Pre-treatment tissue TCR repertoire evenness is associated with complete pathological response in patients with NSCLC receiving neoadjuvant chemoimmunotherapy

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TRASLATIONAL RELEVANCE

The advance of chemoimmunotherapy in locally advanced stages of lung cancer has positioned complete pathologic responses (CPR) as a new relevant clinical entity, with implications for both the differential biology behind these responses and their possible use as an endpoint for assessing therapy efficacy. In this exploratory analysis of the NADIM trial, two biomarkers (Top 1% clonal space and TCR evenness), associated with T-cell repertoire imbalance, outperformed the established biomarkers PD-L1 and TMB, regarding CPR prediction after chemoimmunotherapy. Additionally, some mechanistic insights are revealed, that imply a higher immunogenicity of tumors with high Top 1% clonal space, as well as the presence of a distinctive peripheral immunosurveillance of pre-treatment tissue top 1% clones, in patients achieving CPR. Finally, although these findings have potential clinical impact and are hypothesis generating, they are exploratory and need to be confirmed in additional, larger cohorts.
Purpose: Characterization of the T-cell receptor (TCR) repertoire may be a promising source for predictive biomarkers of pathological response to immunotherapy in locally-advanced non-small cell lung cancer (NSCLC).

Experimental Design: In this study, next-generation TCR sequencing was performed in peripheral blood and tissue samples of 40 NSCLC patients, before and after neoadjuvant chemoimmunotherapy (NADIM clinical trial, NCT03081689), considering their complete pathologic response (CPR) or non-CPR. Beyond TCR metrics, tissue clones were ranked by their frequency and spatiotemporal evolution of top 1% clones was determined.

Results: We have found a positive association between an uneven TCR repertoire in tissue samples at diagnosis and CPR at surgery. Moreover, TCR most frequent-ranked clones (top 1%) present in diagnostic biopsies occupied greater frequency in the total clonal space of CPR patients, achieving an AUC ROC to identify CPR patients of 0.967 (95% CI, 0.897 to 1.000; p=0.001), and improving the results of PD-L1 TPS (AUC 0.767; p=0.026) or TMB (AUC 0.550; p=0.687). Furthermore, tumors with high pre-treatment top 1% clonal space showed similar immune cell populations but a higher immune reactive gene expression profile. Finally, the selective expansion of pre-treatment tissue top 1% clones in peripheral blood of CPR patients suggests also a peripheral immunosurveillance, which could explain the high survival rate of these patients.

Conclusions: We have identified two parameters derived from TCR repertoire analysis that could outperform PD-L1 TPS and TMB as predictive biomarkers of CPR after neoadjuvant chemoimmunotherapy, and unraveled possible mechanisms of CPR involving enhanced tumor immunogenicity and peripheral immunosurveillance.
INTRODUCTION

Lung cancer is one of the main leading causes of death by cancer worldwide. Approximately 85% of cases are non-small cell lung cancer (NSCLC) of which one third are diagnosed at locally advanced or stage III disease. Stage III NSCLC is a heterogeneous disease that includes patients with potentially resectable tumors that could theoretically be cured.

Recently, in NADIM trial (NCT03081689) (1) we have shown that patients treated with neoadjuvant chemoimmunotherapy achieved a progression-free survival (PFS) and overall survival (OS) rates at 2 years of 77% and 90% respectively, while 63% of patients achieve a complete pathological response (CPR). Additionally, the PFS rate in patients with CPR was higher than in non-CPR patients. Although these are encouraging results, with most patients maintaining no evidence of disease status after surgery, some of them do not achieve complete responses and relapse or eventually die. Similar results from the phase III Checkmate 816 trial reinforce the superiority of chemoimmunotherapy compared to chemotherapy alone in the rate of CPR (2). Thus, the identification of biomarkers of CPR to chemoimmunotherapy induction is therefore a priority in the future scenario of resectable NSCLC.

In the context of immunotherapy, both the determination of PD-L1 and tumor mutational burden (TMB) have been carried out to predict which patients are more likely to respond to treatment (3,4). However, the results are not consistent (5–7) and their value in the context of neoadjuvant chemoimmunotherapy is limited (1,2). Thus, it is necessary to explore other biomarkers capable of predicting which patients will benefit most from this treatment (8,9).

The mechanism by which the adaptive immune system responds to immunotherapy and recognizes tumor antigens relies mainly on the highly polymorphic T-cell receptors (TCRs) present in an individual. That is why the characterization of the TCR repertoire in terms of clones, diversity and antigen specificity by sequencing the CDR3 hypervariable region, seems to be a promising approach (10,11). Other authors have previously reported that parameters extracted from the TCR repertoire were associated with response to immunotherapy in different cancer patients (12–15) and in particularly, NSCLC (16–18). However, it has not been demonstrated its predictive value in pathologic response determination in neoadjuvant immunotherapy for NSCLC patients (19). The mechanisms by which this response to immunotherapy occurs by lymphocytes has not been elucidated either, although some authors hypothesize that peripheral lymphocytes are probably involved in the reinvigoration of the response (19,20). For these reasons we propose the study of T-cell receptor repertoire and highlight its importance in mediating the response to immunotherapy.

For the first time, we describe longitudinally and spatially the TCR repertoire in NSCLC patients receiving neoadjuvant chemoimmunotherapy. We also assess its capacity as a possible source for predictive biomarkers of complete pathological response and its relation with a pro-immflamatory state in the tumor microenvironment of CPR patients.
MATERIAL AND METHODS

Study design and sample collection

All the studies presented here regarding TCR repertoire analysis are exploratory in nature and hypothesis generating and therefore will require validation in larger cohorts. 46 patients with resectable stage IIIA from NADIM clinical trial (NCT03081689), were treated with 3 cycles of Nivolumab plus chemotherapy prior to surgery. All patients with enough material were included in the study. Patients were classified in two categories according to their tumor pathological response: CPR patients (i.e. 0% of viable tumor cells in tumor bed or any lymph node analyzed) and non-CPR patients (i.e. patients with any percentage of viable tumor cells in resection specimens). Informed consent for the collection of research samples and study protocol were approved by the clinical research ethics committee of Hospital Puerta de Hierro in accordance with the International Conference on Harmonization Guidelines on Good Clinical Practice and the Declaration of Helsinki. Written informed consent to participate in the study was obtained from all patients.

