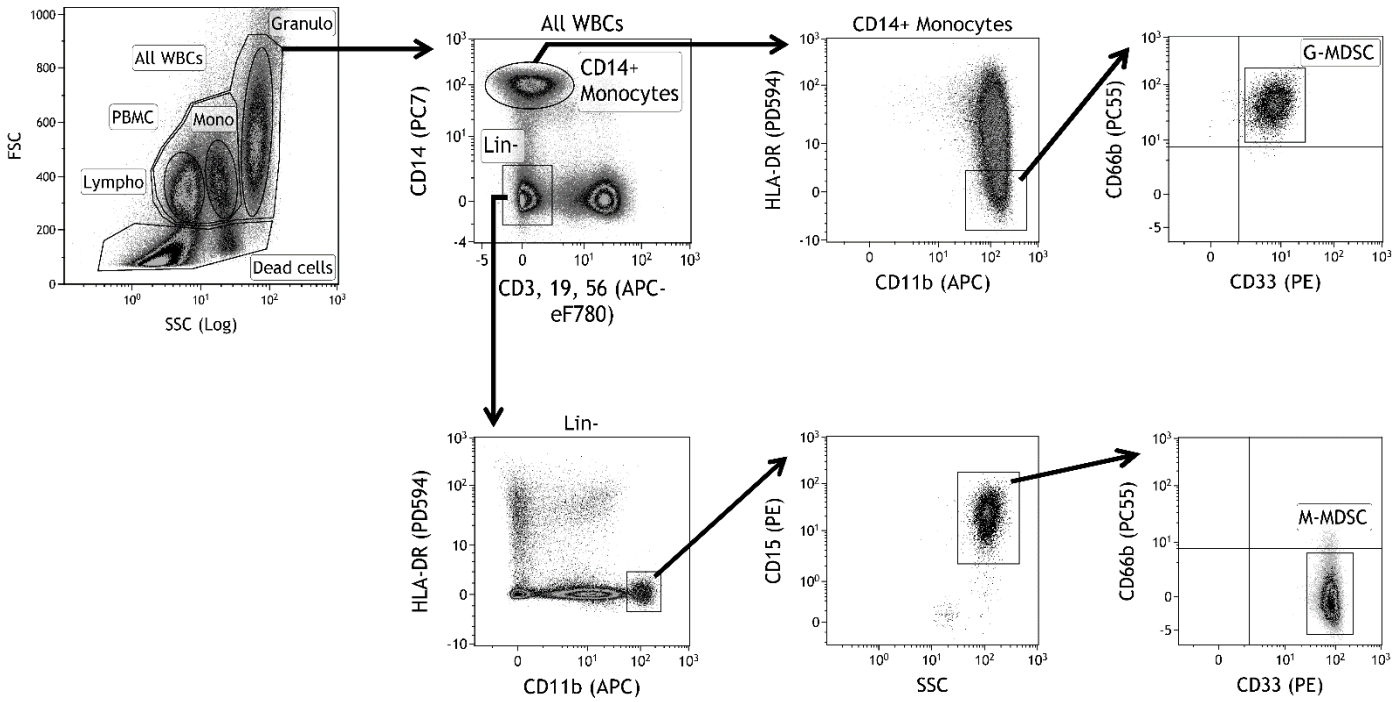


Figure 2.

