

Clinical and technical insights of tumour mutational burden in non-small cell lung cancer

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ABSTRACT

Despite the durable responses provided by the introduction of checkpoint inhibitors in advanced Non-Small Cell Lung Cancer (NSCLC) without actionable targets in a subset of patients, a large proportion of them will progress after immunotherapy. Programmed Death Ligand 1 (PD-L1) was the first biomarker approved for immunotherapy, although it has multiple limitations, thus the development of novel biomarkers is an urgent need. Tumour Mutational Burden (TMB) is an emerging biomarker defined as the total number of mutations per coding area of tumour genome. Targeted gene panels have emerged as a cost-effective approach to estimate TMB. However, there is still an unmet need to fully standardize sample requirements, panel size, and bioinformatic pipelines to ensure that TMB is calculated appropriately. In addition, researchers are also evaluating TMB calculation in liquid biopsy. In this work, we summarize the relevant advances and the clinical utility of TMB in NSCLC.

1. Introduction

Lung cancer is currently the malignant tumour with the highest mortality rate in both sexes worldwide, with 1.8 newly diagnosed cancer cases and 1.6 million cancer-related deaths yearly (Bray et al., 2018). Despite the significant advances made in diagnostic and therapeutic approaches in recent years, mainly the emergence of molecular subsets defined by specific oncogenic aberrations (Lindeman et al., 2018; O'Leary et al., 2019; Planchard et al., 2018; Solomon et al., 2019), the overall 5-year survival remains only 16 %. Despite this, patients are frequently diagnosed in advanced stages and present poor prognosis, probably attributable to inadequate screening programs and the late onset of clinical symptoms (Gridelli et al., 2015).

Recent studies about the cancer genome and tumour microenvironment have led to a new scenario in which morphological and molecular characterization is needed to treat patients in the most personalized way to achieve better outcomes (Cancer Genome Atlas Research Network, 2014; Duréndez-Sáez et al., 2017). The introduction of immune checkpoint inhibitors (ICIs) in clinical practice has significantly improved outcomes of patients with advanced Non-Small Cell Lung Cancer (NSCLC) without actionable targets. In first and second-line treatment, these agents have shown higher activity and more durable responses than chemotherapy (Brahmer et al., 2015; Galvano et al., 2021; Hellmann et al., 2019; Mazieres et al., 2021; Reck et al., 2016; Socinski et al., 2018). Nevertheless, immunotherapy is only effective in a subset of patients; thus, a large proportion of them will progress despite these

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treatments (Gadgeel et al., 2020; Reck et al., 2016; Socinski et al., 2018). Therefore, the search of biomarkers represents a priority, not only to improve efficacy but also to minimize toxicity and make more efficient management of the available resources.

The first approved biomarker for selecting patients to receive treatment with immunotherapy was the expression of Programmed Death Ligand 1 (PD-L1) (Reck et al., 2016). However, PD-L1 has proven not to be perfect since multiple disadvantages have been described, among which are: the use of different antibodies (Dako 22C3, Dako 28-8, Ventana SP142), different platforms, and different assessment scores [for example, Tumour Proportion Score (TPS) and Combined Proportion Score (CPS) with Dako 22C3] for its determination, in addition to the heterogeneity of their expression (Reck et al., 2016). Furthermore, PD-L1 expression has also demonstrated certain limitations in predicting patient responses (Davis & Patel, 2019). For these reasons, the expression of PD-L1 is not robust enough to be the only biomarker for immunotherapy.

Other biomarkers have been studied, such as expression of the major histocompatibility complex (MHC) (Chowell et al., 2019), Interferon (IFN) sensitivity (Saigi, Alburquerque-Bejar, & Sanchez-Cespedes, 2019), CD8 + T-cell tumour-infiltrating lymphocytes (Yu et al., 2019), Interleukin-6 (IL-6) expression (Ni et al., 2018; Tang et al., 2018), Tumour Mutational Burden (TMB) (Penault-Llorca & Radosevic-Robin, 2018), gut microbiota (Bertrand et al., 2018; Zhang et al., 2021) and T Cell Receptor (TCR) repertoire (Anagnostou et al., 2019; Dong et al., 2021; Han et al., 2020). In this review, we focus the content on TMB from a clinical point of view, addressing the clinical trials that have analysed TMB to date, together with other aspects such as the available techniques for TMB assessment. These factors may influence its estimation and its relationship with other biomarkers.

1.1. Definition of tumour mutational burden (TMB)

TMB is defined as the number of somatic mutations found per the tumour genome coding region, generally expressed as mutations per megabase (mut/Mb) (Gubin et al., 2015; Schumacher et al., 2015). The presence of non-synonymous somatic mutations can lead to modifications in the proteins that, in certain cases, can be recognized by the immune system as non-self and become a neoantigen capable of activating the anti-tumour immune response (van Buuren et al., 2014). Genetic alterations that can give rise to neoantigens are: i) single nucleotide variants (SNV), ii) short insertions or deletions of nucleotides (INDELs), and iii) chromosomal translocations. Most studies only include missense mutations to assess TMB (Stenzinger et al., 2019). A neoantigen is a tumour-specific mutated peptide expressed only by tumour cells presented by MHC molecules and recognized by a T cell, which is capable of stimulating an anti-tumour immune response (Schumacher et al., 2018). Thereby, TMB is a predictive biomarker for ICI treatment, as it is an indirect biomarker of the number of neoantigens present in the tumour since a high TMB increases the probability of having a higher number of immunogenic neoantigens (Rizvi et al., 2015, 2018).