Up to 125 samples of peripheral blood (n=65) and tissue (n=60) from these 46 patients were prospectively collected and sequenced to determine their TCR repertoire. TCR sequencing was carried out in PBMCs samples collected before (n=30) and after (n=35) neoadjuvant treatment and in tissue samples obtained at diagnosis (n=22) and at surgery (n=38). RNA was used instead of DNA to decrease material input requirements and maximize valid samples for sequencing. Details of all patients, samples and techniques used are summarized in Supplementary Table S1. Additionally, a summary table with the number of paired samples analyzed for each technique and timepoint is shown in Supplementary Table S2. All molecular techniques were carried out in a blind fashion, only the pre- or post-treatment sample origin was known to the researchers conducting the experiments.

RNA extraction

PBMCs were isolated from blood samples by gradient density centrifugation using Lymphoprep (Alere Technologies) and cryopreserved until use. RNA from cryopreserved PBMCs was extracted using Maxwell RSC simply RNA Cells kit (Promega) as per manufacturer’s instructions. RNA from FFPE samples of biopsy at diagnosis and surgically-resected specimens were extracted with the truXTRAC FFPE DNA kit (Covaris). RNA quantification was carried out using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific Cat. No. Q10210) on Qubit apparatus.

TCR Library preparation

RNA extracted from PBMCs and FFPE samples was used to prepare the libraries for TCR sequencing. cDNA was obtained from RNA using the SuperScript™ IV VILO™ Master Mix (Cat. No. 11756050). RNA input for PBMCs-derived libraries was 25 ng and 100 ng for FFPE-derived libraries. TCR libraries from PBMC samples were done using the Oncomine™ TCR Beta-LR Assay (Cat. No. A35386). For FFPE-derived RNA samples, TCR libraries were done using the Oncomine™ TCR Beta-SR Assay (RNA) (Cat. No. A39359). For PBMCs-derived libraries, equal volumes from 8 samples at 25 pM were pooled for sequencing on an Ion 530 chip. For FFPE-derived libraries, equal volumes from up to 32 samples at 25 pM were combined for sequencing on an Ion 540 chip.

T cell receptor sequencing and data analysis
Once the libraries were templated, they were sequenced in the Ion GeneStudio™ S5 Series (Thermo Fisher Scientific) and analysis was done via Ion Reporter version 5.12 (Thermo Fisher Scientific). TCR convergence is determined as the aggregate frequency of clones, defined as unique TCRB nucleotide sequences, which shared a variable gene and CDR3 amino acid sequence with at least one other clone. Shannon’s diversity was calculated using the formula below, in which $p_i$ is the frequency of clonotype $i$ for the sample with $N$ unique clonotypes:

$$Shannon\ \text{Index} \ H = - \sum_{i=1}^{N} p_i \log_2(p_i)$$

Evenness describes how evenly distributed is the TCR repertoire, approaching 0 if the repertoire is unbalanced by a reduced number of predominant clones and approaching 1 if the repertoire is balanced, with similar frequencies of all the clones. It is calculated by dividing the Shannon’s diversity index by $\log_2(N)$ where $N$ is the total number of detected clones in each sample. Evenness high and low categories were defined using cohort median value ($\geq 0.9077$).

Frequency classifications of the clones in the TCR repertoire were done according to their ranked relative abundance in the total T-cell repertoire, named as total clonal space, for each sample. We divided them into top 1%, top 1-2%, top 2-5% and top> 5% (19). In this way, the top 1% clonal space is defined as the aggregate frequencies of the top 1% most frequent clones. Top 1% high and low categories were defined using cohort median value ($\geq 0.166115$).

When looking at the dynamics, we refer to contracted or expanded clones as those top 1% clones which their clonal frequency in peripheral blood decreases or increases after treatment.

Jaccard’s index was calculated as:

$$J_i = \frac{\text{shared clones} \ (A + B)}{\text{clones in } A + \text{clones in } B - \text{shared clones} \ (A + B)}$$

Tumor microenvironment lymphocytes and gene expression analysis

Tumor immune cells were identified from FFPE diagnostic biopsies of 11 patients through multiplex immunofluorescence and quantified considering tumor or stroma localization as previously described (1,22). The immunofluorescence markers used were grouped into two 6-antibody panels and allowed the quantification of: Total malignant cells or reactive epithelial cells [CK+ (AE1/AE3+)], Malignant cells or reactive epithelial cells PD-L1+ (CK+PD-L1+), Total T cells (CD3+), Cytotoxic T cells (CD3+CD8+), T cells antigen-experienced (CD3+PD-1+), Cytotoxic T cells antigen-experienced (CD3+CD8+PD-1+), T cells PD-L1+ (CD3+PD-L1+), Cytotoxic T cells PD-L1+ (CD3+CD8+PD-L1+), T cells antigen-experienced PD-L1+ (CD3+PD-1+PD-L1+), Cytotoxic T cells antigen-experienced PD-L1+ (CD3+CD8+PD-1+PD-L1+), Total macrophages (CD68+), Macrophages PD-L1+ (CD68+PD-L1+), Cytotoxic T cells activated (CD3+CD8+Granzyme B+), Memory T cells (CD3+CD45RO+), Effector/memory cytotoxic T cells (CD3+CD8+CD45RO+), Regulatory T cells [(CD3+FoxP3+)-(CD3+CD8+FopP3+)], Memory/Regulatory T cells (CD3+CD45RO+FopX3+). Spearman non-parametric test was used for correlations between different cells subpopulations and pre-treatment top 1% clonal space.

A single section of FFPE tissue was used to analyze gene expression through the HTG EdgeSeq Precision Immuno-Oncology Panel (HTG Molecular Diagnostics) following manufacturer’s instructions. Ten out of 11 patients sequenced had valid NGS data for further analysis. Using
the top 1% clonal space cohort median as threshold (≥0.166115), patients with NGS data were divided in two groups (High and low pre-treatment top 1% clonal space).

