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HLA-I diversity and tumor mutational burden by comprehensive next-generation sequencing as predictive biomarkers for the treatment of non-small cell lung cancer with PD-(L)1 inhibitors

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ABSTRACT

Objectives: Immune checkpoint inhibitors (ICIs) improved outcomes in non-small cell lung cancer (NSCLC) patients. We report the predictive utility of human leukocyte antigen class I (HLA-I) diversity and tumor mutational burden (TMB) by comprehensive next-generation sequencing.

Methods: 126 patients were included. TMB high was defined as $\geq$ 10 nonsynonymous mutations/Mb. Patients exhibit high HLA-I diversity if at least one locus was in the upper 15th percentile for DNA alignment scores.

Results: No difference in response rate (RR; 44.4% versus 30.9%; p = 0.1741) or 6-month survival rate (SR; 75.6% versus 77.8%; p = 0.7765) was noted between HLA-I high diversity and low diversity patients. HLA-I high diversity patients did significantly more often exhibit durable clinical benefit (DCB), defined as response or stable disease lasting minimally 6 months (64.4% [29/45] versus 43.2% [35/81]; p = 0.0223).

TMB high patients exhibited higher RR (49.1% versus 25.4%; p = 0.0084) and SR 6 months after start ICI (85.5% versus 70.4%; p = 0.0468) than TMB low patients. The proportion of patients with DCB, did not differ significantly between TMB high and low subgroups (60.0% [33/55] versus 42.3% [30/71]; p = 0.0755).

Patients with combined dual high TMB and HLA-I diversity had higher RR (63.2% versus 22.2%; p = 0.0033), but SR at 6 months did not differ significantly (84.2% versus 64,4%; p = 0.1536). A significantly higher rate of patients experienced DCB in dual high compared to the dual low group (73.7% [14/19] versus 35.6% [16/45]; p = 0.0052). Triple positive patients (high TMB and HLA-I diversity and PD-L1 positive) had higher RR (63.6% versus 0.0%; p = 0.0047) and SR at 6 months (100% versus 66.7%; p = 0.0378) compared to triple-negative patients.

Conclusion: HLA-I diversity was able to predict durable clinical benefit in ICI treated NSCLC patients, but failed to confirm as a predictor of response or survival. TMB confirmed as a predictive biomarker.

Abbreviations: CTLA-4, cytotoxic T-lymphocyte associated antigen 4; DCB, durable clinical benefit; HED, HLA-I evolutionary divergence; HLA-I, human leukocyte antigen class I; ICIs, immune checkpoint inhibitors; IHC, immunohistochemistry; Mb, megabase; MHC, major histocompatibility complex; MSI, microsatellite instability; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein 1; PD-L1, programmed cell death-ligand 1; PFS, progression-free survival; RR, response rate; SR, survival rate; TE, therapy exposure; TMB, tumor mutational burden; TPS, tumor proportion score; TSO500, TruSight Oncology 500; WES, whole-exome sequencing.

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1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. About 85% of patients with lung cancer are diagnosed with non-small cell lung cancer (NSCLC). More than half of NSCLC patients are diagnosed with an advanced disease stage and have a poor prognosis. Several predictive biomarkers correlated to oncogene addiction (e.g., EGFR or ALK) have been identified, mainly in non-squamous NSCLC. In presence of such a biomarker, targeted therapies have resulted in improved response chances and survival outcomes [2–4]. However, in contrast to the crucial advances made for these patients, there remains an unmet need for predictive biomarkers in non-oncogene-driven NSCLC.

In recent years, clinical importance of host immunity response across multiple cancer types has repeatedly been demonstrated. Elucidation of the role of immune checkpoints as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) [5] and programmed cell death protein 1 (PD-1) [6] and subsequent development of immune checkpoint inhibitors (ICIs) led to a paradigm shift in lung cancer treatment. Significant improvement in response rates and overall survival was seen in a "sizable minority" [7]. Only approximately 20% of patients respond to ICIs in an unselected population [8,9]. In addition, these drugs are associated with a substantial cost and potentially severe side effects [10–12]. Hence, there is a need to identify better predictive biomarkers to select patients more likely to respond to these immune checkpoint inhibitors.

Currently, three ICI biomarkers are used in clinical management of patients with solid cancers: programmed cell death ligand 1 (PD-L1) immunohistochemistry (IHC), tumor mutational burden (TMB) [13], and microsatellite instability (MSI) [14,15]. The latter is mainly of interest in colorectal, endometrial and gastric cancer and of less interest in lung cancer, but a regulatory approval of MSI as a tumor agnostic biomarker for treatment with the PD-1 inhibitor pembrolizumab was obtained [16]. Fig. 1 shows a schematic overview of biomarkers relevant to this study and their role in cancer immunity.

At present, tumor proportion score (TPS) by PD-L1 IHC is a routinely used biomarker in treatment decisions for NSCLC patients. PD-L1 is the ligand that binds to the immune checkpoint PD-1 on T cells, leading to T cell inactivation [17]. In recent years, monoclonal antibodies targeting PD-1 or PD-L1 have become a standard treatment strategy for patients with NSCLC [18–21]. In NSCLC patients with a high PD-L1 TPS (≥50%),

