Journal Pre-proof

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PII: S1525-1578(22)00041-1
DOI: https://doi.org/10.1016/j.jmoldx.2022.01.008
Reference: JMDI 1192

To appear in: The Journal of Molecular Diagnostics

Received Date: 1 July 2021
Revised Date: 22 December 2021
Accepted Date: 12 January 2022


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Evaluation of the TruSight Oncology 500 Assay for Routine Clinical Testing of Tumor Mutational Burden and Clinical Utility for Predicting Response to Pembrolizumab

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Funding: The research and all clinical studies included in the analysis were funded by Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA.

Competing interests: All authors are stockholders of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA. BW, JK, MK, GA, LC, PQ, LL, DAG, RC, and DL are employees of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA. MM is a former employee of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA.

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ABSTRACT

Pembrolizumab is approved for treating patients with unresectable or metastatic solid tumors that have high tumor mutational burden (TMB), as assessed by the FDA–approved companion diagnostic FoundationOneCDx, following progression on prior treatment. To expand TMB assessment for enriching response to pembrolizumab, TMB measurement from TruSight Oncology 500 (TSO500) was evaluated in archival pan-tumor samples from 294 patients enrolled in eight pembrolizumab monotherapy studies. TSO500 is a commercial panel-based next-generation sequencing assay with broad availability, quick turnaround time, and a standardized bioinformatics pipeline. TSO500 TMB was evaluated for correlation and concordance with two reference methods, FoundationOneCDx and whole exome sequencing (WES). The TSO500 cut-point for TMB-high (TMB-H) was selected based on the receiver-operating characteristic curve analysis against each reference method’s TMB-H cut-point. Clinical utility of the selected TSO500 cut-point (10 mut/Mb) was assessed by calculating sensitivity, specificity, positive and negative predictive values, and objective response rate enrichment. There was high correlation and concordance of TSO500 TMB with both reference methods. TSO500 TMB was significantly associated with best overall response, and the selected cut-point had comparable clinical utility with respective cut-points for the reference methods in predicting response to pembrolizumab. As a commercial assay with global kit distribution complete with an off-the-shelf software package, TSO500 may provide additional access to immunotherapy for patients with tumors with a TMB score ≥10 mut/Mb.
Keywords (up to 10): Trusight Oncology 500, TSO500, next-generation sequencing, NGS, tumor mutational burden, biomarkers for immune checkpoint blockade, pembrolizumab, immunotherapy, companion diagnostic, whole exome sequencing
INTRODUCTION

Immune checkpoint blockade–based immunotherapies, such as the programmed death 1 (PD-1) inhibitor pembrolizumab, have changed the treatment paradigm for a variety of tumor types, including those with previously poor prognoses, such as advanced melanoma\(^1,2\) and non–small cell lung cancer (NSCLC).\(^3\) Despite impressive clinical outcomes, such as durable antitumor activity and improved survival observed in a subset of patients, many patients do not respond to immunotherapies. To identify patients who are most likely to benefit from immunotherapy, various predictive biomarkers have been investigated. Some of these have been clinically validated, including three predictive biomarkers for use with pembrolizumab: programmed death ligand 1 (PD-L1) expression in specific cancers,\(^4,5\) high microsatellite instability (MSI-H) regardless of tumor type,\(^6\) and high tumor mutational burden (TMB-H) regardless of tumor type.\(^7\)

PD-L1 expression, MSI-H, and TMB-H represent major categories of immunotherapy predictive biomarkers, with PD-L1 indicative of a T cell–inflamed tumor microenvironment and MSI-H and TMB-H as proxies for a high immunogenic neoantigen load.\(^7\)