The Bioconductor DESeq2 packages (version4) (23) was used to perform the differential analysis expression, screening the differential expression genes (DEGs) between high top 1% and low top 1%. To remove those low expressed genes, we applied an expression filter on row count data, selecting genes with, at least, 5 counts. A hierarchical clustering heatmap was performed using pheatmap package in R.

A selection of genes with threshold of absolute fold-change ≥ 1 and p < 0.05 were recruited to perform the gene ontology (GO) enrichment analysis in order to identify significant biological processes among those genes. To analyze the processes involved on each group (high top 1% and low top 1%), two groups were established according to their fold-change values: those values ≥ 1 (up-regulated on high top 1% group), and values ≤ 1 (up-regulated on low top 1%). The significant processes were ordered according to their p-value, selecting the 25 most significant processes for each group. This analysis was performed with R package bcbioRNASeq (24).

**PD-L1 tumor proportion score and TMB assessment**

As previously reported, the baseline TMB of formalin-fixed paraffin-embedded (FFPE) tumor diagnostic samples was assessed using an Ion S5 Sequencer (Thermo Fisher Scientific, Palo Alto, CA, USA) with the Oncomine Tumor Mutation Load Assay (Thermo Fisher Scientific) according to the manufacturer’s instructions. The commercially available PD-L1 immunohistochemistry assay PD-L1 IHC 22C3 pharmDx (Dako, Glostrup, Denmark) was used to assess PD-L1 tumor proportion score (TPS) in FFPE tumor diagnostic samples (1).

**Statistical analysis**

Graphs were drawn using GraphPad PRISM v.6. and SPSS Statistics v25 was used to analyze the data and determine the statistical significance, considered by an overall P value <0.05. Statistical significance was adjusted for multiple testing by Bonferroni correction when appropriate and was indicated in figure caption. Two-tailed, non-parametric Mann-Whitney U test was performed to compare CPR versus non-CPR groups. Wilcoxon matched-pairs signed rank test was adopted to make comparisons between pre- and post-neoadjuvant treatment in paired samples. Spearman rank non-parametric test was used for variable correlations. High and low pre-treatment top 1% clonal space patient groups were defined using the pre-treatment TCR cohort median (n=22). Contingency table analysis when comparing top 1% high and low patients with clinicopathologic features was done using Fisher’s exact test or Chi-square test. The receiver operating characteristic (ROC) curve analysis was done to determine the association of the variables studied with therapy pathological response. Regarding gene expression analysis, a multiple testing correction was performed to reduce the number of false positives, using Benjamini-Hochberg procedure and an adjusted p < 0.05 was considered statistically significant.

**Availability of data**

The datasets supporting the current study are available from the corresponding authors upon request. Raw sequencing data are not freely available due to lack of specific authorization, regarding potential relevant germline information release, present in the original consent form signed by the patients during trial enrollment. However, raw data is available upon request.
and complete TCR clone lists identified in pre-treatment or post-treatment samples can be found in Supplementary File S1 and S2, respectively.

RESULTS

PRE-TREATMENT TISSUE TCR EVENNESS IS ASSOCIATED WITH PATHOLOGIC RESPONSE TO NEOADJUVANT CHEMOIMMUNOTHERAPY

46 patients with resectable stage IIIA were treated with 3 cycles of Nivolumab plus chemotherapy prior to surgery, 41 of them underwent surgical resection. The clinicopathological characteristics of the patients analyzed are shown in Table 1. PBMCs samples collected before (pre-) and after (post-) neoadjuvant treatment and tumor samples obtained at diagnosis (pre-) and at surgery (post-) were subjected to TCR sequencing Supplementary Table 1. From 77 tissue samples collected, we obtained valid TCR data for 60 (78%); 3 samples did not have sufficient tissue for RNA extraction and 14 did not yield enough RNA quantity for further analysis, however, all samples with enough RNA quantity generated valid NGS data. On the contrary, from 65 blood samples collected, we were able to obtain valid TCR data in all cases (100%).

As a first approach, T-cell receptor (TCR) repertoire-derived metrics were analyzed. Regarding blood, there were no differences between CPR and non-CPR patients in both pre- or post-treatment PBMC samples in terms of clones sequenced, convergence, evenness and diversity (Fig. 1A). Also, there were no differences between pre- and post-treatment PBMCs samples in all patients nor stratifying by pathological response for these parameters (Fig. 1A).

However, when comparing tissue samples at diagnosis, we found that CPR patients had significantly lower TCR evenness in the T-cell receptor repertoire than non-CPR patients (p=0.010), reflecting a skewed repertoire in complete pathologic responders (Fig. 1B). No differences were found in the other TCR metrics. In contrast, there were no differences in evenness in surgery samples. Focusing on the effect of neoadjuvant treatment, an increase in evenness (p=0.028), and a decrease in number of clones (p=0.037), between pre- and post-treatment samples seems to be observed in patients who had CPR, that were not considered statistically significant after Bonferroni’s correction (Fig. 1B).

Extensive analysis to define which ranges of clones were responsible for CPR and non-CPR TCR evenness differences was done. The top 1% most common clones occupied higher clonal space, with respect to the total repertoire, in CPR patients than in non-CPR patients (p=0.00015) (Fig. 1C). Opposite results were found for clones below the top 5% most common clones (p=0.001) (Fig. 1C). However, these differences were lost in surgery samples, presenting CPR and non-CPR patients similar clonal space values for all clonal ranges. Evaluating treatment effect, although a decrease in the clonal space occupied by the top 1% seems to occur in post-treatment compared to pre-treatment samples this was not statistically significant (p=0.043) (Fig. 1C).

PRE-TREATMENT TISSUE TCR EVENNESS AND TOP 1% OUTPERFORM TMB AND PD-L1 IDENTIFYING PATIENTS WHO ACHIEVE COMPLETE PATHOLOGICAL RESPONSE.