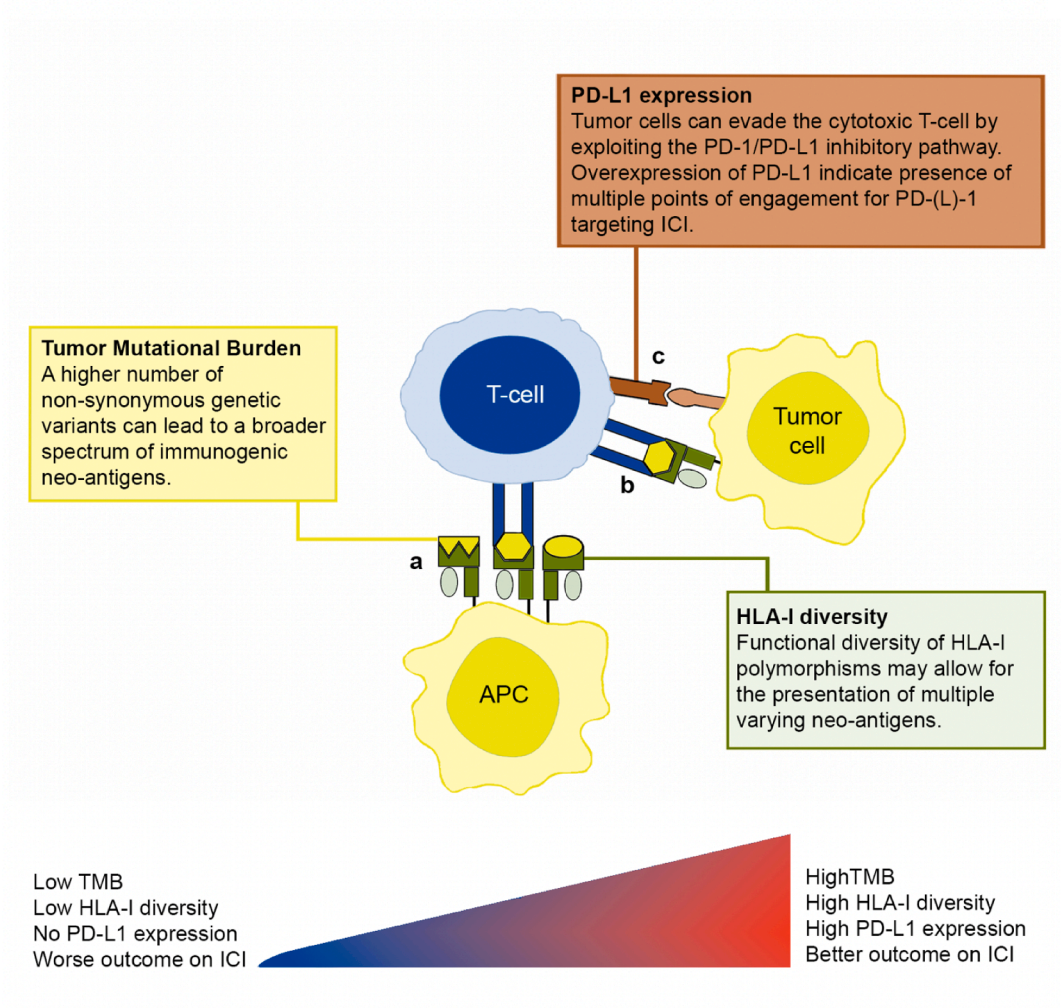


Fig. 1. TMB, HLA-I diversity, and PD-L1 expression as predictive biomarkers for PD-1 inhibitors. (a) Neo-antigens, released from tumor cells, are presented by the HLA-I complex on antigen-presenting cells (APCs) to antigen-specific T-cells. This interaction leads to subsequent activation and proliferation of (cytotoxic) T-cells. A broader range of presented antigens, as well as diversity in HLA-I molecules, can lead to a more differentiated expansion of several antigen-specific T-cells clones. **(b)** After trafficking to and infiltrating in the tumor bed, T-cells can recognize, bind, and finally kill target cancer cells through the interaction between T-cell receptor and corresponding antigen bound to the HLA-I complex. **(c)** Recognition of tumor neo-antigens presented on the HLA-I complex by T-cells consequently induces expression of PD-L1 on tumor cells, facilitating the inhibitory PD-1/PDL-1 axis. PD-(L)1 targeting antibodies reinvigorate T-cells and enhance the anti-tumor effect by blocking this inhibitory feedback mechanism.

pembrolizumab is considered standard of care in majority of patients, but even in patients with high PD-L1 expression, only 45% of patients respond to treatment. Multiple trials underscored the role of PD-L1 expression in treatment decisions, yet many challenges remain. In contrast to typical biomarker dynamics seen in oncogenic-driven tumors, absence of PD-L1 expression does not necessarily preclude patients from exhibiting treatment response. Also, differences exist between testing platforms and scoring of PD-L1 expression [22]. PD-L1 expression should be considered an enrichment factor in the treatment decision process.

TMB is a measure of the number of mutations per megabase (Mb) of DNA, a proxy for the number of cancer neo-antigens that can potentially stimulate the immune system [23]. The gold standard for determining TMB was whole-exome sequencing (WES) [24]. Targeted gene panel sequencing can reproduce TMB measurements comparable to WES, provided that the panel is of sufficient size (greater than1 Mb) and carries at least a few hundred genes [25–27]. The role of TMB as a biomarker has been demonstrated for different solid tumors, but its place in NSCLC treatment decisions is still up for debate. Rizvi *et al.* found that NSCLC patients with a high TMB demonstrated a better response to pembrolizumab, including prolonged durable clinical benefit and progression-free survival (PFS), compared to patients with a low TMB [28]. Furthermore, Samstein *et al.* confirmed the predictive value of TMB both as a continuous variable and as a binary cutoff (determined as the upper 20th percentile of each cancer type) using a targeted 468 cancer gene NGS panel [29]. Because of emerging data related to TMB, an open-label phase 3 trial evaluating nivolumab-based regimens as first-line treatment in patients with advanced NSCLC was amended to include a co-primary endpoint of PFS for nivolumab plus ipilimumab versus chemotherapy among patients with a TMB of at least 10 mutations per Mb [30]. PFS was significantly longer with the combination than with chemotherapy among NSCLC patients with high TMB. However, TMB did not show to be predictive for overall survival [31].

Recently human leukocyte antigen class I (HLA-I) heterozygosity and diversity gained interest as potential predictive biomarker [32,33]. The highly polymorphic HLA complex, encoded by the major histocompatibility complex (MHC), is a group of cell-surface proteins that play a vital role in the adaptive immune system and is mainly involved in presenting antigens on the cell surface to T-cells. Each individual HLA-I genotype comprises pairs of HLA-A, HLA-B, HLA-C alleles. Heterozygosity in these loci and functional diversity of polymorphisms may allow for the presentation of multiple varying tumor-specific neo-antigens. Expression of a broader neo-antigen repertoire may enable a better response to ICIs. Quantification of this diversity could potentially predict outcome. Indeed *Chowell et al.* showed that heterozygosity at all HLA-I loci was associated with better survival outcomes in patients treated with ICI than homozygous patients for at least one locus (HOL) [32]. Heterozygosity however does not always imply functional diversity of polymorphisms. A different genotype can still signal for the same (neo-) epitope. The same investigator evaluated functional diversity through HLA-I evolutionary divergence (HED), a measurement based upon quantification of physiochemical differences between protein amino acid sequences (Grantham distance) [35]. HED was a strong determinant of survival for ICI-treated cancer patients [33]. Besides these findings, the lack of diversity, as implied by the presence of HOL, was associated with a shorter OS and PFS in advanced NSCLC with high PD-L1 expression treated with single-agent immunotherapy [34]. Contrasting to these findings, a recent publication examining samples from 17 clinical trials of more than 3500 cancer patients treated with pembrolizumab, found that germline HLA-I heterozygosity and high HED were not associated with better ICI response [36]. Hence the role of HLA-I diversity remains controversial and merits further investigation.