TMB is the total number of somatic mutations in a tumor genome.\(^8\) TMB-H has been associated with improved immunotherapy responses and improved survival outcomes in multiple tumor types.\(^7,9\) In a pan-tumor setting, TMB-H has higher prevalence than MSI-H and appears to be independent of PD-L1 expression in predicting immunotherapy response.\(^10\) Pembrolizumab has been approved as monotherapy for the treatment of adult and pediatric patients with unresectable or metastatic solid tumors with high TMB scores (≥10 mutations/megabase [mut/Mb] as determined by the US Food and Drug Administration–approved FoundationOneCDx assay) who have no satisfactory alternative treatment options after progression on previous treatment (https://www.fda.gov/drugs/drug-approvals-and-databases/fda-approves-pembrolizumab-adults-and-children-tmb-h-solid-tumors, last accessed on September 21, 2021).
FoundationOneCDx (Foundation Medicine, Inc., Cambridge, MA) is a single-site, panel-based next-generation sequencing (NGS) assay using formalin-fixed, paraffin-embedded (FFPE) tumor specimens for tumor mutation profiling and TMB evaluation. In addition to FoundationOneCDx, TMB has also been determined by whole exome sequencing (WES). However, there are drawbacks to using WES to determine TMB, including the capability to perform WES in a routine clinical diagnostic setting, slow turnaround time, high cost, and a lack of standardized bioinformatics pipelines.

Currently, there are other panel-based TMB assays with commercial kit distribution. One such assay is the TruSight Oncology 500 (TSO500) (Illumina, San Diego, CA), which covers 523 cancer-related genes, including 284 of the 309 genes used by FoundationOneCDx for TMB assessment. Like FoundationOneCDx, TSO500 uses a hybridization capture–based target enrichment strategy and a bridge polymerase chain reaction–based template preparation, with image-intensive sequencing reactions performed with fluorescence-labeled oligonucleotides. TSO500 covers 1.28 Mb exonic regions used for TMB estimation, and the platform provides an off-the-shelf bioinformatics pipeline that can be implemented by end users without modification to obtain TMB results and to identify relevant mutations implicated in various solid tumor types.

Although the clinical utility of FoundationOneCDx and WES-based TMB measurements to identify patients who may benefit from pembrolizumab monotherapy has been demonstrated\textsuperscript{11,12}, the clinical utility of the TSO500 assay for pembrolizumab monotherapy is unknown. In this study, the performance of TSO500 was evaluated to assess TMB and its clinical utility to enrich for response to pembrolizumab monotherapy using FoundationOneCDx and WES as reference methods.

**Materials and Methods**
Clinical Tumor Samples

Table 1 describes the clinical samples used for the TSO500 TMB evaluation. Archival tumor samples from 294 participants with advanced solid tumors enrolled in eight pembrolizumab monotherapy clinical studies, already successfully evaluated by FoundationOneCDx and WES, were utilized in the TSO500 TMB analysis. These samples are a subset of the samples utilized to establish the FoundationOneCDx TMB cut-point of 10 mut/Mb, which was used to determine high tissue TMB (tTMB-H) for the KEYNOTE-158 study (NCT02628067). Samples used in this evaluation were chosen using two criteria: 1) proper consent of the participant to use the sample for exploratory research, and 2) sufficient leftover DNA after use in the FoundationOneCDx and WES assays. The eight clinical studies were KEYNOTE-001 (NCT01295827), KEYNOTE-012 (NCT01848834), KEYNOTE-028 (NCT02054806), KEYNOTE-055 (NCT02255097), KEYNOTE-061 (NCT02370498), KEYNOTE-086 (NCT02447003), KEYNOTE-100 (NCT02674061), and KEYNOTE-199 (NCT02787005). Identifier numbers can be retrieved from https://clinicaltrials.gov/.

All patients provided written informed consent before enrollment in the clinical trials. All study protocols were consistent with the global standards of the International Conference on Harmonization Good Clinical Practices, the Council for International Organizations of Medical Sciences Public Policy Statement, Clinical Trial Ethics Sciences International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS, 2002), the Pharmaceutical Research and Manufacturers of America (PhRMA, 2009) Principles on Conduct of Clinical Trials; applicable local regulatory requirements, and the ethical principles that have their origin in the Declaration of Helsinki.