2. Gene panels to perform TMB

The first studies of TMB were carried out with whole genome or whole exome sequencing techniques, had a high cost and a period of prolonged analytical and post-analytical diagnosis, which could not be applied to clinical practice (Stenzinger et al., 2019). Whole Exome Sequencing (WES), comprises about 40–50 Mb of the genome, and offers an accurate calculation of tumour mutational burden based on coding mutations. Nevertheless, WES implementation into the clinical routine is hazardous due to the DNA input and the bioinformatics analyses required. For these reasons, targeted panels have been developed, usually covering at least 1 Mb and including approximately 300–500 genes, which can identify somatic mutations in cancer-related genes in a single

analysis at a reasonable time and cost (Stenzinger et al., 2019; Wu et al., 2019). The *Foundation One® CDx assay* (Foundation Medicine), *Memorial Sloan Kettering-Integrated capture-based NGS assay* (MSK-IMPACT), and *Oncomine Tumour Mutational Load Assay* (ThermoFisher Scientific, 2022) (Table 1) have shown to reliably estimate TMB in comparison to WES (Campeato et al., 2015; Frampton et al., 2013; Zehir et al., 2017).

Rizvi et al. (2018) used three versions of the MSK-IMPACT platform (covering 0.89, 1.02 and 1.14 Mb in panels of 341, 410 and 468 genes, respectively) to analyse 240 patients with NSCLC treated with immunotherapy, and assessed the correlation of the panel with WES in 49 patients, confirming good correlation in a genome area of at least 1 Mb. The *FoundationOne® CDx Assay* panel was assessed in 102 NSCLC patients in the POPLAR, BIRCH, and FIR trials with atezolizumab; the panel covered 315 cancer-related genes, corresponding to 1.1 Mb of the genome (Kowanetz et al., 2017). In the CheckMate 568 study, the combination of nivolumab and ipilimumab was analysed in NSCLC and the cut-off point was established with TMB ≥ 10 mutations/Mb, using the *FoundationOne® CDx Assay* panel, which covered 324 cancer-related genes (Ready et al., 2019).

Among the advantages of using these platforms, it should be remarked the cost-effectiveness, and the fact that by analysing more restricted regions of the genome, they allow sequencing with greater reading depth, which increases confidence.

3. Factors influencing the estimation of TMB with gene panels

TMB estimation is limited by conditions of the material from which the DNA is extracted, characteristics of the gene panel (size, composition, detection limit) and bioinformatic parameters (allelic frequency threshold, filtering for clonal vs. subclonal mutations, for germline mutations and deamination artefacts) (Anagnostou et al., 2022; Stenzinger et al., 2019) (Fig. 1).

On the one hand, TMB assessment is influenced by DNA quantity and quality, which depends mainly on the type of sample used. Formalin-Fixed Paraffin-Embedded tissue (FFPE) is the most frequently used, although there are also studies using blood to determine TMB. In many cases, the limitation to determine the TMB is given by the amount of tissue obtained by the type of biopsy (transbronchial, endobronchial or imaged-guided), tumour cellularity, and sample exhaustion after other diagnostic determinations (immunohistochemistry, and molecular assays), since in most laboratories these analyses are performed sequentially (Gutierrez et al., 2017; Sholl et al., 2015; Stenzinger et al., 2020). Formaldehyde fixation also introduces changes such as deaminations that may be interpreted as mutations, thus they must be filtered out (Allgauer et al., 2018). Therefore, it is necessary to predefine the diagnostic determinations, in order to optimize the available samples. To do so, the establishment of Molecular Tumour Boards is a feasible option to facilitate communication between clinicians, pathologists, and molecular laboratory staff (Kato et al., 2020; Rolfo et al., 2018).

On the other hand, bioinformatics analysis is of great importance. Indeed, screening for germline mutations is needed, so they are not counted as tumour-specific mutations. Known polymorphisms with low allele frequencies (MAF) and clonal haematopoiesis should also be filtered out (Ptashkin et al., 2018; Sun et al., 2018). Subclonal mutations often occur as a consequence of the treatments received and should also be filtered. To differentiate clonal from subclonal mutations, the detection limit has usually been set as 5 % of the allelic frequency (McGranahan & Swanton, 2017).

While WES only includes missense mutations, some gene panels also include other variant types, as well as synonymous mutations and INDELs (Budczies et al., 2019; Stenzinger et al., 2019). Although synonymous mutations do not produce neoantigens themselves, it is thought that including all mutations in the panels increases the precision of TMB estimation. Furthermore, it has been demonstrated that INDELs have an impact on neoantigen formation (Chalmers et al., 2017).