Despite ubiquitous variety, class I HLA-A and HLA-B polymorphisms expressing for largely overlapping peptide repertoires can be clustered in 9 distinct supertypes [37]. The impact of these HLA-I supertypes on ICI treatment outcome remains unclear. In melanoma patients treated with ICI, HLA-B62 was associated with a worse outcome, whereas B44 was associated with a better outcome [32]. The prognostic relevance of B44 and B62 could however not be confirmed in NSCLC patients. The HLA-A02 supertype has been suggested as potentially prognostically favorable [35].

This study looks at the predictive utility of HLA-I diversity (based on DNA alignment scores) and TMB, determined by a comprehensive 523-cancer gene next-generation sequencing panel (TruSight™ Oncology 500; TSO500). TSO500 has already been correlated well with WGS for TMB determination [38]. We also looked at the predictive potential of biomarker combinations across PD-L1 subgroups and the potential impact of the presence of HOL and HLA-I supertypes A02, B44, and B62.

2. Methods

2.1. Patients

All NSCLC patients with metastatic, locally advanced, or recurrent disease from 4 regional Belgian hospitals (AZ Turnhout, Turnhout; Jessa Hospital, Hasselt; STZH, Sint-Truiden; ZOL, Genk) treated with PD-1-immunotherapy (pembrolizumab, atezolizumab, or nivolumab in first or further lines) from January 1, 2016, to December 31, 2018, were eligible if DNA from previous routine diagnostic testing was still available at the supra-regional laboratory for molecular diagnostics at the Jessa hospital, Hasselt. A total of 127 patients were identified for analysis.

2.2. Oversight

According to the requirements of the Jessa Hospital Hasselt's central ethics committee, this trial took place under approval number 19.53/klin19.01. Data were processed confidentially by the European Data Protection Directive (95/46/EC) and, upon its initiation, the Regulation (EU) 2016/679, also referred to as the General Data Protection Regulation ("GDPR").

2.3. Design and assessments

Relevant demographic data (age, gender, smoking status), disease characteristics (stage, histology, presence of brain metastasis) and treatment patterns (type of therapy, treatment line) were acquired. PD-L1 IHC results were collected only if they had been done as per local standards. No additional IHC was performed.

Clinical and radiographic tumor response assessments took place per local standards. The cutoff date for data extraction was December 31, 2019. Patients were assessed by the investigators for best response [progressive disease (PD), stable disease (SD), partial response (PR), or complete response (CR)] according to RECIST 1.1 (Response Evaluation Criteria in Solid Tumors). No independent response evaluation took place. Response rates (proportion of PR and CR) were calculated and the presence of durable clinical benefit (DCB; defined as CR, PR or SD lasting for 6 months or longer) was assessed. The duration of therapy exposure (in months) was calculated. Survival data were collected at 6 and 12 months after the start of ICI.

2.4. Preparation of study samples

DNA previously extracted for routine therapeutic testing on diagnosis or recurrence tissue samples was used. FFPE biopsies or cell blocks made from cytology samples (e.g., EBUS-TBNA) were obtained either from the primary or a metastatic site at diagnosis or progression.

2.5. Assessment of TMB and HLA-I diversity using the TSO500 assay

Tumor mutational burden was assessed using the TruSight Oncology

500 assay (Illumina Inc., San Diego, CA). This assay uses an integrated workflow to analyze variants in 523 cancer-relevant genes and to assess TMB and MSI. Following DNA extraction, library preparation was carried out using the TSO500 Library Preparation Kit (Illumina, Inc.) according to the manufacturer's protocol. Sequencing was carried out on a NextSeq 500 (Illumina, Inc.). Data analysis was carried out using dedicated Illumina TSO500 Local App V.1.3.0.39 software.

For TMB analysis, results were provided as the number of non-synonymous variants per Mb. Samples with 10 variants/Mb or more were considered TMB high.

After the HLA-I genotype was determined from TSO500 sequencing data, tumor DNA-alignment scores were calculated for all pairwise HLA-A, -B, and -C alleles. For a given pair of each HLA allele, diversity was calculated as the percentile of their score in the distribution of all pairwise scores. A patient was considered exhibiting a high HLA-I diversity if the diversity of at least one locus was in the upper 15th percentile (P15). In one of our first reports on HLA-I diversity the upper 20% (P20) was used [39]. This cut-off was used in line with the observations from melanoma studies showing a beneficial outcome on anti-CTLA-4 treatment for patients with high HLA-I diversity, defined as at least one locus in P20 [40]. The impact of HLA-I diversity on clinical outcome in P20 was however not clear in our ICI treated NSCLC cohort. All data were recalculated to a more stringent P15 (potentially correlating with a more diverse HLA-I repertoire), after which we noted a more clinically meaningful impact for HLA-I diversity on outcome.

Based upon genotyping, patients were attributed if applicable to HLA-I supertypes A2, B44, and B62 and assessed for presence or absence of homozygosity in at least one HLA-I locus.

2.6. Assessment of PD-L1 using immunohistochemistry

PD-L1 immunohistochemistry was performed per local standards (22C3 Dako antibody). A TPS of $\geq$ 1% was considered positive. A TPS of $\geq$ 50% is considered PD-L1 high. PD-L1 staining was not mandatory for inclusion and no additional stainings took place.

2.7. Statistics

The entire data analysis set included 126 patients. Statistical analyses were performed with GraphPad Prism version 7.0a. Results were expressed using descriptive statistics as proportions (response rates, durable clinical benefit, and survival at 6 and 12 months) or a mean (treatment exposure). Fisher's exact test (response rates) and Chi-square-test (durable clinical benefit and survival rates at 6 and 12 months) were used to compare proportions. Kaplan–Meier methodology was used to estimate probability of treatment discontinuation (therapy exposure) and death (overall survival). Hazard ratios and 95% confidence intervals for overall survival and therapy exposure were estimated with a Mantel-Cox regression test. Mann-Whitney test was used to compare means. The significance level of the analyses was set to 5%, and exact p values were reported. Kolmogorov-Smirnov test was applied to identify significant differences in the distribution of variables between subgroups.

3. Results

3.1. Patients

A total of 127 patients who started treatment with PD-1-immunotherapy (first or further lines) from January 1, 2016, to December 31, 2018, were identified for TSO500 analysis. One patient was excluded as TMB could not be determined. Patient, disease and treatment characteristics are described in Table 1. Median age was 66, and patients were predominantly male. Of 126 patients, 64 (50.8%) were treated with pembrolizumab, 59 (46.8 %) with nivolumab, and 3 (2.4%) with atezolizumab. Most patients received at least one previous therapy (64.3%).

Table 1
Patient and disease characteristics.

