**TITLE**

# Assessment of RANK/RANK-L prevalence and clinical significance in NSCLC European Thoracic Oncology Platform Lungscape cohort and SPLENDOUR randomized clinical trial

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**ABSTRACT**

**Background**:

The primary objective of this study is to evaluate the clinical significance of RANK/L expression, in both a retrospective cohort of surgically resected stage I-III NSCLC (Lungscape) and a randomized clinical trial-cohort (SPLENDOUR) of advanced NSCLC treated with chemotherapy alone or in combination with denosumab.

**Methods**:

RANK-L expression was assessed on tissue microarrays (TMAs) in Lungscape and whole sections in SPLENDOUR, using immunohistochemistry, with H-scores values>0 indicating positivity. Prevalence of RANK positivity and its association with clinicopathological characteristics, and patient outcome was explored in a subset of the ETOP Lungscape cohort and in SPLENDOUR. Also investigated were the prevalence of RANK overexpression (proportion of positive cancer cells ≥50%) in the Lungscape cohort, and RANK-L in the SPLENDOUR trial.

**Results**:

In the Lungscape cohort, RANK expression was assessed at a median follow-up of 46 months (N=488 patients; 4 centers); 35% were female, 44/49/6% adenocarcinomas (AC)/squamous cell carcinomas (SCC)/other, 48/27/25% with stage I/II/III. Median RFS/TTR/OS were 58/Not reached/74 months. Prevalence of RANK expression was 31% (95%CI:27%-35%); significantly higher in AC: 50% (95%CI:43%-57%) vs SCC: 12% (95%CI:8%-16%) (p<0.001); more frequent in females (42% vs 25%, p<0.001) and tumors ≤4cm (35.3% vs 23.3%, p=0.0065). No association with outcome was found.

In the SPLENDOUR trial (463 patients), the prevalence of membranous and cytoplasmic RANK positivity was 34% (95%CI:30%-38%) and 9% (95%CI:7%-12%), respectively, while prevalence for RANK-L was 5% (95%CI:3%-7%) and 36% (95%CI:31%-40%), respectively. Cytoplasmic RANK-L positivity was more common among females (47% vs 31%, p=0.001) and in non-SCC histology (45% vs 10%, p<0.0001). At the pre-specified 1% significance level, no prognostic or predictive effect was found.

**Conclusions**:

Both cohorts indicate that RANK expression is more common in adenocarcinoma/non-squamous NSCLC and in female patients. No prognostic effect is found, and in the clinical trial involving addition of denosumab to chemotherapy no predictive effect is detected.

## INTRODUCTION

Lung cancer remains the leading cause of cancer mortality. Non-small cell lung cancer (NSCLC) accounts for 85% of primary lung malignancies. Recently, the knowledge of lung carcinogenesis has been rapidly improving and life expectancy of individuals with lung cancer has significantly increased following the emergence of targeted therapies and immunotherapy.

Common cell survival signalling pathways are activated by carcinogens as well as by inflammatory cytokines, growth factors and immune modulators, which contribute substantially to cancer development. Accordingly, prognostic and predictive biomarkers have been identified.

Nuclear factor-kappaB (NF-kappaB) has been shown to be involved in multiple steps in carcinogenesis and resistance to chemotherapy or radiotherapy. There is today considerable evidence that NF-kappaB is constitutively activated in a variety of solid tumors, including prostate, breast, cervical, pancreatic and lung cancer [1]. Animal models and cell culture systems have established the links between NF-kappaB and lung carcinogenesis [2, 3], highlighting the significance of evaluating the NF-kappaB signalling pathway as a potential biomarker and therapeutic target in lung cancer.