Another aspect that is a limitation of the gene panels is the size. A

Table 1
Comparison of different gene panels used to determine TMB in tumour tissue.

Gene Panel	FoundationOne® CDx (Marabelle et al., 2020)	MSK -IMPACT (Cheng et al., 2015; Schoenfeld et al., 2020; Zehir et al., 2017)	TruSight Oncology 500 (TSO500) (Pestinger et al., 2020;Zhao et al., 2020)	Oncomine Tumour Mutational Load (Chaudhary et al., 2018)
NGS platform	Illumina	Illumina	Illumina	IonTorrent (ThermoFisher)
Number of genes (Mb coverage)	324 (0.8 Mb)	468 (1.139 Mb) 410 (1.017 Mb) 341 (0.897 Mb)	523 (1.94 Mb)	409 (1.65 Mb)
Type of mutations	SNVs, INDELs, CNVs, rearrangements	SNVs, short INDELs (<30 bp), CNV and structural rearrangements	SNVs, INDELs, CNVs, gene fusions	SNVs, INDELs
Sample requirements	10 FFPE scrolls (>20 % tumour cells)	5–20 FFPE scrolls (>20 % tumour cells)	2 × 5-µm FFPE scrolls (40 ng DNA/ 40 ng RNA) (≥10 % tumour cells)	20 ng DNA

CNV, Copy Number Variant; FFPE, Formalin-fixed paraffin-embedded tissue; INDEL, Insertion/deletion; Mb, Megabase; NGS, Next Generation Sequencing; SNV, single Nucleotide Variant.

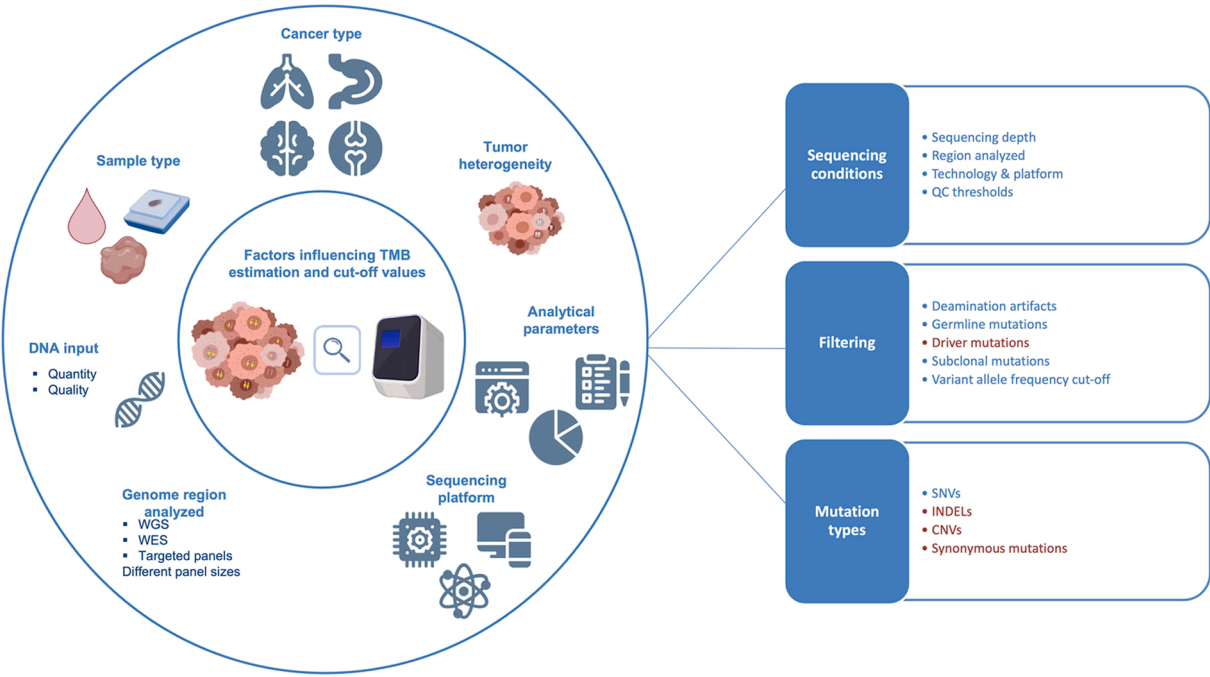


Fig. 1. Key aspects of Tumour Mutational Burden (TMB) estimation. This scheme summarizes the main issues affecting TMB estimation, such as cancer and sample type, DNA input, tumour heterogeneity, genome region analyzed, sequencing platform, and analytical parameters. All of these aspects hinder the establishment of a cut-off value and enhance the standardization initiatives to facilitate its implementation in clinical practice as a biomarker for immunotherapy. Maroon letters indicate parameters that are only taken into account in certain pipelines. CNV, Copy Number Variant; DNA, Deoxyribonucleic acid; FFPE, Formalin-fixed paraffin-embedded tissue; INDEL, Insertion or Deletion; QC, Quality Control; SNV, Single Nucleotide Variant; TMB, Tumour Mutational Burden; WES, Whole Exome Sequencing; WGS, Whole Genome Sequencing.

size of 1.5–3 Mb is adequate for a good approximation to the TMB, while sizes of less than 1 Mb are not adequate (Allgäuer et al., 2018; Buchhalter et al., 2019).