FFPE Tissue DNA Extraction
For the clinical FFPE samples used in the TSO500 evaluation, at least 20% tumor content was required. For all clinical studies except KEYNOTE-061, FFPE tissue were extracted by Foundation Medicine, Inc. (Cambridge, MA), and first evaluated by FoundationOneCDx and then by TSO500. For KEYNOTE-061, FFPE DNA extraction was performed by Almac Diagnostics (Craigavon, United Kingdom) and first evaluated by WES and then by TSO500; samples from KEYNOTE-061 were not evaluated using FoundationOneCDx. All DNA samples used for WES were extracted by Almac Diagnostics; the TMB analysis has been described previously.\(^7\)

**TSO500 NGS Library Preparation and TMB Analysis**

TSO500 was evaluated with 48 to 50 ng of leftover FFPE DNA material except for two samples, in which 35 and 37 ng of DNA were utilized, respectively. DNA fragmentation was performed using the Covaris (Woburn, MA) M220 focused-ultrasonicator with the settings of 75 W for peak incident power, 25% as duty factor, 1000 for cycles per burst, and treatment time of 6 minutes per sample at 4°C. The TruSight Oncology 500 DNA Kit, For Use with NextSeq (48 samples) (Illumina; catalog number 20028214) was used for the TSO500 NGS library preparation. The library preparation was performed manually according to the manufacturer’s protocol, with 24 samples per batch. The Qubit dsDNA HS Assay Kit (Thermo Fisher; catalog number Q32851) was used to quantify the hybridization capture-enriched NGS libraries before library normalization. Only two TSO500 libraries had concentrations less than 3 ng/μl (1.5 and 2.1 ng/μl, respectively); for these two samples twice the volume of the bead-normalized libraries was used in NGS sequencing. NGS sequencing was performed on a NextSeq 550 instrument (Illumina) with eight libraries per sequencing run.

TSO500 TMB was reported using TruSight Oncology 500 Local App version 1 (Illumina). The manufacturer’s quality control criteria were used to determine whether a TSO500 TMB result
was valid, including NGS library concentration ≥1 ng/μL, median insert size ≥70 bp, median exon coverage ≥50 count, and percentage of exons with coverage of at least 50 count ≥90%.

**FoundationOneCDx and WES TMB Bioinformatics Pipelines**

FoundationOneCDx TMB was obtained with the FoundationOneCDx pipeline (version 3.2.0). The WES pipeline has been described previously.7

**Discretization of Continuous TSO500 TMB Values for Concordance Analysis**

The following formula was used by the TruSight Oncology 500 Local App for the calculation of TSO500 TMB: the count of the total TMB variants (synonymous and non-synonymous non-hotspot somatic coding variants that are either single-nucleotide variants or small insertions/deletions with variant allele frequency [VAF] ≥5%), divided by the size of the coding regions that passed quality control criteria as defined by the manufacturer. Thus, for each given count of the TSO500 total TMB variants, there could be a range of TSO500 TMB values because of the slight variability of the size of the coding regions passing quality control criteria. A tight distribution of the size of the coding region was observed in the analysis, with the median at 1.278 Mb, the first quartile at 1.277 Mb, the third quartile at 1.279 Mb, the 2.5% percentile at 1.266 Mb, and the 97.5% percentile at 1.279 Mb. To simplify the concordance analysis, 1.278 Mb was used as the size of the coding region in the TMB calculation.

**Concordance Analysis of TSO500 TMB Against Reference Methods, and Selection of TSO500 TMB Cut-Point**

Concordance of TMB status (TMB-H or non-TMB-H), determined by potential TMB cut-points of TSO500 and the established TMB cut-points of the reference methods, was assessed by calculating the area under the receiver-operating characteristic (AUROC) curve, positive percentage agreement (PPA), and negative percentage agreement (NPA). PPA was calculated
as the proportion of patients identified as TMB-H by a reference method (FoundationOneCDx or WES) who were also identified as TMB-H by TSO500. NPA was calculated similarly using the proportion of patients with non–TMB-H for all assays. Overall percentage agreement (OPA) was estimated as the proportion of participants identified as TMB-H or non–TMB-H by TSO500 and a reference TMB method (FoundationOneCDx or WES). The reference cut-points used to define TMB-H and non–TMB-H statuses were 10 mut/Mb for FoundationOneCDx (https://www.accessdata.fda.gov/cdrh_docs/pdf17/P170019S016B.pdf, last accessed on September 21, 2021) and 175 mutations per exome (mut/exome) for WES. To facilitate the selection of the optimal TMB cut-point used by TSO500 for TMB-H status determination, the commonly adopted Youden index (YI) criteria, which aims to optimize the average of PPA and NPA, were applied.