The Receptor Activator of NF-κB, RANK, (also known as TNFRSF11A) and the ligand RANK-L (also known as TNFSF11), a tumor necrosis factor (TNF) family member that signals through its receptor RANK, was first discovered for its role in modulating the interactions between T-cells and dendritic cells. Subsequently, its receptor RANK-L has been shown to activate osteoclasts, promote lymphocyte maturation and function, and enable mammary gland and secondary lymph node organogenesis[4-6]. A link between RANK-L signalling and cancer has been established in recent years. Perhaps owing to its bone remodelling capabilities, the RANK-L-RANK pathway facilitates bone metastasis formation [7, 8]. Also, it promotes seeding of breast tumor cells into the lungs in a T-regulatory–cell-dependent manner and participates in development of progestin-dependent mammary tumors [9-11]. A similar role in carcinogenesis, in correlation with sex hormones, was suggested regarding lung cancer in animal models [12].

Targeting RANK using denosumab, a fully human monoclonal IgG2 antibody binding with a high affinity to RANK-L is a proven strategy to prevent skeletal complications due to bone metastases in advanced lung cancer [13] and other solid tumors. A retrospective subgroup analysis of patients with lung cancer from this trial [13] suggested a potential survival benefit related to denosumab administration as compared to zoledronate [14]. SPLENDOUR (ETOP-5-12/EORTC 08111) was an open label phase III trial with the primary objective to evaluate whether the

addition of denosumab to standard first-line platinum-based doublet chemotherapy in advanced NSCLC improves overall survival (OS). The study was stopped early, and no difference in outcome was found.

Whether measuring the expression of RANK/L in NSCLC might serve as a prognostic or predictive biomarker has not been previously addressed and no standard method for its determination has been described. One report described a frequent and high expression of RANK on lung tumors, associated with worse prognosis, in particular in females. No correlation was observed between RANK expression and smoking status, but a potential correlation with the presence of KRAS mutation was hypothesized [12].

The primary objective of the current study is to evaluate the prevalence of RANK(L) (over)expression, as well as its association with selected patient or tumor characteristics and its clinical significance, in NSCLC. The study addresses these questions from two distinct NSCLC cohorts. The first is from the ETOP Lungscape, a clinically annotated biobank of resected stages I-III NSCLC while the second from the SPLENDOUR (ETOP-5-12/EORTC 08111) clinical trial in advanced NSCLC.

# METHODS

## Lungscape RANK cohort

### *Study design*

Lungscape RANK was a cohort study of surgically resected, stage I-III NSCLC cases from a subset of the Lungscape cohort [15]. Clinical and molecular data were obtained from the Lungscape iBiobank database (https://etopdata.etop-eu.org). Mandatory clinical parameters and the 7$^{th}$ TNM staging exactitude were centrally reviewed. The study has been conducted according to the Lungscape master and RANK sub-study protocols; with adherence to country specific ethics and regulatory requirements.

### *RANK IHC scoring and definition of RANK expression/overexpression*

Each site participating in the study provided 4 freshly-cut slides from one or more TMAs containing 2 - 4 FFPE cores for each case accepted in the Lungscape program. IHC staining was performed at a laboratory facility designated by Amgen, in order to allow the establishment of optimal IHC procedures and uniform examination of all samples. RANK IHC assay sensitivity was

achieved on the commercial platform by use of a combination of RANK antibodies in a 50:50 ratio, using RANK cocktail 2.5 ug/ML each and RANK close N1H8 ug/mL.

For that purpose, 4 sections of each TMA were cut and sent to the Amgen designated facility, Clarient/Neogenomics, that is CLIA certified . The designated laboratory performed IHC analysis for RANK on the provided TMA slides, including reading of slides for RANK staining. For each of the four cores, staining intensity was measured in different localizations of the cell, i.e., in both cytoplasm and membrane, and overall. RANK expression and overexpression were defined based on the staining intensity measured overall in the cells. A Lungscape case is considered to have positive RANK expression (RANK(+)) if the H-score of at least one core s non-zero, while RANK overexpression (RANK(++)) corresponds to the cases that at least one of the four cores shows ≥50% of positive staining intensity.