Composition is another important factor, as panels must contain a combination of driver gene mutations in addition to those genes used for TMB analysis, in order to provide the maximum amount of clinically relevant information in one assay. For NSCLC, the panel should include targetable alterations such as mutations in the following genes: Epidermal growth factor Receptor (*EGFR*), *B-Raf* proto-oncogene (*BRAF*), Human Epidermal receptor growth factor 2 (*HER2*), Kirsten rat sarcoma virus (*KRAS*), and *MET* Proto-Oncogene (*MET*). It should also include positive and negative predictive factors of response to immunotherapy, with a particular interest in defects in DNA repair genes, microsatellite instability, and defects in homologous recombination caused by Breast cancer type 2 susceptibility protein (*BRCA2*) mutations. Phosphatase and tensin homolog (*PTEN*) loss, Mouse double minute 2 homolog (*MDM2*), amplifications, DNA Polymerase Delta 1 Catalytic Subunit (*POLD1*), DNA polymerase epsilon catalytic subunit

(*POLE*), Janus Kinase 1 (*JAK1/2*), and Serine/threonine kinase 11 (*STK11*) mutations also influence the response to anti-PD1 agents as we will see in the next section, and should also be included in the gene panels. Nevertheless, it must also be noted that the inclusion of driver genes may entail a TMB overestimation, thus perhaps these genes could be included in the panel for tumour characterization, but excluded in the analysis for TMB calculation (Buchhalter et al., 2019).

Friends of Cancer Research and Quality Assurance Initiative Pathology (QuIP) have participated in a project to harmonize TMB assessment, establishing several recommendations and guidelines for TMB standardization (Merino et al., 2020; Vega et al., 2021). Regarding the bioinformatic pipelines and statistical methods used for TMB calculation, they highlight that: 1) TMB should be reported in mutations per megabase, independently of its calculation from WES or targeted gene panels; 2) analytical accuracy, precision, and sensitivity should be assessed by standardized analytical validation studies, and 3) panel TMB values should be aligned to an external reference standard derived from WES. Furthermore, they proposed a Uniform TMB Calculation Method

for analysis of WES TMB, which consists of 300X median coverage (as lower coverage is associated with reduced sensitivity for identification of low-frequency variants), a read depth equal to or higher than 25, and variant count equal or higher than 3. In addition, this project proposed the exclusion of synonymous variants and a variant allele frequency cut-off of 5 % (Stenzinger et al., 2019, 2020).

In addition to the difficulty of TMB assessment, there is another point of discussion. TMB is a continuous biomarker, with highly heterogeneous values among different tumour types, which difficult the standardization of a cut-off value, since each platform has used a different cut-off value.

4. Clinical trials that have assessed the role of TMB

The role of TMB as a predictive biomarker of response to immunotherapy (Goodman et al., 2017; Topalian et al., 2016; Yarchoan et al., 2017), has been described in various phase III trials (Table 2).

Rizvi et al. analysed the role of TMB using WES in patients with pembrolizumab first-line (1L) monotherapy. Patients with durable clinical benefit (DCB, defined as partial response or stable disease for at least 6 months) had a median of 302 non-synonymous mutations, while patients without DCB had a median of 148 mutations ($p = 0.006$). Similarly, an elevated TMB was related to increased progression-free survival (PFS) (median PFS 3.4 months vs. Not Reached [NR]; Hazard Ratio [HR] 0.15, 95 % CI [0.04–0.59]) (Rizvi et al., 2018).

Regarding nivolumab, the CheckMate 026 trial evaluated the role of nivolumab vs. chemotherapy in treatment-naïve NSCLC patients. In this study, an exploratory analysis of TMB was performed, which was classified into 3 levels: low (between 0 and 100 mutations), medium (100–242 mutations) and high (above 243 mutations). Patients with

high TMB presented increased RR (47 % vs. 28 %) and PFS (median PFS 9.7 months vs. 5.8 months; HR 0.62, 95 CI [0.38, 1.0]) with nivolumab than with platinum-based chemotherapy (Carbone et al., 2017).