Clinical Utility of TSO500

Best overall response (BOR) was determined using Response Evaluation Criteria in Solid Tumors, version 1.1, by independent central review. Statistical significance of the association of TMB measured by TSO500 with BOR was assessed using logistic regression adjusted for Eastern Cooperative Oncology Group performance status and tumor type. The clinical utility of the selected TSO500 TMB cut-point for discriminating responders from non-responders was assessed by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), objective response rate (ORR) enrichment, and prevalence.

Availability of Data and Material

Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA (MSD) is committed to providing qualified scientific researchers access to anonymized data and clinical study reports from the company’s clinical trials for the purpose of conducting legitimate scientific research. MSD is also obligated to protect the rights and privacy of trial participants and, as
such, has a procedure in place for evaluating and fulfilling requests for sharing company clinical trial data with qualified external scientific researchers. The MSD data sharing website (available at: http://engagezone.msd.com/ds_documentation.php, last accessed on October 20, 2021) outlines the process and requirements for submitting a data request. Applications will be promptly assessed for completeness and policy compliance. Feasible requests will be reviewed by a committee of MSD subject matter experts to assess the scientific validity of the request and the qualifications of the requestors. In line with data privacy legislation, submitters of approved requests must enter into a standard data-sharing agreement with MSD before data access is granted. Data will be made available for request after product approval in the United States and the European Union or after product development is discontinued. There are circumstances that may prevent MSD from sharing requested data, including country or region-specific regulations. If the request is declined, it will be communicated to the investigator. Access to genetic or exploratory biomarker data requires a detailed, hypothesis-driven statistical analysis plan that is collaboratively developed by the requestor and MSD subject matter experts; after approval of the statistical analysis plan and execution of a data-sharing agreement, MSD will either perform the proposed analyses and share the results with the requestor or will construct biomarker covariates and add them to a file with clinical data that is uploaded to an analysis portal so that the requestor can perform the proposed analyses.

Results

Reproducibility of TSO500 TMB

Twenty participants had their FFPE DNA samples analyzed multiple times. Among these 20 replicate sets, two sets used DNA extracted from different tissue blocks from the same subject, three sets used DNA extracted from different sections of the same tissue block, and the rest
used DNA from the same extraction. The replicate samples from the same participant were analyzed in different batches of the TSO500 NGS library preparation. The inter-run reproducibility of TSO500 TMB was high, indicated by <20% coefficient of variation (CV) for samples with an average of TSO500 TMB ≥6 mut/Mb (Supplemental Figure S1). Because of the high reproducibility of TSO500 TMB, only the replicate with the highest TMB value was selected for each participant for downstream analysis.

Correlation of TSO500 TMB With FoundationOneCDx TMB and WES TMB
There was high correlation of TSO500 TMB with FoundationOneCDx TMB and WES TMB, demonstrated by both the Pearson correlation coefficient $r$ and the Spearman rank correlation coefficient $\rho$, especially for samples with TMB above the FoundationOneCDx (10 mut/Mb) and WES (175 mut/exome) cut-points (Table 2, Figure 1). Because the Spearman rank correlation coefficient is non-parametric and less sensitive to outliers, it was a more robust measure than Pearson correlation in this analysis. Thus, although the Pearson correlation was provided for completeness, it should be interpreted with caution.

Concordance of TMB Status Determined by TSO500 and Reference Methods
The high AUROC values of the TSO500 TMB in predicting TMB-H status as determined by FoundationOneCDx (AUROC with 95% confidence interval [CI], 0.99 [0.98 to 1.00]) and WES (AUROC with 95% CI, 0.95 [0.92, 0.97]) demonstrated high concordance of TSO500 TMB with the two reference TMB methods (Figure 2). The YI criteria for TSO500 TMB against the FoundationOneCDx cut-point of 10 mut/Mb and against the WES TMB cut-point of 175 mut/exome were both 10.17 mut/Mb, rounded to 10 mut/Mb (Figure 2). Analysis of the PPA, NPA, and OPA values for potential TSO500 TMB cut-points adjacent to the YI of the respective ROC curves suggested that the proposed pan-tumor TSO500 TMB cut-point of 10 mut/Mb
corresponded to the cut-points for both FoundationOneCDx TMB (10 mut/Mb) and WES TMB (175 mut/exome) (Supplemental Tables S1 and S2).