## SPLENDOUR cohort

### *Study history and translational objectives*

SPLENDOUR was an open-label phase III trial with the primary objective to assess the efficacy of the addition of denosumab to standard first-line platinum-based doublet chemotherapy in advanced stage IV NSCLC, stratified by bone metastases (presence versus absence), ECOG PS (0/1 versus 2), histology (squamous versus non-squamous), and geographic region (Eastern versus Western versus Southern Europe). A total of 514 patients, 259 in chemotherapy-denosumab and 255 in the chemotherapy-alone arm, were randomized in the trial from 11-December-2014 to 10-January-2018. The trial is registered with ClinicalTrials.gov, number NCT02129699. The primary efficacy analysis failed to demonstrate an OS improvement of the chemotherapy-denosumab treatment combination compared to chemotherapy-alone[16]. This finding was also verified by the combined analysis of SPLENDOUR and AMGEN-249 trials [17].

The translational research objective of the SPLENDOUR trial, presented here, is to investigate the prognostic role of membranous and cytoplasmic RANK/RANK-L (RANK(L)) expression as well as its predictive role as marker of response to denosumab treatment.

### *Material collection and testing*

Patients' availability of tumor tissue for translational research, was one of the trial's eligibility criteria. FFPE tumor tissue was collected at baseline from a biopsy of the primary tumor or metastasis or from archival tissue from tumor resection, or a paraffin-embedded cell block and subsequently submitted to, catalogued, and maintained at a central laboratory (Institute of Pathology at the University Hospital Lausanne – CHUV).

In the frame of the present study, cytoplasmic and membranous immunohistochemistry (IHC) staining was performed for both RANK and RANK-L, using Mouse anti-RANK, clone N2B10/N1H8, Monoclonal mouse anti-RANKL, clone M366 and Mouse IgG1 Isotype Control (Clone 11711).

Primary analysis for both biomarkers (RANK or RANK-L with cytoplasmic or membranous staining) was performed using as cut-off an H-score value of 0 (negative cases: "0 H-score" versus positive cases: ">0 H-score"). An alternative cut-off of 10 for positivity was used as sensitivity analysis only for the membranous RANK and cytoplasmic RANK-L (not meaningful for the other cases, due to very few cases above that cut-off value).

## Statistical Methodology

The prevalence of RANK(L) (over)expression is reported as a percentage with a corresponding 95% exact binomial confidence interval (CI). Differences in clinicopathological characteristics by biomarker status were assessed based on the Fisher's exact test, for categorical characteristics, or Mann-Whitney test, for continuous ones.

In the Lungscape cohort, where additional cancer-related biomarkers were available (MET[18], ALK[19], PTEN[20], PD-L1[21, 22] IHC as well as MET, EGFR, KRAS & PIK3CA genes[23]), their association with RANK (over)expression status was evaluated by histology type (adenocarcinoma and squamous cell carcinoma; Fisher's exact test) and overall (stratified by histology, Cochran-Mantel-Haenszel statistic; homogeneity, Breslow-Day test).

In Lungscape, the primary long-term outcomes of interest were OS, recurrence-free survival (RFS; time from surgery to first recurrence or death), and time to recurrence (TTR, time from surgery to recurrence). In the SPLENDOUR trial, OS was the primary endpoint, with progression-free survival (PFS) a secondary one.

In both studies, the time-to-event endpoints were estimated by the Kaplan-Meier method, while Cox proportional hazards (PH) models were used to assess the clinical significance of RANK(L) biomarkers.

More particularly, in Lungscape, the effect of RANK (over)expression on outcome was explored through Cox proportional hazard models, adjusting for the following clinicopathological variables of interest: sex, ethnicity, smoking history, age, adjuvant chemotherapy, adjuvant radiotherapy, previous history of cancer, performance status (PS), stage, localization of primary tumor, tumor size categories, histology, year of surgery, surgery technique, surgery anatomy, and the following biomarkers/gene mutations: MET IHC, MET gene, ALK IHC, PTEN, EGFR, KRAS, PIK3CA and PD-L1 (cutoff 5%). The significant outcome prognostic factors were derived based on the backwards

elimination method (removal $p \geq 0.10$) and their effect was expressed through corresponding Hazard Ratios (HRs) and 95%CIs.