The ability of these two potential tissue biomarkers to potentially predict the pathologic response of these patients was analyzed. The AUC ROC for TCR evenness to distinguish between CPR and non-CPR was 0.844 (95% CI, 0.667 to 1.000, p=0.011). An evenness value lower than 0.863 showed 50% sensitivity and 100% specificity identifying patients that will...
achieve CPR, and therefore, will be potentially free of disease at the time of surgery (Fig. 2A). Determination of top 1% clonal space categorize CPR and non-CPR patients even with more accuracy than evenness, showing an AUC of 0.967 (95% CI, 0.897 to 1.000, p=0.001) (Fig. 2A). Top 1% clonal space higher than 0.1842 showed 80% sensitivity and 100% specificity identifying patients that will achieve CPR after chemoimmunotherapy. Out of the 19 total patients from our cohort with valid data, we are able to classify correctly 8/10 of them with CPR using this cut-off. No association between pre-treatment tissue top 1% clonal space using the cohort median as threshold with age, sex, smoking status, histology or nodal stage at diagnosis, was found (Table 1).

We next compared the ability of TCR evenness and top 1% clones with PD-L1 TPS and TMB to potentially predict CPR patients. The AUC ROC for PD-L1 TPS and TMB to distinguish CPR and non-CPR patients were 0.767 (95% CI, 0.578 to 0.955, p=0.026) and 0.550 (95% CI, 0.308 to 0.792, p=0.687), respectively (Fig. 2A). These results indicate that, both evenness and top 1% showed better sensitivity and specificity to classify patients achieving CPR after neoadjuvant chemoimmunotherapy than the commonly used PD-L1 and TMB.

Regarding survival, although the number of patients is limited and due to the efficacy of the therapy the number of events is small, patients with high pre-treatment tissue top 1% clonal space seem to show a better prognosis both in their PFS (p=0.053) and OS (p=0.059) (Fig. 2B). Thus, in the low top 1% group 5 patients have progressed and 3 of them have died, while in the high top 1% group only 1 patient has progressed without any death. These patterns are not observed for evenness, PD-L1 or TMB using median values as thresholds (Supplementary Fig. S1A).

We also assessed the relationships between TCR evenness, top 1%, PD-L1 TPS, and TMB analyzing the correlations between them. Evenness and top 1% clonal space were strongly negatively correlated (R=−0.886, p<0.001) (Fig. 2C). However, there was no correlation between top 1% clonal space and PD-L1 or TMB (R=0.382, p=0.087; R=0.230, p=0.329) (Fig. 2D) nor between evenness and PD-L1 or TMB (R=−0.359, p=0.109; R=−0.241, p=0.306) (Supplementary Fig. S1B) strengthening the independent value of these biomarkers.

The presence of any mutations of potential clinical relevance, specifically KEAP1 (n=3), EGFR (n=1), TP53 (n=9), KRAS (n=3) and HNF1A (n=4) in this cohort, was not associated with changes in tumor top 1% clonal space levels (Supplementary Fig. S1C, Supplementary Table S1 and S3).

TISSUE TCR EVENNESS AND TOP 1% CLONAL SPACE ARE INDEPENDENT OF MAIN TECHNICAL PARAMETERS.

To assess the robustness of tissue TCR repertoire evenness and top 1% clonal space parameters, we tested whether different common technical factors, (i.e read depth, identified clones, tissue origin and library preparation) could influence these putative biomarkers. No correlation between reported read count and evenness (R=−0.079; p=0.748) or tissue top 1% clonal space (R=0.384; p=0.104) was observed (Supplementary Fig. S2A). Additionally, there were no differences in read counts between samples of CPR and non-CPR patients at diagnosis (p=0.182) or surgery (p=0.777) (Supplementary Fig. S2B). Also, no correlation was
found between the number of clones detected and evenness ($R=0.149; p=0.542$) or top 1% clonal space ($R=0.193; p=0.429$) (Supplementary Fig. S2C).

Furthermore, these biomarkers were not strongly influenced by biopsy’s origin. No differences were found between tumor or lymph nodes biopsies, for both evenness or top 1% clonal space (Supplementary Fig. S2D). Also, the differences seen in the frequency of the top 1% were maintained regardless of the origin of the sample. Patients with CPR have a higher frequency of the top 1% than non-CPR patients in both tumor tissue samples ($p = 0.030$) and in lymph node samples ($p = 0.029$) (data not shown). Finally, we also checked that these metrics and the clonal reproducibility did not significantly vary between different libraries made from the same sample or within technical sequencing replicates from the same library. TCR evenness and top 1% were stable across technical replicates (Supplementary Fig. S3A). Clonal reproducibility was almost perfect between sequencing replicates, reaching a Jaccard’s index near 1 for all clonal ranges. However, the clonal reproducibility between libraries was only maintained for top 1% and top 1-2%, decreasing for top 2-5% and being almost gone for clones below the 5% most common clones (Supplementary Fig. S3B).

**CPR patients showed a selective expansion of pre-treatment tissue top 1% clones in peripheral blood**

In view of the importance of the top 1% frequency-ranked clones present in pre-treatment tissue samples we studied the relevance of this top 1% in the different compartments, deepening in the study of their individual dynamics in peripheral blood and tissue considering whether they were expanded or contracted during neoadjuvant treatment.

As a first approach in blood, we found no statistically significant differences between CPR and non-CPR patients in the clonal space occupied by the top 1% clones of diagnostic tissue in pre- or post-treatment PBMCs samples (pre-treatment, $p=0.234$; post-treatment, $p=0.059$). Similarly, there was also no significant changes after treatment in the clonal space of this pre-treatment tissue top 1% clones in blood for both CPR and non-CPR patients (Fig. 3A).

We also evaluated the percentage of peripherally-expanded or contracted pre-treatment tissue top 1% clones. Patients achieving CPR showed statistically significant lower percentage of expanded clones and consequently, higher percentage of contracted clones compared to non-CPR patients ($p=0.005$) (Fig. 3B). However, there were no differences in the clonal space occupied by expanded or contracted pre-treatment tissue top 1% clones between pathologic response groups in pre- or post-treatment PBMC samples nor in their fold changes associated (Fig. 3C).