With the TSO500 TMB cut-point selected at 10 mut/Mb, there was a high overlap of TMB-H calls between TSO500 and the two reference methods (Supplemental Figure S2) in the 268 participants with evaluable TMB data from all three TMB assays.

**Similar Correlation and Concordance of TSO500 TMB and FoundationOneCDx TMB Against WES TMB**

TSO500 TMB and FoundationOneCDx TMB had comparable correlation and concordance against WES TMB. Analysis of 268 patient samples that had evaluable TMB values from all three platforms showed that TSO500 TMB and FoundationOneCDx TMB had highly similar Pearson correlation coefficient $r$ and Spearman rank correlation coefficient $\rho$ against WES TMB (Supplemental Table S3). The AUROC values of TSO500 TMB or FoundationOneCDx TMB in predicting TMB-H status determined by WES TMB were highly similar (TSO500 vs WES AUROC with 95% CI, 0.955 [0.928, 0.982]; FoundationOneCDx vs WES AUROC with 95% CI, 0.934 [0.895, 0.973]) (Figure 3).

**Association of TSO500 TMB With Clinical Response and Clinical Utility of TSO500 TMB**

When assessed as a continuous variable, TMB measured by the TSO500 assay was significantly associated with BOR, with a one-sided $p$-value of 3.68E-05 (i.e., higher TSO500 TMB was associated with improved clinical response), adjusted for Eastern Cooperative Oncology Group performance status and the six major tumor types as color coded in Figure 1. Clinical utility metrics such as sensitivity, specificity, PPV, NPV, and ORR enrichment, were similar between TSO500 and FoundationOneCDx, and between TSO500 and WES, at the respective cut-point for each TMB assay (Table 3, Figure 4).
In this study, the TSO500 assay was evaluated for TMB assessment using FFPE samples collected from about 300 patients with at least twelve different types of advanced solid tumors enrolled in eight clinical trials of pembrolizumab monotherapy. The samples are a subset of the samples used to establish the FoundationOneCDx TMB cut-point for the KEYNOTE-158 study. Using FoundationOneCDx and WES as TMB reference methods and FoundationOneCDx as the standard for panel-based TMB assays, TSO500 can reliably classify the TMB-H status as determined by the reference methods.\textsuperscript{15,16} TMB measured by TSO500 is highly correlated and concordant with TMB measured by the FoundationOneCDx assay and by WES.

The high success rates of TSO500 NGS library preparation and sequencing in this evaluation are likely due to the quality of the clinical FFPE samples used in the study. These samples were successfully analyzed by FoundationOneCDx and WES, which was a requirement for profiling with TSO500 to enable the concordance analysis. Subsequent use of this assay with clinical samples demonstrated that TSO500 is robust for good-quality FFPE DNA samples with a DNA input of 20 ng or even lower (data not shown). A simple solution to obtain successful TSO500 analysis for poorer quality FFPE DNA samples is to increase DNA input.

In both the subset used for this TSO500 evaluation and the original sample set for the FoundationOneCDx TMB evaluation, the head and neck squamous cell carcinoma (HNSCC) tumor type had the highest representation (31\% of samples in the TSO500 evaluation). Figure 1 shows that the HNSCC and non-HNSCC samples have similar TMB distributions and thus the relatively high presence of HNSCC is not likely to introduce tumor type-specific bias. The relatively high presence of HNSCC might add rigor to a pan-tumor TMB evaluation due to its complex biology, as HNSCC can be caused by various factors such as alcohol and tobacco usage, human papilloma virus associated oropharyngeal cancer, and Epstein-Barr virus.
associated nasopharyngeal cancer. Melanoma, on the other hand, with a much higher prevalence of TMB-H compared to other tumor types, was not utilized in the TSO500 evaluation because the only sample with enough leftover DNA material failed WES analysis and its very high TMB score as determined by FoundationOneCDx might introduce bias. NSCLC, another tumor type with a relatively high TMB-H prevalence, has a relatively low representation (6%) in the TSO500 sample set, yet it accounted for about 20% of TMB-H calls by both TSO500 and WES.