In the frame of the randomized SPLENDOUR study, the prognostic role of each biomarker was assessed by applying univariate Cox models, for OS and PFS, separately in each treatment arm (Chemotherapy and Denosumab, Chemotherapy). A biomarker would be called prognostic if a statistically significant difference was found between positive/negative biomarker status, separately in each treatment arm. The predictive effect of the biomarkers was also assessed through the testing of the interaction of each biomarker with treatment on OS and PFS. In this case, multivariable Cox models including treatment and the RANK(L) biomarkers, were adjusted for sex, age, ECOG PS, histology, bone metastasis and region.

The proportional hazards assumption of the Cox models was tested, using the Schoenfeld residuals.

A stringent 1% significance level was chosen due to the issue of multiple testing for interpreting the results of these translational analyses. Of note, all these analyses should be considered exploratory, and no adjustment for multiple testing was performed.

Statistical analyses were carried out in SAS version 9.4 (SAS Institute, Cary, NC) and performed for Lungscape at the ETOP statistical center, Frontier Science Foundation-Hellas, Athens, Greece and for SPLENDOUR at the European Organisation for Research and Treatment of Cancer-EORTC.

## RESULTS

### Lungscape: Assessment of prognostic effect of RANK (over)expression

***Analysis Cohort***

In the frame of the ETOP Lungscape study, RANK IHC was scored by a board-certified central Lungscape pathologist. The analysis cohort consisted of 488 surgically resected stage I-III NSCLC patients (88% of 556 total in 4 Lungscape centres (Medical University Gdansk, University Hospital Basel, University Hospital Heidelberg and Roswell Park Cancer Institute ), operated from 2003 up to 2011. Clinicopathological characteristics of the analysis cohort, that includes 216 (44%) adenocarcinomas and 241 (49%) squamous cell carcinomas, are presented in Table 1.

The median age of the analysis cohort was 66 years, consisting primarily of male patients (65%), of Caucasian ethnicity (98%), former or current smokers (81%; 61% and 21%). Adjuvant

chemotherapy and radiotherapy were administered to 21% and 7% of the patients, respectively. Disease stage distribution was: I 48%, II 27% and III 25%, while 60% of the tumors were of up to 4cm size. With respect to the surgery anatomy, lobectomy was the most frequent type (71%), 79% of the surgeries were open thoracotomies and the majority of surgeries (85%) were performed after 2006.

***RANK (over)expression prevalence and association with clinico-pathological characteristics and other molecular biomarkers***

The prevalence of RANK expression was 30.5% (95%CI: 26.5% - 34.8%), significantly higher in adenocarcinomas 50.0% (95%CI: 43.1% - 56.9%) versus squamous cell carcinomas 11.6% (95%CI: 7.9% - 16.4%) ($p<0.001$, Table 1).

Among the 149 positive cases, 86 (58%) were positive only in cytoplasm, 54 (36%) positive in both membrane and cytoplasm, while 9 (6%) only in membrane (Supplementary Table S1, Supplementary Figure S1).

Maximum RANK score intensity in cytoplasm was 1+ for 25% of the cases, 2+ for 3% and 3+ for only 0.2%, while analogously in membrane scores were 11% 1+, 1% 2+ and 0.4% 3+ (Supplementary Table S2). The distribution of H-scores is further illustrated in Supplementary Figure S2. In cytoplasm, 93% of H-scores were up to 25 (71% 0), with an overall average H-score of 6), while in membrane 97% were up to 25 (87% 0), with 2.3 average value.

The prevalence of RANK overexpression was 9.6% (95%CI: 7% - 13%) and was also significantly more common in adenocarcinomas (17.6%; 95%CI: 13% - 23%) versus squamous cell carcinomas (2.5%; 95%CI: 0.9% - 5.3%) ($p<0.001$, Table 1).