Since the percentage of expanded clones in CPR patients was lower than in non-CPR, but the clonal space remained similar, we decided to evaluate the individual contribution of those clones to the clonal space. To do this, we normalized the clonal space by the number of clones that were expanded or contracted, thus comparing the average clonal space per clone between CPR and non-CPR patients. The tissue top 1% clones that were expanded in the blood of CPR patients seems to have larger average size per clone than their non-CPR counterparts; both in the pre- ($p=0.022$) or post- ($p=0.051$) PBMCs’ clonal space (Fig. 3D).

Concerning their implication in tissue, the clonal space occupied by the pre-treatment top 1% clones in tissue samples was significantly reduced after treatment for all patients ($p=0.005$).
However, stratifying by pathological response only CPR patients showed a decrease (CPR patients, p=0.013; non-CPR patients, p=0.208) (Fig. 3E). Additionally, the space occupied by the pre-treatment top 1% tissue clones seems to be higher in CPR patients compared to non-CPR patients after treatment, though no statistically significant differences were seen (p=0.237).

When looking at their intratumoral dynamics, we observed that more than 80% of them were contracted in post-neoadjuvant treatment tissue. Unlike blood, no differences were observed in tissue top 1% dynamics between CPR and non-CPR patients (Fig. 3F). Same as previously shown with PBMCs samples, the clonal space occupied by expanded clones nor the fold change between pre- and post- treatment tissue, showed differences between CPR and non-CPR patients (Fig. 3G). Additionally, there was a higher clonal space occupied by contracted clones in tissue pre-treatment in CPR patients compared to non-CPR (p=0.002) (Fig. 3G). However, no differences were seen in the average clonal space occupied by each clone in pre- and post- treatment tissue, in CPR or non-CPR patients (Fig. 3H).

TUMORS WITH HIGH PRE-TREATMENT TOP 1% CLONAL SPACE SHOWED AN IMMUNE REACTIVE GENE EXPRESSION PROFILE

In order to understand what characterizes the tumors with high or low top 1% clonal space, we analyzed the relationship of this biomarker with the pre-treatment tumor immune infiltrate and the gene expression profile.

In terms of immune cell content, no association was found between top 1% clonal space and tumor-, stroma- or total- levels of any immune cell subpopulations analyzed (data not shown). Only a weak correlation was found between T lymphocytes infiltrating the tumor (CD3+ cells) and the clonal space of the top 1% (R= 0.609, p=0.047) that was not considered statistically significant after multiple comparisons correction (Fig. 4A). However, stratifying between high and low top 1% clonal space tumors, using cohort median as cutoff, no differences were found in CD3+ levels (Fig. 4A).

Despite the low number of cases analyzed, the RNA-seq results showed a differential gene expression profile for tumors with high or low top 1% clonal space, as shown in the hierarchical heatmap (Fig. 4B). Nearly 200 genes were differentially expressed in high compared to low top 1% tumors (Fig. 4C). Specifically, 139 genes were upregulated, including IFNg (Log2FC 1.14, adjusted p-value 0.022), IL2 (Log2FC 1.78, adjusted p-value < 0.001) and IL13 (Log2FC 2.16, adjusted p-value < 0.001). Conversely, 53 genes were downregulated, including VEGFC (Log2FC -1.39, adjusted p-value 0.008), MAPK1 (Log2FC -1.30, adjusted p-value 0.008) or IGF1R (Log2FC -1.31, adjusted p-value < 0.001). A complete list of differentially expressed genes can be found at Supplementary Table S4.

Finally, to identify the main biological processes of differentially expressed genes in tumors with high pre-treatment top 1% clonal space, Gene Ontology (GO) enrichment analysis was carried out Supplementary Table S5. Top 25 terms for upregulated genes include processes such as; positive regulation of immune effector process (GO:0002699), positive regulation of cell killing (GO:0031343) or regulation of receptor signaling pathway via JAK-STAT (GO:0046425). On the other hand, top 25 terms for downregulated genes include processes such as; epithelial cell proliferation (GO:0050673), cell cycle arrest (GO:0007050), positive regulation of angiogenesis (GO:0045766), negative regulation of extrinsic apoptotic signaling pathway (GO:2001237) or positive regulation of protein serine/threonine kinase activity (GO:0071902) (Fig. 4D).
SHARED AND NEWLY EMERGENT POST-TREATMENT TOP 1% CLONES ARE NOT INFORMATIVE OF PATIENTS RESPONSE.

In order to reveal possible response mechanisms, we decided to analyze also the role of the clones belonging to the top 1% post-treatment tissue according to whether they belong to the pre-treatment top 1%, pre-treatment non-top 1%, or were newly emerging clones (NEC) (i.e. not detected in pre-treatment tissue).

Post-treatment tissue top 1% clonal space analysis showed no statistically significant differences between these categories (p=0.073), however the median clonal space of non-top 1% pre-treatment clones doubles the clonal space of NEC in post-treatment tissue (p=0.09) (Fig. 5A). Anyhow, no differences between CPR and non-CPR were observed in the relative space of these clone subgroups (Fig. 5B).

To determine the specific relevance of the pre-treatment top 1% clones, their clonal space was compared with the rest of the clones (i.e. the sum of the non-top1% and NEC clones), showing the latter category larger values, occupying near 70% of the post-treatment top 1% clonal space (p=0.010) (Fig. 5C).

Regarding NEC, their clonal space was compared to that occupied by pre-treatment shared clones. The clonal space of shared clones was higher than that of NEC, occupying near 80% of the post-treatment top 1% clonal space (p=0.020) (Fig. 5D).