Patient demographics (N = 126)	
Median age – years (range)	66 (48–85)
Male sex – no. (%)	74 (58.7%)
Female sex – no. (%)	52 (41.3%)
Current or former smokers – no. (%)	112 (88.9%)
Never smokers – no. (%)	14 (11.1%)
Histological features – no. (%)	
Adenocarcinoma	104 (82.5%)
NSCLC not otherwise specified (NOS) and other	13 (10.3%)
Squamous cell carcinoma	9 (7.1%)
CNS disease (brain or leptomeningeal metastasis) – no. (%)	
No CNS disease	89 (70.6%)
CNS disease	37 (29.4%)
Disease stage – no. (%)	
Stage II and III	14 (11.1%)
Stage IVA	22 (17.5%)
Stage IVB	90 (71.4%)
Previous therapy – no. (%)	
No previous therapy	45 (35.7%)
1 previous line of therapy	63 (50.0%)
2 or more previous lines of therapy	18 (14.3%)
Treatment – no. (%)	
Pembrolizumab	64 (50.8%)
Nivolumab	59 (46.8%)
Atezolizumab	3 (2.4%)

As shown in Table 2, TMB was low in 71 (56.3%) and high in 55 (43.7%) patients. The highest noted TMB was 57 mutations/Mb. Median TMB was 9, and lowest was 0 variants/Mb. In 45 (35.7%) patients, the diversity of at least one HLA-I locus was in the upper 15th percentile and thus considered as high HLA-I diversity; 81 patients (64.3%) had a low HLA-I diversity. PD-L1 status was unknown in 18 patients (14.3%), 24 (19.0%) were PD-L1 negative and 84 (66.7%) were positive of which 54 patients (42.8%) had a TPS of $\geq$ 50%. Distribution of TMB and HLA-I diversity across PD-L1 subgroups is shown in Table 3. The Kolmogorov-Smirnov test revealed no significant differences in the distribution of variables between subgroups.

3.2. Treatment response

Response to ICI treatment was evaluated for response rate (RR; the proportion of patients with a partial or complete response) and for durable clinical benefit (DCB), defined as a partial or complete response or stable disease lasting for 6 months or longer. Fig. 2 depicts response rates across different biomarker groups.

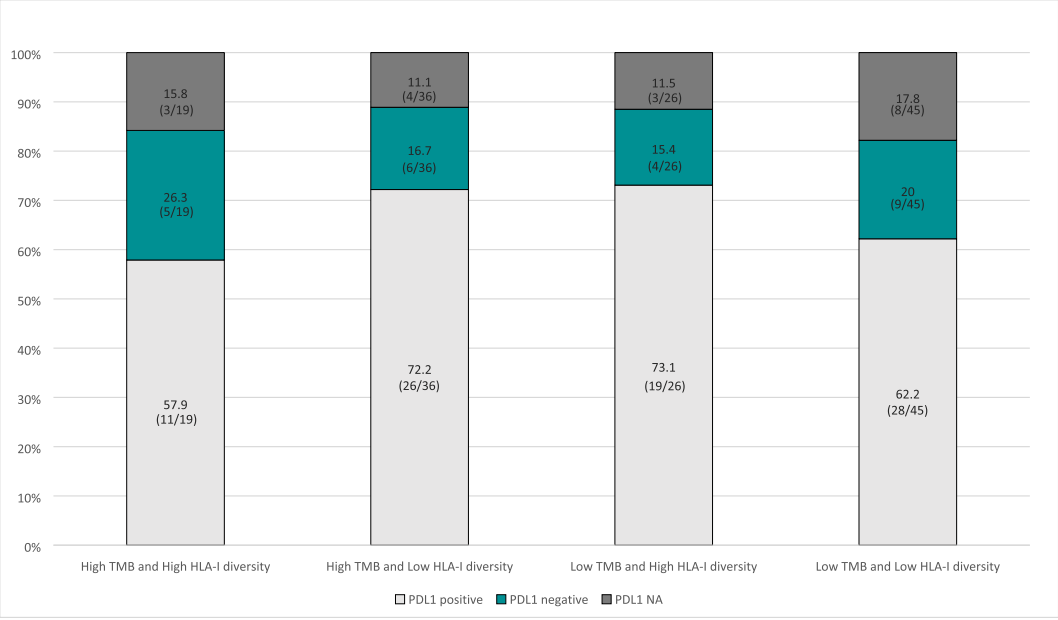
3.2.1. HLA-I diversity and TMB as a predictive biomarker

No statistically significant difference in RR was seen between HLA-I high and low diversity patients (44.4% [20/45] versus 30.9% [25/81]; p = 0.1741). RR was significantly higher in TMB high than in TMB low patients (49.1% [27/ 55] versus 25,4% [18/ 71]; p = 0.0084). Response prediction became more robust by combining TMB and HLA-I diversity

Table 2
Prevalence of TMB, HLA-I diversity, and PD-L1 TPS.

TMB – no (%)	
< 10 mutations/Mb	71 (56.3%)
$\geq$ 10 mutations/Mb	55 (43.7%)
HLA-I diversity – no. (%)	
Low HLA-I diversity	81 (64.3%)
High HLA-I diversity	45 (35.7%)
PD-L1 TPS – no. (%)	
Not available	18 (14.3%)
< 1%	24 (19.0%)
$\geq$ 1%	84 (66.7%)
$\geq$ 50%	54 (42.9%)

Table 3
Distribution of PD-L1 status [positive, negative, or not available (NA)] according to TMB and HLA-I diversity status is displayed (in %). Kolmogorov-Smirnov test revealed no significant differences in the distribution of variables between subgroups.



(dual high or dual low), with a significantly higher RR in dual high compared to dual low patients (63.2% [12/19] versus 22.2% [10/45]; p = 0.0033).

HLA-I diversity high patients did exhibit significantly more often durable clinical benefit (DCB) compared to patients with low HLA-I diversity (64.4% [29/45] versus 43.2% [35/81]; p = 0.0223). The number of patients that experienced DCB did not differ significantly between TMB high and low subgroups (60.0% [33/55] versus 42.3% [30/71]; p = 0.0755). Also, a significantly higher proportion of patients experiencing DCB was seen in the dual high compared to the dual low group (73.7% [14/19] versus 35.6% [16/45]; p = 0.0052).

3.2.2. *HLA-I diversity and TMB in different PD-L1 subgroups*

The predictive utility of HLA-I and TMB was evaluated in different PD-L1 subgroups. Striking differences were noted between triple-positive (TMB high, high HLA-I diversity and PD-L1 TPS of $\geq$ 1%) and triple-negative patients (TMB low, low HLA-I diversity and negative PD-L1). Not a single triple-negative patient exhibited response. Response rate in triple-positive patients was significantly higher compared to triple-negative patients (63.6% [7/11] versus 0.0% [0/9]; p = 0.0047). Despite numerical differences, the proportion of patients exhibiting DCB did not differ significantly between the triple-positive and negative group (81.8% [9/11] versus 44.4% [4/9]; p = 0.0813).