Kowanetz et al. examined the association between atezolizumab efficacy and TMB assessed by the F1 panel. They evaluated TMB in pre-treatment tumour specimens from 102 1L and 465 second-line (2 L) patients enrolled in three atezolizumab trials: POPLAR (Mazieres et al., 2021), BRICH (Peters et al., 2017), and FIR trials (Spigel et al., 2018). They found that median TMB was similar in 1 L and 2 L patients (9/Mb and 9.9/Mb respectively). In 1 L and 2 L PD-L1-selected patients, atezolizumab efficacy was increased in those with higher TMB. In 1 L PD-L1 selected patients, PFS HR 0.58 (95 % CI 0.36–0.94) in the subgroup with TMB ≥ 9.9 mutations/Mb and PFS HR 0.54 (CI 95 % 0.3–0.97) in the subgroup with TMB ≥ 13.5 mutations/Mb). In 2 L PD-L1 selected patients, PFS HR 0.64 (CI 95 % 0.5–0.8) with TMB ≥ 9.9 mutations/Mb) and PFS HR 0.5 (CI 95 % 0.38–0.67) with TMB ≥ 17.1 mutations/Mb. In 2 L unselected patients (from POPLAR trial) the atezolizumab benefit vs. docetaxel were also enhanced in patients with increased TMB (PFS HR 0.49 (0.25–0.93) in patients with TMB ≥ 9.9 mutations/Mb and PFS HR 0.49 (CI 95 % 0.19–1.3) with TMB ≥ 15.8 mutations/Mb)(Kowanetz et al., 2017).

The role of TMB in immunotherapy combinations has also been evaluated. The combination of nivolumab and ipilimumab has shown to increase the rate and duration of response in patients with NSCLC. In the CheckMate 227 trial, one of the co-primary endpoints was PFS in patients with a pre-defined TMB of ≥ 10 mutations/Mb, which was higher in patients treated with the combination, regardless of PD-L1 status compared with patients treated with platinum-based chemotherapy (PFS 7.2 months vs. 5.5 months; HR 0.58 CI 95 % [0.41, 0.1]; $p < 0.001$). In addition, TMB was also analysed in PD-L1 < 1 % patients,

Table 2

Clinical trials which evaluated TMB as immunotherapy biomarker.

Publication	Number of patients	Patients who underwent TMB	ICI	Gene platform	Numer of genes	Median TMB	Results
Rizvi et al. <i>J Clin Oncol</i> 36:633–641 (Rizvi et al., 2018)	759	398 (52 %)	Anti-PD-L1 alone or in combination with anti-CTLA-4	MSK-IMPACT	Version 1: 341 Version 2: 410 Version 3: 468	7.4 Mut/Mb	Higher TMB in pts. with DCB than NCB (median 8.5 vs. 6.6 Mut/Mb, $p = 0.0062$) Higher TMB in pts. with CR/PR than SD (median 8.5 vs. 6.6 Mut/Mb, $p = 0.0151$)
BIRCH/FIR (Kowanetz et al., 2017; Peters et al., 2017; Spigel et al., 2018)	567 (102 1st line, 465 2nd line)	473 (83 %) (100 % 1st line) 80 % 2nd line)	Atezolizumab	FoundationOne® CDx Assay	315	1 L: 9 Mut/Mb 2 L: 9.9 Mut/Mb	1st line pts. with high TMB (≥ 13.5 /Mb) OS HR 0.45 CI 95 % (0.17, 1.16); PFS HR 0.54 CI 95 % (0.3, 0.97) 2nd line pts. with high TMB (≥ 17.7 /Mb) OS HR 0.7 CI 95 % (0.49, 1.00); PFS HR 0.5 CI 95 % (0.38, 0.67)
POPLAR (Kowanetz et al., 2017; Mazieres et al., 2021)	287	92 (32 %)	Atezolizumab	FoundationOne® CDx Assay	315	9.9 Mut/Mb	Pts. with high TMB (≥ 15.8 /Mb) OS HR 0.5 CI 95 % (0.15, 1.67); PFS HR 0.49 CI 95 % (0.19, 1.3)
CheckMate026 (Carbone et al., 2017)	541	312 (57.7 %)	Nivolumab	WES	–	Low burden: 0–100 mutations Medium burden: 100–242 mutations High burden: > 243 mutations	TMB high RR Nivolumab 48 % vs. docetaxel 28%; mPFS 9.7 vs. 5.8 m, HR 0.62, CI 95 % (0.38, 1.00)
CheckMate 227 (Hellmann et al., 2018)	1739	1004 (57.7 %)	Nivolumab+ipilimumab	FoundationOne® CDx Assay	324	10Mut/Mb	TMB high (PFS 7.2 vs. 5.5 m, HR 0.58, CI 95 % (0.41, 0.81) $p > 0.001$)
CheckMate 568 (Ramalingam et al., 2018)	288	98 (34 %)	Nivolumab+Ipilimumab	FoundationOne® CDx Assay	324	10Mut/Mb	PFS TMB high (7.1 months [95 % CI, 3.6–11.3 months]) vs. TMB low (2.6 months [95 % CI, 1.4–5.4 months])

TMB, Tumour Mutational Burden; ICI, Immune checkpoint inhibitors; OS, Overall Survival; PFS, Progression-Free Survival; RR, Response Rate; m, months; CI, Confidence Interval; Mut/Mb, Mutations/Megabase; WES, Whole Exome Sequencing; HR, Hazard Ratio; Pts, patients; CR, Complete Response; PR, Partial Response; SD, Stable Disease.

and allowed the selection of patients who benefit from longer responses with the combination vs. chemotherapy (PFS at one year of 45 % vs. 18 % with TMB high vs. low, respectively) with 93 % of responses maintained per year (Hellmann et al., 2018).