To analyse in blood the dynamics of the top 1% clones of post-treatment samples, we selected those patients from whom we had paired samples of the 4 compartments (tissue and blood at the pre- and post-treatment timepoints, n=12). Clonal space of tissue shared clones were not altered in blood after treatment (Supplementary Fig. S4A), being this behaviour similar between CPR and non-CPR patients (Supplementary Fig. S4B). Concerning NEC, an increase in their clonal space in blood after treatment was observed (p=0.018) (Supplementary Fig. S4C). Analysing it by response, it seems that this increase is mostly produced in non-CPR patients, showing a trend for higher NEC clonal space in post-treatment blood compared to CPR patients (p=0,020). (Supplementary Fig. S4D).

DISCUSSION

There is a need for predictive biomarkers since neither PD-L1 nor TMB are capable of accurately predict the response to chemoimmunotherapy in patients with advanced NSCLC (5–7), or neoadjuvant chemoimmunotherapy in locally advanced patients (1,2). In the current study, we performed T-cell receptor sequencing in peripheral blood and tissue samples from patients enrolled in NADIM trial (NCT03081689), at both pre- and post- neoadjuvant treatment timepoints. With this approach, we have shown for the first time a relationship between an uneven TCR repertoire in tissue samples at diagnosis and the complete pathologic response in NSCLC patients treated with chemoimmunotherapy.

We hypothesized that this uneven repertoire in CPR patients could be caused by a small number of dominating clones in the total repertoire. When looking to the most frequency-ranked clones, we saw that the top 1% clones occupied higher clonal space in complete responder patients than in non-complete responders. Altogether, we have demonstrated that these two novel biomarkers derived from the TCR repertoire analysis could predict, with higher accuracy than PD-L1 and TMB, patients that will be free of disease at time of surgery due to their association with CPR after neoadjuvant chemoimmunotherapy.
In this way, the study of the pre-treatment tissue top 1% clonal space as potential biomarker is encouraging, it reflects tumor immunogenicity and is strongly associated with tumor response being technically robust(21) and affordable considering turnaround times and costs. Thus, if its value is confirmed in larger cohorts with longer follow-ups for survival analysis, we believe that the analysis of the top 1% clonal space could be implemented in the clinic. This could allow the personalization of the follow-up and treatment of patients with low top 1% clonal space, who would presumably not achieve CPR, as well as would enable studies to determine the value of surgery in patients that will achieve CPR, that likely would present high top 1% clonal space at diagnosis. However, limitations similar to those of TMB, such as standardization between platforms and laboratories, as well as democratization of NGS access, would have to be solved for an effective application in clinical practice. The role of the most-frequent clones as biomarkers was also assessed in other studies, in which a higher frequency of these top-ranked clones in peripheral blood was associated to higher PFS rates at 9 months in patients treated with immunotherapy (12) and that a higher frequency of the top 1% clones present at resection was associated with MPR (19). However, this is the first time that baseline TCR parameters showed and association with CPR after chemoimmunotherapy.

We have shown that tumors with higher top 1% clonal space presented, despite having similar levels of PD-L1, TMB or TILs populations, a higher pro-inflammatory profile reflected by up-regulation of biological processes such as leukocyte-mediated immunity, cell killing and T cell activation among others. This stronger immunogenic profile could explain the repertoire imbalance and greater pathologic responses, since in such a permissive microenvironment the specific activation and clonal expansion after antigen recognition of some anti-tumoral T cell clones would be more easily allowed (25,26). In turn, it seems that this repertoire imbalance does not depend exclusively on the TMB of these tumors, existing modifying elements between the mutations and their ability to elicit an immune response (i.e. neoantigen presentation (27,28), or immunosuppressive microenvironment factors (29)), that the study of TCR repertoire would consider, but TMB alone would not.

Concerning the mechanism and involvement of pre-treatment tissue top 1% clones in the response, there are different options that are compatible with each other.

On the one hand, it is possible that these clones were not involved in the response. Thus, new clones or clones belonging to the non-top 1% category would participate in the response, as would support: the increase of tissue NEC in blood post-treatment, the loss of clonal space of top 1% pre-treatment clones in post-treatment tissue, and the rise of clonal space of pre-treatment non-top 1% clones and NEC in top 1% clonal space of post-treatment tissue (which combined account for more clonal space than that of the pre-treatment top 1% clones). However, NEC in the top 1% post-treatment tissue seem to occupy half of the space that pre-treatment non-top 1% clones do, indicating a greater importance of reinvigoration of pre-existing tumor clones below the top 1% than of the emergence of new clones. In any case, the fact that no differential behavior is observed between responses, reinforces that the NEC and non-top1% clones do not seem to have a specific role to achieve CPR, however it does not rule out their possible participation in responses.

On the other hand, we have shown how this top 1% remained in tissue post-treatment and was also present in the blood. Thus, the possible role of these clones in the response may be exerted through tissue or peripheral mechanisms. In terms of tissue mechanisms, despite this drop of pre-treatment tissue top 1% clones, they still account for 10-20% of the total post-treatment clonal space (showing a tendency to be more relevant in CPR patients) and occupy...
near 30% of the post-treatment top 1% clonal space. Furthermore, this role may have been underestimated given that, at the time of surgery, there was a reduction of T-cells probably related to tumor clearance (1). Thus, we cannot rule out the importance of reinvigoration of top 1% in tumor elimination (30). Regarding the role of pre-treatment tissue top 1% clones in the peripheral response, we have seen that in our patients the clones identified in tissue are found in blood and are maintained after treatment. Furthermore, CPR patients showed a selective expansion of pre-treatment tissue top 1% clones in peripheral blood compared to non-CPR patients. This could indicate peripheral immunosurveillance, responsible for eliminating possible relapses at systemic level, which could explain the high survival rate of patients with CPR (1) and the absence of deaths in the high top 1% group. In this regard, other authors have previously described the clonal changes in the peripheral TCR repertoire after immunotherapy, and identified clones that expanded or contracted peripherally after treatment (12,15,19,20).

Finally, further studies are needed to overcome the limitations of our study, including but not limited to: the number of patients, the lack of in-between samples (19), the lack of a control group and a validation cohort, and the phenotype and antigen specificity of the sequenced T cells (31).