In the PD-L1 positive subgroup, RR for high TMB and HLA-I diversity patients was higher compared to low TMB and HLA-I diversity patients (63.6% [7/11] versus 32.1% [9/28]; p = 0.1461), but this result was not statistically significant. There was however a statistically significant higher proportion of patients experiencing DCB in PD-L1 positive patients with a high TMB and HLA-I diversity (81.8% [9/11] versus 35.7% [10/28]; p = 0.0095). Between TMB and HLA-I diversity high and low patients, in the PD-L1 intermediate group (TPS of 1%-49%), a trend towards better outcomes was noted, but we could not show statistically significant differences in RR (75.0% [3/4] versus 16.7% [1/6]; p = 0.1905) or rate of DCB (100% [4/4] versus 16.7% [1/6]; p = 0.3894). Notably, sample sizes were very small.

Finally, PD-L1 negative patients, showed a significantly higher RR (60.0% [3/5] versus 0.0% [0/9]; p = 0.0275) in the dual high compared to the dual low group. No significant difference was noted in DCB.

3.3. *Treatment exposure and survival analysis*

Kaplan-Meier analysis was performed for treatment exposure. Hazard ratios (HR) for treatment discontinuation due to death, progression or toxicity, were calculated for several biomarker combinations (Fig. 3).

Despite a notable numerical discrepancy, mean therapy exposure did not differ significantly (12 months [range: 0.5–42] versus 4 months [range 0.5–35]; p = 0.0652) between HLA-I diversity high and low patients. HR for treatment discontinuation was 0.73 [(CI 95%; 0.44–1.03) p = 0.0709]. Mean treatment exposure was 8 months (range: 0.5–42) in TMB high and 4 months (range: 0.5–33) in TMB low patients, this difference was not significant (p = 0.1119). HR for treatment discontinuation was 0.63 [(CI 95%; 0.43–0.92) p = 0.018]. There was also a clinically meaningful and statistically significant different mean therapy exposure in dual high versus dual low patients (15 months [range: 0.5–42] versus 3.5 months [range: 0.5–33[; p = 0.0136). HR for treatment discontinuation was 0.51 [(CI 95%; 0.31–0.82) p = 0.0054]. Similarly, a significant result was seen in triple positive compared to negative patients [14 months (range: 0.5–42) versus 4 months (range: 0.5–11); p = 0.0136]. HR for treatment discontinuation was 0.34 [(CI 95%; 0.12–0.97) p = 0.0027].

At time of data cut-off, 49 patients were still alive with a median follow-up of 14 months (Fig. 4). Survival rate (SR) at 6 months after starting ICI did not differ between HLA-I high and low diversity group (75.6% [34/45] versus 77.8% [63/81]; p = 0.7765) nor at 12 months (62.2% [28/45] versus 63.0% [51/81]; p 0.9343).

The SR at 6 months was significantly higher in the TMB high than in the low group (85.5% [47/55] versus 70.4% [50/71]; p = 0.0468). At 12 months the difference was not statistically significant (70.9% [39/55] versus 56.3% [40/71]; p 0.0935).

The number of patients in the dual positive compared to the dual negative group alive at 6 months [89.5% (17/19) versus 73.3% (33/45); p = 0.1536] and 12 months after starting ICI (73.7% (14/19) versus 57.8% (26/45); p = 0.2298) did not differ significantly despite numerically relevant differences. The SR at 6 months after starting ICI was significantly higher in the triple-positive than in the triple-negative group (100% [11/11] versus 66.7% [6/9]; p = 0.0378). At 12 months however the difference did not reach significance (81.2% [9/11] versus