In 2020, the FDA approved Pembrolizumab as a treatment for High TMB solid tumours, using 10 mut/Mb as a cut-off value. Of note, this was the first time TMB was used as a biomarker to approve a therapy by the FDA (Marcus et al., 2021). However, given the aforementioned limitations of TMB, this approval has been considerably disputed (Addeo et al., 2021; Prasad & Addeo, 2020), especially the cut-off value of 10mut/Mb, highlighting the need to reinforce research on TMB and other predictive biomarkers for immunotherapy for all tumour types.

5. Relation of TMB with other biomarkers

Hitherto, the most plausible approach to predict response to immunotherapy may be a combination of the currently approved biomarkers, together with others that are still under evaluation. Given the controversial approval of TMB as an agnostic biomarker for treatment with pembrolizumab, it is rationale to combine TMB with other biomarkers, in order to better reflect the molecular heterogeneity of different types of tumours. Below are some examples of alterations and makers that might be used in combination with TMB to predict response to immunotherapy:

Since PD-L1 expression determined by IHC is currently the most widespread immunotherapy biomarker, various groups have studied its relation with TMB. In the CheckMate 026 trial, it was observed that patients with elevated TMB and PD-L1 (≥ 50 %) had a higher response rate (RR) (Carbone et al., 2017). Rizvi et al. (2018) showed in their cohort that patients with elevated TMB and positive PD-L1 ($\geq 1\%$) presented greater clinical benefit than those with only one positive biomarker. By contrast, Kowanetz et al. (2017) showed that TMB and PD-L1 (≥ 5 %) were independent factors of efficacy with atezolizumab and the association of TMB and response to atezolizumab occurred in both PD-L1 selected and non-selected patients. In the CheckMate 227 trial, elevated TMB was an independent biomarker of response to combination immunotherapy regardless of PD-L1 status (≥ 1 %) (Hellmann et al., 2018). Recently, Ricciuti et al., showed that increased TMB is associated to clinical benefit with ICI, independently of PD-L1 expression. They also found an association of TMB and higher infiltration of CD8 + PD1 + T cells, as well as PDL1 tumour expression, MHC II antigens presentation and upregulation of immune-related pathways (Ricciuti et al., 2022).

Another gene with increasing interest in NSCLC is *STK11*, which is mutated in 16.7 % of lung adenocarcinoma. *STK11* plays a role in the regulation of cellular metabolism and growth and its inactivation is associated with a “cold” tumour immune microenvironment. (Facchinetti et al., 2017). Mutations in *STK11* have been associated with lack of benefit from PD-1/PD-L1 blockade (Shire et al., 2020). It has to be remarked that *STK11* mutations were significantly enriched among TMB intermediate/high, PD-L1 negative tumours (Skoulidis et al., 2018). Furthermore, *STK11* is frequently co-mutated with *KEAP1* (Papillon-Cavanagh et al., 2020).

The relation of mutations in Kelch-like ECH associated protein 1 gene (*KEAP1*) and TMB has also been explored. *KEAP1* encodes a protein that mediates a cytoprotective response to oxidative stress and xenobiotics. *KEAP1* mutations are frequent in lung cancer (23 % of lung adenocarcinoma and 31 % of squamous cell carcinoma) (Cancer Genome Atlas Research Network, 2014; “Comprehensive genomic characterization of squamous cell lung cancers,” 2012). Mutations in *KEAP1* are associated with poor clinical outcomes with chemotherapy (Goeman et al., 2019). Recently, alterations in *KEAP1* have been associated with reduced efficacy of immunotherapy (Aggarwal et al., 2020; Scalera et al., 2022). Other studies suggested that the presence of mutations in *KEAP1* is a prognostic poor factor regardless of the treatment (Papillon-Cavanagh et al., 2020; Rizvi et al., 2019). Regarding its potential association with

TMB, *KEAP1* alterations have been associated with high TMB (Chen et al., 2020; Marinelli et al., 2020).

Regarding co-mutations, *STK11* was found to be co-mutated with *KEAP1*, and it has been demonstrated that these double-mutant patients presented worse outcomes than single mutant or wild-type patients. *STK11* was also found co-mutated with *KRAS*, these double mutants presented as well worse OS and PFS with immunotherapy and chemotherapy (Papillon-Cavanagh et al., 2020).

Higher TMB has also been found in patients harboring mutations in AT-Rich Interactive Domain-containing protein 1 A (*ARID1A*) gene. Some studies have found that patients with alterations in *ARID1A* have longer PFS, thus highlighting its potential as a predictive biomarker for immunotherapeutic treatment (Okamura et al., 2020; Sun et al., 2020).

Another gene of interest is Tumour Protein P53 (*TP53*), which has been associated with high TMB, as well as with improved responses and outcomes to immunotherapeutic treatments. *TP53* has been found co-mutated with other genes such as *KRAS* or Ataxia telangiectasia mutated (*ATM*), noting that these co-mutational patterns may predict prognosis more accurately (Dong et al., 2017) Chen et al. found that patients with *ATM* and *TP53* co-occurring mutations presented higher TMB, better benefit rates with immunotherapy, and improved overall survival (Chen et al., 2019).