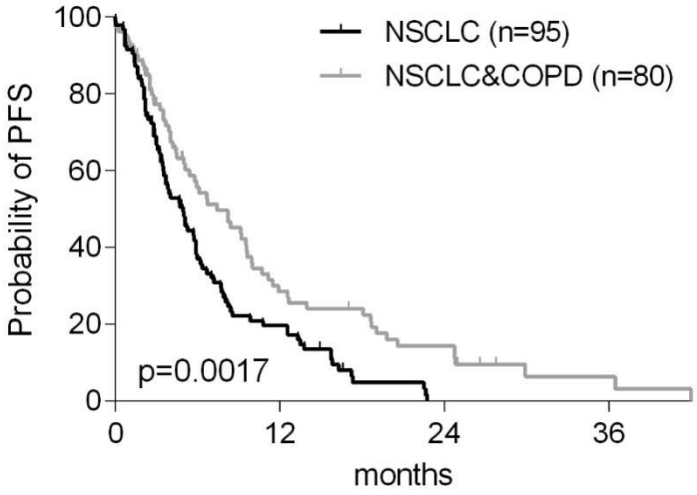
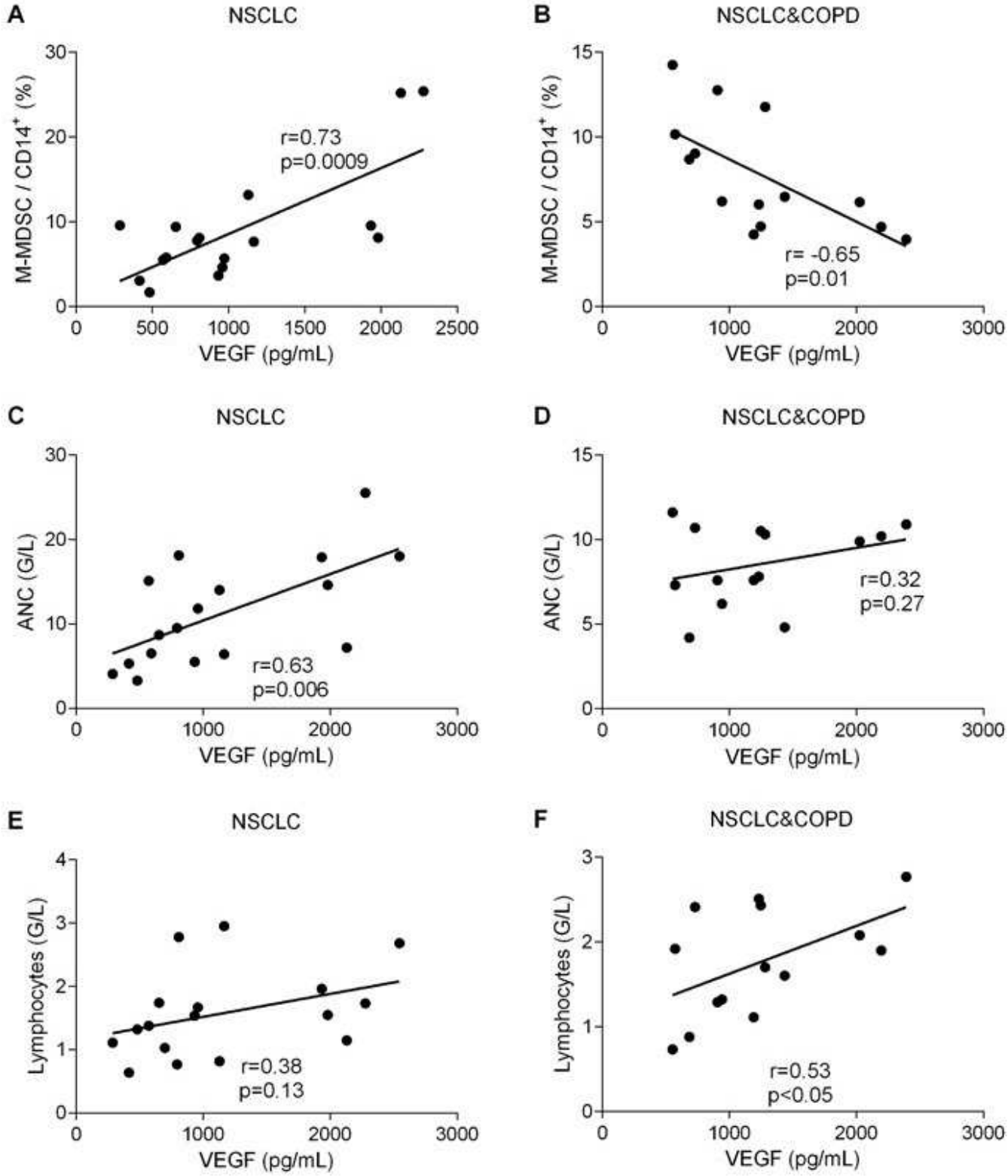

NSCLC (n=95)
NSCLC&COPD (n=80)
Probability of PFS
100
80
60
40
20
0
p=0.0017
0
12
24
36
months

Figure 3.


A
NSCLC
M-MDSC / CD14+ (%)
r=0.73
p=0.0009
VEGF (pg/mL)
B
NSCLC&COPD
M-MDSC / CD14+ (%)
r= -0.65
p=0.01
VEGF (pg/mL)
C
NSCLC
ANC (G/L)
r=0.63
p=0.006
VEGF (pg/mL)
D
NSCLC&COPD
ANC (G/L)
r=0.32
p=0.27
VEGF (pg/mL)
E
NSCLC
Lymphocytes (G/L)
r=0.38
p=0.13
VEGF (pg/mL)
F
NSCLC&COPD
Lymphocytes (G/L)
r=0.53
p<0.05
VEGF (pg/mL)