Furthermore, RANK expression was statistically significantly (at 1%) associated with sex and tumor size (Table 1), irrespective of histology. More particularly, positive RANK expression was higher in females (42%) rather than males (25%, $p<0.001$), and in tumors ≤4cm than >4cm (35% vs 23%, respectively; $p=0.0065$). Also, significant associations were detected between surgery characteristics and RANK expression (surgery anatomy and surgery technique, both $p<0.001$).

RANK overexpression was more prevalent in never smokers (18% vs 8% in former/current smokers, $p=0.0078$) (Table 1).

Among the specific cancer-related biomarkers evaluated in Lungscape, a significant association with RANK expression overall was detected only with IHC PTEN. Positive RANK expression was significantly more prevalent in patients with IHC PTEN expression compared to those with PTEN

loss (37% vs 22%, p<0.001, stratified by histology). The same relationship existed for adenocarcinomas alone (58% vs 38%, respectively, p=0.0054) (Supplementary Table S3).

RANK overexpression associations, were also examined and although based on small number of cases, the results are consistent. The mentioned significant association of IHC PTEN with RANK expression is also found with RANK overexpression both in adenocarcinomas (25% RANK overexpression among IHC PTEN expression vs 7% in IHC PTEN loss, p<0.001), and overall (p<0.001). No further significant association was detected (Supplementary Table S4).

***Clinical significance of RANK (over)expression: Association with outcome***

In the analysis cohort of 488 Lungscape patients, at a median follow-up time of 46.2 months (IQR: 35.4 - 55.3), the majority of patients (67%) were alive, with 283 (58%) without evidence of disease. In total, 205 (42%) RFS events were observed, with median RFS 57.9 months [95%CI: 52.9 - Not Estimable (NE)]. The median OS was 74.0 months (95%CI: 61.0 - NE), while median TTR was not reached.

None of the three outcome variables (overall or separately for adenocarcinomas or squamous cell carcinomas) differed significantly by RANK expression status (all log-rank p-values non-significant >5%; OS in Figures 1A-B, S3). No significant effect of RANK expression was found in the corresponding multivariable Cox models adjusted for clinicopathological characteristics.

Regarding RANK overexpression, a difference (log-rank p-value=0.037), not significant at 1%, was only found in the OS of adenocarcinomas (Figures 1C-D). This effect was also identified in the overall multivariable Cox model, stratified by tumor size and adjusted for ethnicity, stage, PS, previous cancer history and surgery technique, with $HR_{RANK\ express\ \_vs\_not}$=1.73 (1.07 - 2.80, p=0.026, Table S5).

Finally, no statistically significant difference was found in the rate of bone metastases by RANK expression or overexpression: 9.4% for positive RANK expression vs 8.0% for negative, p=0.60 and 10.6% for RANK overexpression vs 8.2% for not, p=0.58 (Table S6).

SPLENDOUR: *Assessment of prognostic and predictive effect of RANK(L) expression*

***Analysis Cohort***

Information on RANK(L) measurements was available for 463 patients, 229 in chemotherapy-alone and 234 in chemotherapy-denosumab arm, among the total 514 patients randomized in the SPLENDOUR trial (Supplementary Figure S4).

***Prevalence of membranous and cytoplasmic RANK(L) measurements and association with clinico-pathological and other baseline characteristics***

The prevalence of membranous RANK positivity (H-score>0) was 33.9% (exact binomial 95%CI: 29.6% - 38.4%) while for cytoplasmic was only 8.9% (95%CI: 6.4% - 11.8%) The corresponding percentages for membranous RANK-L and cytoplasmic RANK-L were 4.8% (95%CI: 3.0% - 7.1%) and 35.6% (95%CI: 31.3% - 40.2%), respectively (Tables 2, S7).

RANK(L) expression according to patients' baseline characteristics is summarized in Table 2, for membranous RANK and cytoplasmic RANK-L. Since a low positivity rate was found for cytoplasmic RANK and membranous RANK-L, leading to a small number of positive cases, results are provided only for descriptive purposes (Table S7).