Interestingly, the co-occurring mutations in *KEAP1*, *STK11* and other related genes such as Polybromo 1 (*PBRM1*) and Transcription activator BRG1 (*SMARCA4*), which are less frequently mutated) suggest an immunologically cold phenotype of tumours, associated with poor clinical outcomes with immunotherapy and showing a subpopulation of lung carcinoma that may be misclassified as immune responsive by TMB (Marinelli et al., 2020).

TMB has also been linked with microsatellite status. PD-1/PD-L1 blockade has been described as highly effective in microsatellite instability-high (MSI-high)/mismatch repair-deficient (dMMR) tumours (Le et al., 2017). Approval of pembrolizumab in tumours MSI-H represented the first genomically driven pan-solid tumour authorization granted by the FDA in 2017 (Marcus, Lemery, Keegan, & Pazdur, 2019). Goodman et al. showed that patients with MS-stable/TMB-high tumours had significantly longer survival than MS-stable/TMB-low/intermediate, suggesting that TMB-high patients, regardless of MSI status, may respond to checkpoint blockade (Goodman et al., 2019). By contrast, it has also been reported that TMB high in MS-stable tumours of different cancer types had limited benefit with immunotherapy (Kwon et al., 2021; McGrail et al., 2021).

Furthermore, TMB was significantly associated to mutational signatures denoting genomic instability, APOBEC activity, and tobacco smoking (Mashtalir et al., 2018; Yu et al., 2019). Newell et al., in a recent study of multiomic profiling of patients with melanoma treated with immunotherapy, have developed a model combining TMB and a signature of IFN γ -related genes that predicts treatment response (Newell et al., 2021). Banchereau et al. found that NSCLC tumours were enriched in B/plasma cell and type I IFN signatures. In addition, they found that in PD-L1 negative tumours, increased expression of DNA Damage Repair (DDR) and cell cycle related genes were associated with response, but this signature had only a slight correlation with TMB (Banchereau et al., 2021).

The possible role of TMB with targeted therapy has also been explored. Regarding lung cancer patients with *EGFR* mutations, a negative relationship has been observed between TMB and response to tyrosine kinase inhibitors (TKI) therapy (Blakely et al., 2017; Offin et al., 2019). Contrary to its association with immunotherapy, it has been described that patients with medium or low TMB have a better response to targeted therapy. Regarding resistance mechanisms, Offin et al. showed that *EGFR*-TKI resistant patients presented higher TMB, although it was lower in patients in which the resistance mechanism is mediated by T790M. It has been hypothesized that the finding of higher TMB in patients who develop resistance to *EGFR*-TKIs could suggest the use of immunotherapy after progression (Offin et al., 2019).

6. The future: TMB in liquid biopsy

Hitherto, the analysis of TMB in tissue using NGS is considered the standard technique, although tissue samples present certain limitations, as described previously. Especially in NSCLC, biopsies obtained by Endobronchial Ultrasound Bronchoscopy (EBUS) are small in size and may contain low-quality DNA. To overcome these limitations, the analysis of TMB in circulating-free DNA (cfDNA) is being developed, since it is less invasive, reproducible, and can be assessed not only prior to the start of treatment but also throughout it for monitoring (Fenizia et al., 2018; Wan et al., 2017; Yu et al., 2021).

Different panels of TMB in liquid biopsy (bTMB) have been developed, such as *FoundationOne® Liquid CDx assay*, *GuardantOMNI™ assay* and *NCC-GP150* (Chaudhuri et al., 2017; Friedlaender et al., 2020; Ma et al., 2021; Qiu et al., 2019; Wang et al., 2019). Some studies have also shown that TMB calculated by WES was consistent between tissue and plasma samples (Koeppel et al., 2017; Schuurbiers et al., 2022).

The role of bTMB has also been assessed in clinical trials (POPLAR, OAK, MYSTIC, BFAST, B-FIRST) as shown in Table 3. Gandara et al. showed that higher bTMB (bTMB ≥ 16 Mut/Mb) is associated with increased PFS and OS in NSCLC patients treated with atezolizumab in second-line, with patients from POPLAR and OAK trials (Gandara et al., 2018). In addition, in MYSTIC trial, exploratory analyses suggested that bTMB ≥ 20 Mut/Mb (using *GuardantOMNI™ assay*) was associated with better OS with immunotherapy (durvalumab with tremelimumab) vs. chemotherapy, although it was an overall negative study (Rizvi et al., 2020; Si et al., 2021). There is a lack of studies regarding the role of bTMB as a predictive marker of response to treatment with immunotherapy in combination with chemotherapy. However, Garassino et al. presented in ASCO 2020, that bTMB with a cut-off value of 15mut/Mb was correlated to tTMB of 175mut/exome, and that bTMB predicted PFS for patients treated with pembrolizumab plus chemotherapy. Aggarwal et al., assessed bTMB using a panel of 500 genes in patients treated with pembrolizumab and found that patients with durable clinical benefit presented higher bTMB. In addition, patients with a high TMB (cut-off value of 16mut/Mb) had improved PFS and OS, and predictivity was improved if TMB was combined with the analysis of mutations in genes such as *HER2*, *STK11*, *KEAP1* or *P TEN* (Aggarwal et al., 2020). Recently,