CONCLUSIONS
In conclusion, baseline tissue T-cell receptor evenness and top 1% clonal space are associated with complete pathological response to neoadjuvant chemoinmunotherapy. Additionally, although we cannot rule out the role of new clones and the tissue reinvigoration of pre-treatment clones, we describe the relevance of the peripheral selective expansion of tissue top 1% clones to achieve complete pathological response. Future studies are warranted in larger cohorts overcoming our limitations to validate the relevance of the TCR repertoire analysis.

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5. REFERENCES
Neoadjuvant chemotherapy and nivolumab in resectable non-small-cell lung cancer


Table 1. Clinicopathologic characteristics of patients and their association with pre-treatment tissue top 1% clonal space.

<table>
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<th>Clinicopathologic characteristics</th>
<th>Complete molecular cohort (n=44)</th>
<th>Pre-treatment TCR analysis cohort (n=22)</th>
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FIGURE AND TABLE LEGENDS

**Table 1:** Clinicopathologic characteristics of patients and their association with pre-treatment tissue top 1% clonal space. The data shown correspond to number and (%) or median and (IQR). Complete molecular cohort consisted in all patients from who at least one molecular analysis was done (TMB, PD-L1, TILs, HTG or TCR determination). Pre-treatment TCR cohort was composed of all patients from who TCR metrics were determined in pre-treatment tissue sample. Patients with TCR determination in pre-treatment tissue were classified as top 1% high or low according to cohort median (0,166115). a p-value was calculated using the U-Mann Whitney test. b p value was calculated using Fisher’s exact test. c p-value was calculated using Chi-square test.

**Figure 1:** PRE-TREATMENT TISSUE TCR EVENNESS IS ASSOCIATED WITH PATHOLOGIC RESPONSE TO NEOADJUVANT CHEMOIMMUNOTHERAPY

A. Differences in metrics derived from TCR repertoire analysis (such as number of clones, evenness, convergence and Shannon’s diversity index) in peripheral blood samples between CPR and non-CPR patients at pre- and post- neoadjuvant treatment. Pre-treatment (pre-T) CPR patients, n=15; pre-T non-CPR patients, n=12; post-treatment (post-T) CPR patients, n=19; post-T non-CPR patients, n=13. B. Differences in metrics in tissue samples between CPR and non-CPR patients. Pre-T CPR patients, n=10; pre-T non-CPR patients, n=9; post-T CPR patients, n=24; post-T non-CPR patients, n=14. Comparisons were done between CPR and non-CPR patients and between pre- and post- treatment timepoints. C. Clonal space occupied by each of the percentage rank (top 1%, top 1-2%, top 2-5% and top> 5%) of the total repertoire in tissue samples. Comparisons between CPR and non-CPR patients are shown for each rank. Pre-T CPR patients, n=10; pre-T non-CPR patients, n=9; post-T CPR patients, n=24; post-T non-CPR patients, n=14. Each patient is represented by a black symbol. P<0.0125 was considered statistically significant after Bonferroni’s correction for multiple tests. Only significant differences after Bonferroni’s correction are shown.

**Figure 2:** PRE-TREATMENT TISSUE TCR EVENNESS AND TOP 1% COULD BE BETTER PREDICTORS OF PATHOLOGIC RESPONSE THAN TMB AND PD-L1

A. Receiver operating characteristic (ROC) curve analysis for TMB (square, n=23), PD-L1 (asterisk, n=25), evenness (triangle, n=19) and top 1% (dot, n=19) determined in pre-treatment tissue samples. B. PFS and OS percent survival stratified by pre-treatment top 1% clonal space high and low patients (n=22). C. Correlation between clonal space occupied by top 1% clones and evenness in pre-treatment tumor samples (n=22; CPR, n=10; non-CPR, n=9; non-resected, n=3). D. Correlation between clonal space occupied by the top 1% clones and PD-L1 in pre-T tissue samples (n=21; CPR, n=10; non-CPR, n=8; non-resected, n=3) and correlation between frequency of the top 1% clones and TMB in pre-treatment tissue samples (n=20; CPR, n=9; non-CPR, n=8; non-resected, n=3). Each patient is represented by a dark grey (CPR), light grey (non-CPR) or white (non-resected) symbol. The black line indicates the linear regression line, and the dotted lines indicate the upper and lower boundaries of the 95% confidence interval.

**Figure 3:** CPR PATIENTS SHOWED A SELECTIVE EXPANSION OF TISSUE TOP 1% CLONES IN PERIPHERAL BLOOD
A. Clonal space occupied by the top 1% tissue pre-T clones in pre- and post-treatment PBMC samples. Comparisons between CPR and non-CPR patients in pre- and post-treatment timepoints are shown (n=13; CPR, n=6; non-CPR, n=7). B. Percentage of top 1% tissue pre-T clones that were peripherally expanded or contracted (as known as dynamic clones). Comparisons between CPR and non-CPR patients are shown (n=13; CPR, n=6; non-CPR, n=7). C. Clonal space occupied by dynamic clones in peripheral repertoire: PBMCs pre-T and PBMCs post-T. Expressed as frequency of clonal space occupied by the top 1% tissue pre-T clones and fold change between pre- and post-treatment timepoints. Comparisons between CPR and non-CPR patients are shown (n=13; CPR, n=6; non-CPR, n=7). D. Median contribution of peripherally-expanded (n=13; CPR, n=6; non-CPR, n=7) or contracted clones (n=12; CPR, n=6; non-CPR, n=6) in pre- and post-treatment peripheral blood. Comparisons between CPR and non-CPR patients are shown. E. Clonal space occupied by the top 1% tissue pre-T in pre- and post-treatment tissue samples. Comparisons between CPR and non-CPR patients in pre- and post-treatment timepoints are shown (n=18; CPR, n=10; non-CPR, n=8). F. Percentage of top 1% tissue pre-T clones that were intratumorally expanded or contracted. Comparisons between CPR and non-CPR patients are shown (n=18; CPR, n=10; non-CPR, n=8). G. Clonal space occupied by top 1% dynamic clones in tissue: tissue pre-T and tissue post-T. Expressed as frequency of clonal space occupied and fold change between pre- and post-timepoints. Comparisons between CPR and non-CPR patients are shown (n=18; CPR, n=10; non-CPR, n=8). H. Median contribution of intratumorally-expanded (n=16; CPR, n=10; non-CPR, n=6) or contracted clones (n=18; CPR, n=10; non-CPR, n=8) in pre- and post-treatment peripheral blood. Comparisons between CPR and non-CPR patients are shown. Each patient is represented by a symbol. P<0.0125 was considered statistically significant after Bonferroni’s correction for multiple tests. Only significant differences after Bonferroni’s correction are shown.