Treatment arms were well balanced between positive/negative RANK(L) patients.

Membranous RANK did not appear to be associated with any of the examined characteristics (a differentiation by histology was present with higher RANK positivity among non-squamous, p=0.04, not significant at 1%).

Cytoplasmic RANK-L expression was significantly associated with sex, histology as well as region. More particularly, RANK-L positivity was more common among females (47% vs 31% in males, p=0.001), Western Europe compared to Southern Europe (41% vs 25%, p<0.001), and in in non-squamous histology (45% vs 10% in squamous, p<0.0001).

***Clinical significance of RANK(L): Association with Overall and Progression-free Survival***

The evaluation of the clinical significance of RANK(L) biomarkers, was based on the 463 patients with available membranous and cytoplasmic RANK(L) results of the total 514 randomised in the SPLENDOUR trial [16], with median follow-up time 24.6 months (IQR: 14.0-30.4), 329 recorded deaths (median OS 8.1 months (95%CI 7.4-9.9)) and 413 PFS events (median PFS 4.7 months (95%CI 4.2-5.0)). In the overall cohort, as well as within each treatment arm, no significant effect on OS was seen for membranous or cytoplasmic RANK expression (membranous $HR_{+vs-}$=1.11, 95%CI 0.88-1.39, p=0.38; cytoplasmic $HR_{+vs-}$=0.83, 95%CI 0.57-1.22, p=0.35) (results by treatment arm in Figures S5A-B, S6 A-B).

Similarly, no significant effect was detected for membranous RANK-L expression (overall $HR_{+vs-}$=1.36, 95%CI 0.85-1.26, p=0.20; by treatment arm in Supplementary Figures S7A-B).

In the case of the more frequent cytoplasmic RANK-L, a not significant at 1% OS differentiation appeared only in the Chemotherapy only arm, with RANK-L(+) having a better prognosis

compared to RANK-L(-) patients ($HR_{+vs-}$=0.71, 95%CI 0.51-0.98, p=0.037) (by treatment arm in Supplementary Figures S8A-B).

Results for PFS, analogous with OS, are provided in Supplementary Figures S9-S12. In addition, similarly to OS a PFS differentiation (not significant at 1%) by cytoplasmic RANK-L expression was found in the Chemotherapy only arm, ($HR_{+vs-}$=0.73, 95%CI 0.55-0.97, p=0.029).

Thus, overall, membranous or cytoplasmic RANK(L) expression levels cannot be considered as prognostic factors, none of them reaching 1% significance level or found significant in both treatment arms.

No biomarker was found predictive at the 1% level. The interaction effect of each one of the biomarkers with treatment on OS/PFS was not significant (p>0.01), in separate analysis by biomarker (Supplementary Figures S13-S14) as well as in multivariable analysis adjusting for other baseline characteristics (Supplementary Tables S8-S9). Only cytoplasmic RANK-L (Figure 2) had a marginally significant p-value for interaction (p<0.05), and this finding is reported here (interaction p=0.039/0.099 in the separate OS/PFS analysis, p=0.019/0.065 in multivariable OS/PFS). More particularly, cytoplasmic RANK-L(-) patients would tend to benefit more from the combination of chemotherapy with denosumab treatment, compared to chemotherapy alone, than RANK-L (+) patients (adjusted OS $HR_{chemo+deno\ vs\ chemo}$=0.80, 95%CI: 0.61-1.04 in RANK-L(-) and $HR_{chemo+deno\ vs\ chemo}$=1.29, 95%CI: 0.89-1.88 in RANK-L (+), interaction p=0.019) .

In the frame of sensitivity analysis, using the alternative cut-off of H-score=10 for defining positive membranous RANK (20% prevalence) and cytoplasmic RANK-L (31% prevalence), survival results were consistent with the main analysis (interaction of cytoplasmic RANK-L with treatment on OS: p=0.03).