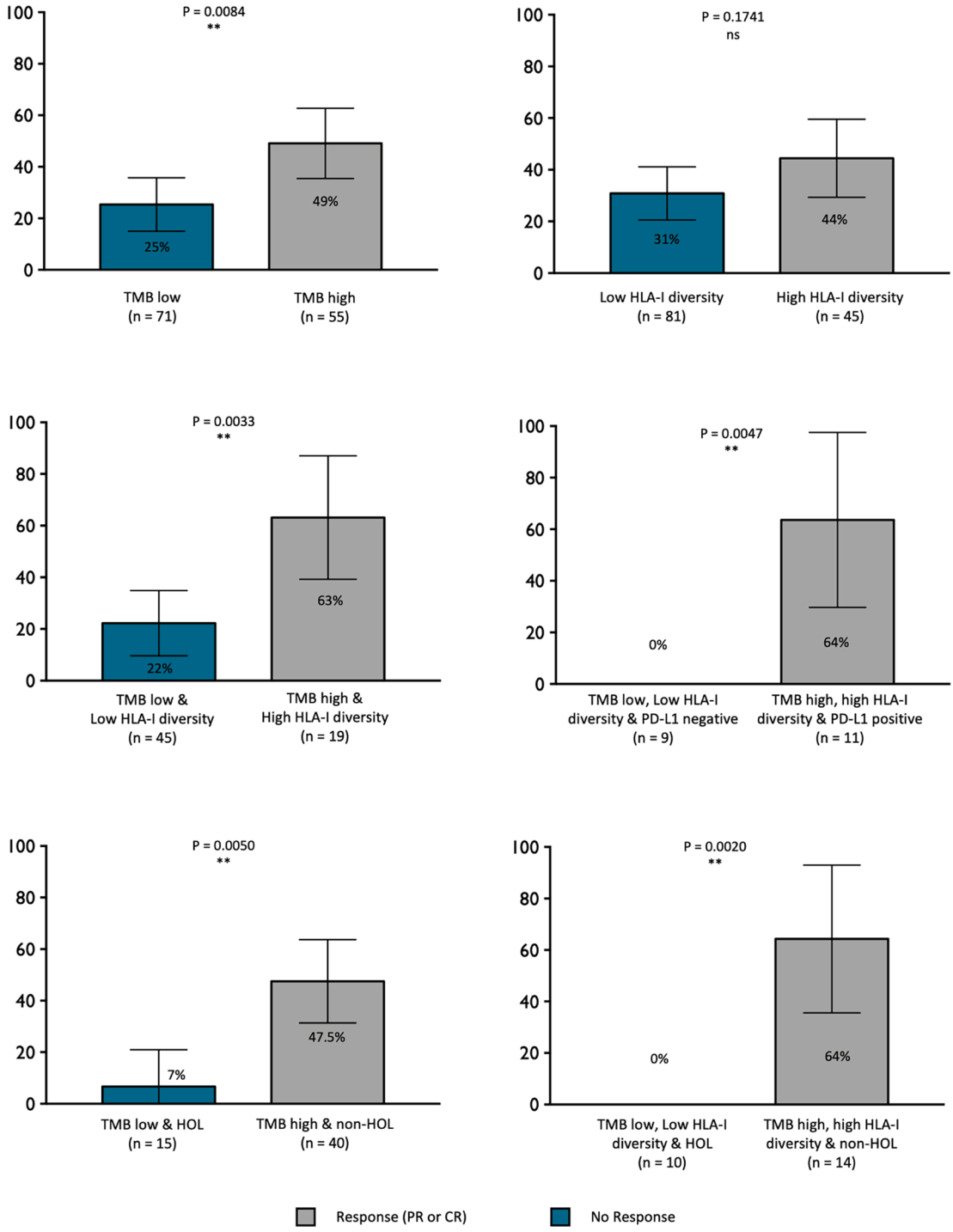


Fig. 2. Response rates (RR) of NSCLC patients treated with immune checkpoint inhibitors (ICI) in first or further lines for different single or combined biomarkers: tumor mutational burden (TMB); humane leucocyte antigen class I (HLA-I) diversity; combined TMB and HLA-I status; combined PD-L1, TMB, and HLA-I status; TMB in absence or presence of homozygosity in at least one locus (HOL). ** indicate significant p-values; ns = non significant; bars indicate confidence intervals.

44.4% [4/9]; p = 0.0813).

Kaplan-Meier survival estimates showed a trend towards improved overall survival (OS) for the TMB high group with a hazard ratio (HR) for death of 0.66 [(CI 95%; 0.42 – 1.03); p = 0.071)]. Median OS was 17 months (95% CI, 12 to 23 months) in TMB high patients and 13 months (95% CI, 10 to 16 months) in TMB low patients. The dual positive group also showed a trend towards better OS [HR 0.57; 95% CI (0.30 – 1.07); p = 0.103)] but failed to show statistical significance. Median OS was 20 months (95% CI, 7 to 29 months) in dual high patients and 13 months (95% CI, 9 to 17 months) in dual low patients. Sample sizes were too small to perform adequate Kaplan-Meier analysis across PD-L1 subgroups.

3.4. Impact of HLA-I homozygosity and HLA-I supertypes on clinical outcomes

Additionally, we looked at the impact of HLA-I supertypes and presence or absence of homozygosity in at least one HLA-I locus (HOL) on outcome in our cohort. HOL was detected in 23.8% of patients. Potentially prognostic favorable HLA-I supertype A02 was noted in

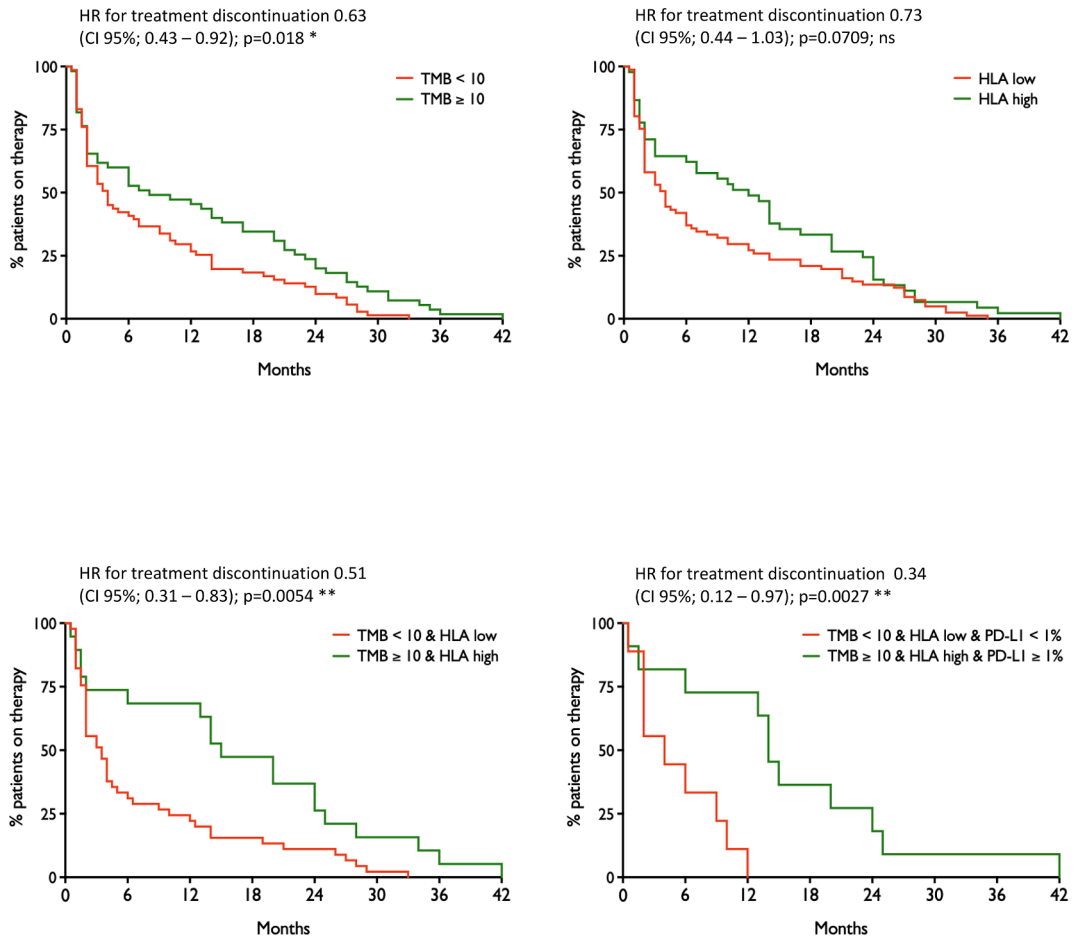


Fig. 3. Kaplan Meier estimates for treatment exposure in different biomarker combinations: tumor mutational burden (TMB), Humane Leucocyte Antigen class I (HLA-I) diversity, and programmed death-ligand 1 (PD-L1). Hazard Ratio (HR) was calculated for permanent treatment discontinuation due to death, progression or toxicity.* and ** Indicate significant p-value; ns = non significant.

48.4% and B44 in 13.5% of patients. Potentially prognostic unfavorable supertype B62 was seen in 11.9% of patients.