The proposed TMB cut-point of TSO500, based on the ROC curve analysis against the FoundationOneCDx cut-point of 10 mut/Mb and against the WES cut-point of 175 mut/exome, is 10 mut/Mb. This TSO500 TMB cut-point has clinical utility metrics similar to those of the FoundationOneCDx and WES TMB cut-points. One exception is that the FoundationOneCDx TMB had a lower TMB-H prevalence than TSO500 and WES among the evaluated samples. The lower prevalence might be due to the smaller size of the gene-coding region used by FoundationOneCDx in the TMB assessment (0.79 Mb) compared with 1.28 Mb used by TSO500 and the whole exon region (about 30 Mb) used by WES. Although the prevalence of TMB-H called by TSO500 is numerically higher than FoundationOneCDx, this difference is not statistically significant, as indicated by the Fisher’s exact test’s p value of 0.11. In addition, Figure 4 shows the overlapping of the 95% confidence intervals of ORR between TSO500 and FoundationOneCDx for each assay’s TMB-H group. Therefore, TSO500 and FoundationOneCDx TMB assays have similar clinical utilities.

There are several advantages to the TSO500 assay including that FFPE DNA repair steps are not needed for poorer quality FFPE samples to generate reliable TMB data and the off-the-shelf software provided by Illumina. In TSO500, unique molecular identifiers are used in the NGS library preparation to reduce sequencing noise and FFPE deamination artifacts through a
bioinformatics step called *duplex collapsing with reads from complementary strands*. Illumina also implemented an additional bioinformatics step that uses a likelihood ratio–based, variant-filtering method to further reduce FFPE false-positives. By implementing unique molecular identifiers and likelihood ratio filtering, TSO500 achieves high specificity in variant calling at a 5% VAF, resulting in the high correlation and concordance that are comparable to those of FoundationOneCDx against WES TMB.

Although the TSO500 assay is highly reproducible, in one replicate set that used the same DNA extraction, the %CV of this TMB-H sample is above 10% (Supplemental Figure S1). The relatively higher %CV of this replicate set was attributed to the inconsistent results of the TSO500 Local App’s “germline filter by proxy” algorithm. Four mutations in one run were called as somatic and thus counted as TMB variants; but they were called as germline and not counted as TMB variants in another run. The samples were later re-analyzed with TSO500 Local App version 2.0 and the problem of inconsistent germline filtering persisted.

One disadvantage of TSO500 is its labor intensive manual NGS library preparation procedures. TSO500 requires more than 2 days of hands-on time for NGS library preparation, followed by 24-hour sequencing on the NextSeq for eight NGS libraries. There are many reagents and experimental steps in the manual TSO500 NGS library preparation that can be susceptible to operator error when no automated methods are available. To implement the bioinformatics pipelines, the TSO500 Local App software requires information technology support and resources to first install the software and then to run the scripts to process and analyze the NGS data. To overcome the challenges associated with manual NGS library preparation and the TSO500 Local App software, Illumina has introduced automation kits that can be run on third-party vendors’ liquid handling systems and the NextSeq Local Run Manager software, which includes the TSO500 analysis module for on-instrument analysis.
This analysis compared the concordance and clinical utility of three NGS assays (panel-based TSO500 and FoundationOneCDx, and WES) to evaluate TMB from FFPE samples. Other studies have also compared panel-based NGS assays to identify tumors that are TMB-H. Several of them are in silico studies that have used the WES data from The Cancer Genome Atlas (TCGA) and down-selected the genes on various panels to be used for TMB prediction.\textsuperscript{16,17,18} The report from the Friends of Cancer Research Consortium\textsuperscript{16} relied on platform-specific analysis pipelines for each diagnostic platform, whereas other studies used the same bioinformatic approach across different panels.\textsuperscript{17,18} These in silico studies involved large numbers of samples across tumor types, but are limited in that the analysis does not include pre-analytical or, in some cases, bioinformatic variables. Additional studies have compared NGS panels beginning with FFPE samples,\textsuperscript{19,20,21} but these studies tend to be small in terms of sample size and/or tumor types. The power of the study described here is the use of a relatively large number of samples, diverse tumor types, and three assay-specific analytical pipelines that may best represent performance in a real-world setting.