Of note, in an exploratory post-hoc analysis, no statistically significant difference was found in the rate of bone metastases (post randomization) by RANK/RANKL membranous or cytoplasmic expression, overall and by treatment arm, while differentiations between arms were also not significant (Table S10).

## DISCUSSION

Current available literature to accurately describe the prevalence of RANK(L) expression in lung cancer is scarce, despite biological hypotheses correlating the downstream NF-kB pathway activation to oncogenesis, as well as to poor prognosis. Our study demonstrated a prevalence of

RANK expression of 50% in lung adenocarcinoma and 12% in lung squamous cell carcinoma. Expression was commonly weak; overexpression was only present in 10% of cases and mostly restricted to lung adenocarcinoma.

Another study on human lung cancer found RANK not to be expressed in healthy human lung tissue, but expressed in 60-80% of 120 NSCLC tissues using 3 independent cohorts. The incidence and intensity of expression were also greatest in adenocarcinomas, with 72% of lung adenocarcinomas staining positive for RANK with a mean H-score of 20, as compared to 61% of positivity with an H-score of 20 in squamous cell carcinoma [12]. RANK expression was significantly associated with the presence of *KRAS* mutations while no correlation was observed between RANK expression and smoking status and tumor stage.

A more rapid progression of lung cancer in women has been suggested in historical studies [24]. Earlier studies on mammary tumors have suggested the RANK-L/RANK system to be partly regulated by sex hormones [5, 25]. Rao and colleagues demonstrated that female sex hormones can promote lung cancer progression via the RANK pathway [12]. In our study prevalence of RANK expression, using the combined membranous and cytoplasmic staining, was slightly lower, using however a different staining technology and platform. No correlation with KRAS mutation could be detected. However, we were able to confirm the higher prevalence in non-squamous and female's tumors, suggesting a potential role of sex determinants impacting this oncogenic pathway.

RANK-L inhibition using the monoclonal antibody denosumab has been approved for the treatment of osteoporosis and to prevent skeletal-related events in patients with metastatic cancer. Also, it has been linked to delayed re-occurrence of breast tumors in an adjuvant setting, and reduction of tumor growth in patient-derived lung cancer xenografts [12, 26]. A post-hoc exploratory analysis suggested that denosumab might improve survival of metastatic lung cancer[14], which could not be confirmed by our randomized phase III trial SPLENDOUR [16]. Our subsequent combined analysis of two randomised trials evaluating the addition of denosumab to standard first-line chemotherapy in advanced NSCLC, using individual patient data, also did not find any statistically significant improvement in PFS or OS with the combination of denosumab and chemotherapy versus chemotherapy alone, in the overall population and across the important clinical subgroups as defined by age, gender, PS and presence of bone metastases, histology, and region.

In our Lungscape cohort, the expression of RANK was not correlated with outcome in any clinical subgroup in radically resected stage I-III NSLC. Also, using any positivity or a threshold of 10 by H-score, we could not identify a subgroup of biomarker-positive patients, be it RANK or RANK-L, benefiting from the addition of denosumab to chemotherapy in patients treated in SPLENDOUR.

A disadvantage of the study is that given the pre-set methodology of staining, the emphasis of the data lies on 1+ intensity of the staining. It cannot be excluded that a threshold of 2+ intensity, which was not available for the trial samples, might yield relevant trustworthy prognostic or predictive associations.

Novel immunotherapy-based combinations, particularly those targeting non-redundant immunosuppressive mechanisms, may improve clinical responses in advanced tumors. In pre-clinical models, the addition of a RANK-L-specific antibodies to anti-PD-1 or anti-CTLA-4 antibodies increased T-cell effector function and increased $CD8^+$ T-cell infiltration, leading to increased anti-tumor immunity [27]. Small real-world studies of metastatic melanoma and NSCLC patients treated concurrently with denosumab and immunotherapies indicate promising clinical responses [28]. While potential biological mechanisms of synergy have been suggested [29], prospective controlled data are required to confirm the clinical utility of such combinations.

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