The presence of HOL has been associated with worse outcomes on ICI treatment in different types of cancer, including NSCLC with high PD-L1 expression treated with single-agent immunotherapy [32 35]. In our cohort, the presence of HOL could not be correlated with significantly worse outcomes. When we looked more specifically to patients with high PD-L1 expression (≥50%), we noted a trend towards worse clinical outcomes in the presence of HOL compared to patients who were heterozygous on all HLA-I loci, in terms of RR (23.1% [3/13] versus 48.8% [20/41]; p = 0.1218), and therapy exposure (8.5 months [range: 1.5–24] versus 12 months [range: 0.5–33]; p = 0.4135). The SR at 6 months after starting ICI did not differ between patients with HOL and those without HOL (84.6% [11/13] versus 90.2% [37/41]; p = 0.5737).

TMB appeared to have a stronger negative predictive utility in patients with HOL. When comparing patients that are TMB low in the presence of HOL to patients that are TMB high in the absence of HOL, the RR was significantly worse (6.7% [1/15] versus 47.5% [19/40]; p = 0.005). A trend towards worse OS was noted for TMB low patients with HOL (HR 1.79; 95% CI 0.8089 – 3.963; p = 0.0964). Also, SR 6 months after starting ICI was lower (73.3% [11/15] versus 85.0% [34/40]; p = 0.4339). However, this result was not statistically significant.

Finally, we looked at the predictive utility of HLA-I diversity in presence or absence of HOL. No significant differences were noted in outcomes between patients with low HLA-I diversity in the presence of HOL compared to patients with a high HLA-I diversity that are heterozygous at all HLA-I loci. Interestingly though, the predictive utility of TMB improves when combined. Dual low patients (low TMB and HLA-I diversity) with HOL have worse clinical outcomes than dual high patients without HOL as indicated by significantly lower RR (0% [0/10] versus 64.3% [9/14]; p = 0.0020) and mean therapy exposure (4 months [range: 1.5–14] versus 18 months [range: 0.5–36]; p = 0.0208). No survival differences were noted.

In our cohort no significant differences were seen between HLA-I supertypes. HLA-A02 supertype was not associated with a significantly higher RR compared to non-A02 patients (37.7% [23/61] versus 33.8% [22/65]; p = 0.7116) nor were there significantly more patients alive 6-months after starting ICI (80.3% [49/61] versus 73.8% [48/65]; p = 0.3877). Reduced mean therapy exposure in B62 compared to non-B62 patients might imply a worse outcome in this subgroup (3.5 months [range 0.5 to 20] versus 11 months; range [0.5 to 42]; p = 0.3189), however this difference was not statistically significant. The predictive utility of TMB confirmed across HLA-I supertypes of potential prognostic relevance. HLA-02 patients that are TMB high have a significantly higher RR compared to non-HLA02 patients that are TMB low (55.6% [15/27] versus 27.0% [10/37]; p = 0.0370). The same is seen in patients with HLA-I B44 supertype that are TMB high compared to non-B44 TMB low patients (RR 66.7% [6/9] versus 26.4% [14/53]; p = 0.0225). TMB might gain negative predictive utility in patients with potentially unfavorable HLA I- supertype B62. In HLA-I B62 patients with a TMB low status, RR was lower compared to non-B62 TMB high patients (12.5% [1/8] versus 45.8% [22/48]; p = 0.1225), but the result was statistically non-significant.

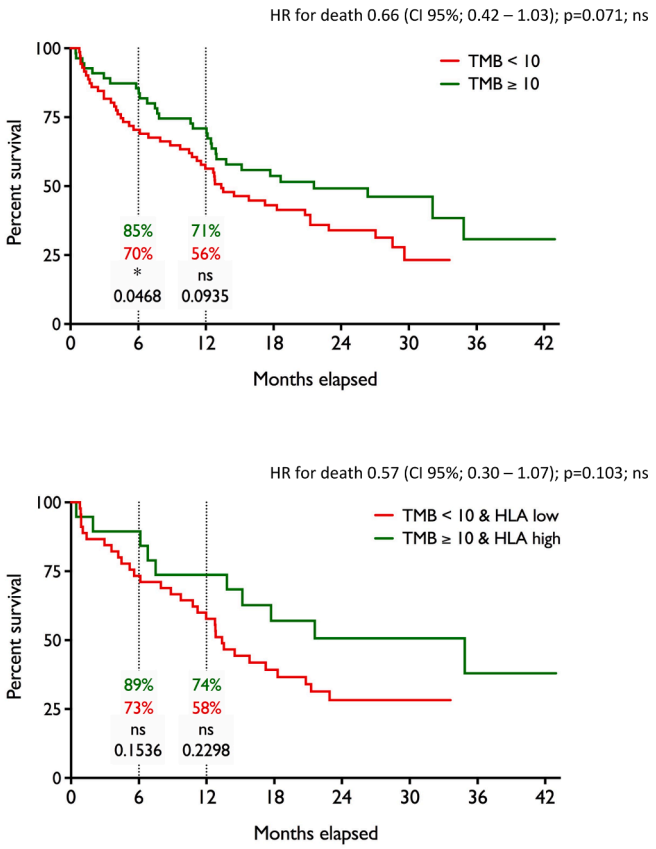


Fig. 4. Kaplan Meier survival estimates for TMB high compared to TMB low (top panel) as well as TMB and HLA-I high diversity compared to TMB and HLA-I low diversity (bottom panel) NSCLC patients treated with ICI. 6 and 12 month survival rates are depicted.* Indicate significant p-value; ns = non-significant; HR = hazard ratio.

4. Discussion

The introduction of ICIs led to a paradigm shift in cancer treatment for several tumor types, particularly NSCLC. The response rate (RR) is low in unselected patients, leading to an ongoing quest for better biomarkers. The predictive utility of TMB has been demonstrated in ICI-treated NSCLC. Recently, the role of HLA-I diversity has gained interest but controversy due to conflicting data exists. The need for complex and expensive procedures (such as WES), hindered the introduction of TMB and HLA-I diversity in the standard diagnostic repertoire. Comprehensive NGS panels have however shown consistent congruency with WES and can be readily incorporated in daily practice. We explored the use of HLA-I diversity and TMB as biomarkers, using a 523-cancer gene NGS panel (TSO500) in NSCLC patients treated with ICIs. To our knowledge, this is the first study to report the predictive utility of HLA-I diversity based upon DNA-sequence alignment scores as determined by a comprehensive NGS assay. TMB confirmed as predictive biomarker for treatment response and survival outcome. HLA-I diversity was able to predict durable clinical benefit in ICI treated NSCLC patients but was not able to predict response or survival. When combined, HLA-I diversity and TMB showed enhanced ability for response prediction. In TMB high patients, RR of 49% was noted, increasing to 63% when combined with HLA-I high diversity. Predictive utility of both biomarkers also appears to be independent of PD-L1 status as relevant differences can be seen in PD-L1 positive (≥1%), intermediate (1%-49%), and negative patients (<1%), with a RR of respectively 63.6%, 75% and 60% for combined high TMB and HLA-I diversity patients.