Figure 4: IMMUNE CELLS AND GENE EXPRESSION ANALYSIS OF TUMORS WITH HIGH OR LOW TOP 1% CLONAL SPACE.

A. Correlation between CD3+ tumor infiltrating lymphocytes (cells per mm2) and top 1% clonal space in pre-treatment tissue. Comparisons between high top 1% and low top 1% patients are shown. P<0.001 was considered statistically significant after Bonferroni’s correction for multiple tests. B. Hierarchical clustered heatmap showing the expression patterns of all genes analyzed across tumors with high (pink) and low (cyan) top 1% clonal space. The red boxes indicate the up-regulated genes, and the blue boxes indicate down-regulated genes. C. Volcano plot showing the Log10 of adjusted p-value and Log2 fold change of all genes studied. Red (upregulated) and blue (downregulated) dots represent genes with Log2 fold change >|1| and statistically significant (adjusted p-value <0.05). D. Dot plots of top 25 enriched GO pathways for down-regulated and up-regulated genes in tumor with high top 1% clonal space vs tumors with low top 1% clonal space.

Figure 5: COMPOSITION OF TOP 1% CLONES OF SURGICAL SPECIMENS.

A. Relative top 1% post-treatment clonal space occupied by clones shared between pre- and post-treatment, belonging to the top 1% pre-T tissue or non-top 1%, and by NEC (n=18; CPR, n=10; non-CPR, n=8). Comparisons between shared top 1% pre-T clones, shared non-top 1% pre-T clones and NEC were done. B. Relative top 1% post-treatment clonal space occupied by clones shared between pre- and post-treatment, belonging to the top 1% pre-T tissue or non-top 1%, and by NEC stratified by response (n=18; CPR, n=10; non-CPR, n=8). Comparisons between CPR and non-CPR patients were done. C. Comparison of the relative top 1% post-
treatment clonal space occupied between shared top 1% clones and non-top 1% plus NEC (n=18). D. Comparison of the relative top 1% post-treatment clonal space occupied between total shared clones and NEC (n=18). Each patient is represented by a symbol. P<0.05 was considered statistically significant. Only significant differences are shown.
Figure 1

A. PBMCs samples

B. Tissue samples

C. Clonal space in Tissue

Figure 1: Clones, Evenness, Convergence, and Shannon's diversity for PBMCs and tissue samples before and after treatment. The figures show the distribution of clones and their convergence, along with Shannon's diversity indices for different samples and treatments.
**Figure 2**

A  
![Graph A](image1.png)

B  
![Graph B](image2.png)

C  
![Graph C](image3.png)

D  
![Graph D](image4.png)
Figure 4

A

CD3 (n/mm²) in tumor area pre-T

Top 1% pre-T

0.0 0.1 0.2 0.3 0.4

R = 0.609

p = 0.047

CD3 (n/mm²) in estroma area pre-T

Top 1% pre-T

0.0 0.1 0.2 0.3 0.4

R = 0.361

CD3 (n/mm²) in total area pre-T

Top 1% pre-T

0.0 0.1 0.2 0.3 0.4

R = 0.484

B

Differential expression

C

Upregulated Biological Processes on Tumors with high Top 1%

regulation of T cell activation
cell killing
regulation of immune effector processes
leukocyte-cell cell adhesion
regulation of lymphocyte mediated immunity
regulation of T cell activation
regulation of cell killing
regulation of leukocyte mediated immunity
regulation of mononuclear cell proliferation
regulation of leukocyte mediated cytotoxicity
regulation of receptor signaling pathway via MAPK
receptor signaling pathway via JAK-STAT
positive regulation of leukocyte mediated immunity
positive regulation of T cell killing
tyrosine phosphorylation of STAT proteins
positive regulation of receptor signaling pathway via JAK-STAT
positive regulation of tyrosine phosphorylation of STAT proteins

Downregulated Biological Processes on Tumors with high Top 1%

gland development
cell cycle arrest
regulation of MAP kinase activity
positive regulation of phospholipid turnover
peptidyl-prolyl cis-trans isomerase activity
prostaglandin biosynthesis
epithelial cell proliferation
protein autophosphorylation
positive regulation of MAP kinase activity
ornithine decarboxylase
ERK1 and ERK2 cascade
positive regulation of protein tyrosine kinase activity
positive regulation of anorgonogenesis
positive regulation of eukaryotic development
regulation of leukocyte migration
tumor metastasis
army morphogenesis
tumor development
mesenchyme morphogenesis
atrophic development
cell migration involved in heart development
fibroblast growth factor signaling pathway
smooth muscle tissue development
glomerulus development

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Figure 5

A

B

C

D

Relative top 1%
post-treatment clonal space

Shared
Top 1%
Pre-T clones
Shared
non-Top 1%
Pre-T clones
NEC

Relative top 1%
post-treatment clonal space

Shared
Top 1%
Pre-T clones
Shared
non-Top 1%
Pre-T clones
NEC

Relative top 1%
post-treatment clonal space

Shared
Top 1%
Pre-T clones
Shared
non-Top 1%
Pre-T clones
NEC

Relative top 1%
post-treatment clonal space

Shared
Top 1%
Pre-T clones
Shared
non-Top 1%
Pre-T clones
NEC

CPR
non-CPR
CPR
MPR
IPR
Pre-treatment tissue TCR repertoire evenness is associated with complete pathological response in patients with NSCLC receiving neoadjuvant chemoimmunotherapy

Marta Casarrubios, Alberto Cruz-Bermúdez, Ernest Nadal, et al.

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