in the phase 2 B-FIRST trial, bTMB with a cut-off value of 16mut/Mb was associated with higher ORR and longer OS, although PFS was not statistically significant using this cut-off value (Kim et al., 2022). Peters et al. showed lack of predictive value of bTMB in the primary analysis of BFAST cohort C randomized trial, using a cut-off value of 16mut/Mb. Only patients with non-squamous histology presented improved PFS with atezolizumab than with chemotherapy. Nevertheless, it was found a benefit in OS with atezolizumab in patients with bTMB ≥ 16 at increased follow-up (Peters et al., 2022). In addition, authors performed an exploratory analysis comparing the TMB cut-off value of different assays, and obtained a different optimal cut-off value for PFS than for OS, highlighting the difficulty of establishing a threshold to determine bTMB high or low.

In conclusion, all the studies comparing the analysis of TMB in tissue and liquid biopsy emphasize the need for further research in order to set an optimal cut-off value to increase predictive value of TMB analysis in plasma samples (Chalmers et al., 2017; Gandara et al., 2018; Wang et al., 2019).

7. Conclusion and future perspective

Assessment of TMB in tissue has been a milestone in the search of biomarkers in NSCLC. Although TMB was initially determined by WES, recent studies have demonstrated that targeted gene panels can accurately assess TMB in a time and cost-effective manner. Moreover, TMB is a surrogate biomarker of immunogenic neoantigens, and several basic and clinical studies have demonstrated its utility as a predictive biomarker for immunotherapy in NSCLC patients.

However, there are still challenges to be achieved. As the studies carried out to date have estimated TMB in tissue with different platforms and different analytic requirements and parameters, standardization of key aspects of panel-based TMB estimation is still an urgent need. Therefore, prospective clinical trials assessing TMB are required to further validate this biomarker and to enhance its value in clinical decision-making. We should also focus our efforts on the development of TMB in liquid biopsy, which may allow monitoring the response to immunotherapy of NSCLC patients.

In conclusion, TMB has a considerable potential for the optimal

Table 3
Clinical trials which evaluated bTMB as immunotherapy biomarker.

Publication	Number of patients	ICI	Gene platform	Panel size	TMB cut-off	Results
B1FIRST (Kim et al., 2022)	119 patients	Atezolizumab monotherapy in locally advanced or metastatic stage IIIB–IVB non-small cell lung cancer	bTMB Clinical Trial Assay and comparison with FoundationOne Liquid Companion Diagnostic assay (CDx)	bTMB CTA N.S and F1 1.1 Mb (311 genes)	≥ 16 (mut/Mb)	bTMB ≥ 16 was associated with higher ORR, and ORR improved as bTMB cutoffs increased.
BFAST Cohort C (Peters et al., 2022)	145 patients in the atezolizumab arm and 146 in the chemotherapy arm	Atezolizumab or platinum-based chemotherapy in stage IIIB–IV NSCLC	bTMB Clinical Trial Assay and comparison with FoundationOne Liquid Companion Diagnostic assay (CDx)	bTMB CTA N.S and F1 1.1 Mb (311 genes)	≥ 16 (mut/Mb)	bTMB ≥ 16 was not associated with improved PFS, it was only found a benefit of atezolizumab vs. chemotherapy in non-squamous histology. Optimal TMB cut-off values and follow-up were different for PFS and OS.
MYSTIC (Rizvi et al., 2020; Si et al., 2021)	809	Durvalumab with Tremelimumab	GuardantOMNI assay	2.145 Mb (500 genes)	≥ 20 mut/Mb	Patients with bTMB ≥ 20 mut/Mb improved OS for durvalumab plus tremelimumab vs. chemotherapy (HR, 0.49; 95 % CI, 0.32–0.74).
OAK (Gandara et al., 2018)	583	Atezolizumab	Designed panel	1.125 Mb of genome coding region	≥ 16 mut/Mb	Patients with bTMB ≥ 16 had improved PFS benefit (HR: 0.65 (95 % CI: 0.47–0.92); $P = 0.013$) with atezolizumab versus docetaxel.
POPLAR (Gandara et al., 2018)	211	Atezolizumab	Designed panel	1.125 Mb of genome coding region	≥ 16 mut/Mb	At the bTMB cut-point of ≥ 16 , the PFS HR was 0.57 (95 % CI: 0.33–0.99)

TMB, Tumour Mutational Burden; OS, Overall Survival; PFS, Progression-Free Survival; RR, Response Rate; m, months; CI, Confidence Interval; Mut/Mb, Mutations/Megabase; WES, Whole Exome Sequencing; HR, Hazard Ratio; Pts, patients; CR, Complete Response; PR, Partial Response; SD, Stable Disease; NS, Not Specified.

management of NSCLC patients, since it is not only useful to select the best treatment but also provides in-depth information about tumour biology.

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Declaration of interest

The authors have no conflicts of interest to declare.

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