One of the most remarkable findings was the disadvantageous outcome of ICI treatment in triple-negative patients (TMB low, low HLA-I diversity, and negative PD-L1 expression), with not a single documented response and significant differences in treatment exposure and survival. While this finding needs further prospective validation, the combination of PD-L1, TMB, and HLA-I diversity holds great potential as a negative predictive biomarker to identify patients that will probably lack clinical benefit from ICI monotherapy. Whether these findings can be extrapolated to NSCLC patients treated with combination chemo-and immunotherapy is a clinically relevant question. Combination chemo- and immunotherapy has shown survival benefit in PD-L1 negative, low, and high patients compared to platinum doublet chemotherapy in squamous and non-squamous NSCLC [41,42]. It is interesting to investigate whether the above findings can be extrapolated to patients treated with combination chemo-immunotherapy. Perhaps TMB and HLA-I profiling can be used to discriminate better patients that might or might not benefit from adding a PD-(L)1 inhibitor to first-line platinum doublet chemotherapy. Treatment with ICI combinations (e.g., PD-(L)1 and CTLA-4 inhibitors) in the first line is extensively investigated. TMB already has predictive utility in this setting [43]. Biomarker-driven studies combining, among others, TMB and HLA-I diversity merit further investigation to identify which patients could benefit from ICI combinations.

In this trial, we also looked at the impact of homozygosity in at least one locus (HOL) and different HLA-I supertypes. The clinical importance of HOL and HLA-supertypes in NSCLC remains unclear. *Chowell et al.* observed reduced survival in ICI treated cohort of melanoma and NSCLC patients with HOL. In the same study, melanoma patients with HLA-B62 had a worse outcome, whereas HLA-B44 was associated with longer survival [32]. They did not evaluate the role of HLA supertypes in the NSCLC cohort specifically. *Abed et al.* confirmed the deleterious outcome associated with HLA-I homozygosity in PD-L1 high (TPS ≥ 50%) NSCLC patients treated with single-agent ICI [35]. The same researchers demonstrated a statistically significant association between HLA-A02 supertype and improved survival, but they could not confirm the negative prognostic impact of HLA-B44 and HLA-B62. In our study, no apparent differences in clinical outcome between HLA-I supertypes were noted. The role of HLA-supertypes remains unclear and needs further investigation. We were also not able to confirm HLA-I homozygosity to be associated with a worse outcome. However, our results suggest that in patients with HLA-I homozygosity in at least one locus, TMB seems to have a more robust negative predictive utility, demonstrated by low response rates (6.9%) and a clear trend towards worse survival.

Previous studies reporting on the association of HLA-I diversity and outcome of ICI treatment used blood-based germline HLA-genotyping, while we used DNA extracted from tumor tissue. Acquired genetic deficiencies leading to downregulation of HLA-I, impaired or reduced antigen presentation on tumor cells, or loss of heterozygosity can lead to resistance to ICI treatment [44–46]. These (epi-)genetic changes might explain differences between our results and those from other trials looking at HLA-I diversity. Unfortunately, blood-based germline HLA-genotyping was not performed in our cohort. The use of tissue DNA has some clinical challenges. Sufficient tissue is crucial to perform the required analysis, and limited tissue availability and tumor heterogeneity are common in lung cancer patients. Not only are we often faced with small biopsies or cytology specimens, residual tissue after routine diagnostic immunohistochemistry, PD-L1 analysis, and therapeutic molecular testing may also be scarce. Comprehensive genomic analysis by extended NGS panels can optimize the therapeutic testing strategy, enabling detection of targetable somatic genomic alterations and determine predictive immune-biomarkers such as TMB and HLA-I diversity using a single test.

Another possible confounder is the timing of tissue collection. Most samples were obtained at diagnosis and not necessarily at progression. Although the discussion on the impact of previous chemotherapy or radiotherapy on TMB is ongoing, there is known variation in PD-L1 expression [47 –48].

Finally, we should also consider that TMB and HLA-I diversity are, in

fact, continuous variables. Our study used a dichotomous model. A true false approach has advantages in clinical decision-making over complex linear regression models, but this strategy introduces possible bias. First, interpreting continuous results in a dichotomous fashion can increase the likelihood of false positive results [49]. Second, the extent of variation in outcome between subgroups can be underestimated [50]. For example, are patients with a TMB value close to or below cutoff (e.g., 9 and 10) likely to exhibit that different biology in the tumor microenvironment (TME) solely based upon TMB as a proxy for neo-antigen load? Many other confounders, such as cancer-specific genomic alterations, changing immune interactions in the TME and host factors, influence the outcome of ICI treatment. Integration of multiple biomarkers in a prediction model should be the focus of future research.

5. Conclusion

This study reports on the predictive utility of HLA-I and TMB diversity determined by a comprehensive NGS gene panel in NSCLC treated with immune checkpoint inhibitors. To our knowledge, this is the first study to report the predictive utility of HLA-I diversity based upon DNA-sequence alignment scores as determined by NGS in lung cancer. NSCLC patients with high HLA-I diversity or high TMB, alone or in combination, show more favorable clinical outcomes to PD-1 inhibiting immunotherapy. HLA-I diversity was able to predict durable clinical benefit in ICI treated NSCLC patients but could not predict response or survival. The predictive utility of TMB for treatment response and survival outcome was confirmed. Combined TMB, HLA-I diversity, and PD-L1 can identify NSCLC patients likely to respond and more strikingly identify patients who lack benefit from ICI treatment. Potentially prognostic unfavorable HLA-I supertypes and presence of HOL appear to improve negative predictive utility of TMB.

We conclude that HLA diversity and TMB should be considered part of comprehensive genomic profiling in NSCLC patients, but the role of HLA-I as predictive biomarker for ICI treatment remains controversial.

CRediT authorship contribution statement

Kristof Cuppens: Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Paul Baas:** Conceptualization, Methodology, Writing – review & editing. **Ellen Geerdens:** Investigation, Resources, Writing – review & editing. **Bert Cruys:** Methodology, Formal analysis, Data curation, Writing – review & editing. **Guy Froyen:** Conceptualization, Investigation, Resources, Writing – review & editing. **Lynn Decoster:** Investigation, Writing – review & editing. **Michiel Thomeer:** Investigation, Writing – review & editing. **Brigitte Maes:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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