\textbf{Conclusion}

In summary, TSO500 can be used to reliably assess TMB in clinical FFPE samples, and TMB assessed by TSO500 is highly correlated and concordant with TMB measured by FoundationOneCDx and WES. Similar to the validated and FDA-approved TMB cut-point of 10 mut/Mb assessed by FoundationOneCDx, the TSO500 TMB cut-point of 10 mut/Mb is predictive of response to pembrolizumab monotherapy. With completely developed NGS chemistry and bioinformatics pipelines for reliable TMB assessment, TSO500 could be used in clinical laboratories around the globe for TMB evaluation, which may provide greater access to immunotherapy for patients with solid tumors that have a TMB score of 10 mut/Mb or higher.
Acknowledgments: The authors thank the patients and their families and caregivers as well as the primary investigators and site personnel for participating in the studies. The authors also thank Gary Fox, Guochun Xie, Karena Yu, and Drew Roberts for technical support and Jared Lunceford for guidance. Medical writing and/or editorial assistance was provided by Dana Francis, PhD, of ApotheCom (Yardley, PA). This assistance was funded by Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA.

Author contributions: Conception, design, or planning of the study: PQ, DL, BW, and DAG; acquisition of the data: RC, LC, BW, GA, MK, and MM; analysis of the data: DL, BW, and JK; interpretation of the results: PQ, DL, BW, MK, JK, and LL; drafting of the manuscript: DL, BW, and JK; critically reviewing or revising the manuscript for intellectual content: all authors; approval of the final manuscript: all authors.

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Figure 1: Correlation of TMB assessed by TSO500 with FoundationOneCDx and WES

A: Scatter plot of TSO500 TMB with FoundationOneCDx TMB (N=269 with evaluable TMB scores by both assays); the vertical line corresponds to the established FoundationOneCDx TMB cut-point of 10 mut/Mb. B: Scatter plot of TSO500 TMB with WES TMB (N=293 with evaluable TMB scores by both assays); the vertical line corresponds to the established WES TMB cut-point of 175 mut/exome. Data are plotted on a log$_{10}$ scale to better visualize the correlation at lower TMB scores. Both plots are colored coded for major tumor types (HNSCC, head and neck squamous cell carcinoma; NSCLC, non–small cell lung cancer; TNBC, triple-negative breast cancer).
Figure 2: Selection of the TSO500 TMB cut-point that corresponds to the established FoundationOneCDx TMB and WES TMB cut-points

A: ROC curve of TSO500 TMB against the TMB-H status as determined by the established FoundationOneCDx TMB cut-point of 10 mut/Mb (N=269 with evaluable TMB scores by both assays). B: ROC curve of TSO500 TMB against the TMB-H status as determined by the established WES TMB cut-point of 175 mut/exome (N=293 with evaluable TMB scores by both assays). The Youden index in both 2A and 2B is 10.17 mut/Mb, corresponding to a count of 13 TSO500 total TMB variants over the exon regions covered by TSO500.
**Figure 3: Concordance of TSO500 and FoundationOneCDx TMB with WES TMB**

ROC curves TSO500 TMB and FoundationOneCDx TMB against the TMB-H status as determined by the established WES TMB cut-point of 175 mut/exome (N=268 with evaluable TMB scores by all three assays). The ROC curve of TSO500 TMB versus WES TMB-H labeling is drawn in teal and the ROC curve of FoundationOneCDx TMB versus WES TMB-H labeling is drawn in black.
Figure 4: ORR enrichment between TSO500 TMB and FoundationOneCDx TMB, and between TSO500 TMB and WES TMB

ORR and the associated 95% confidence interval in the TMB-H and non–TMB-H populations determined by the selected TSO500 cut-point compared with the ones determined by A) FoundationOneCDx and B) WES TMB cut-points. The population in Figure 4A or 4B includes participants with evaluable TMB scores by TSO500 and the corresponding reference assay. Cut-points for distinguishing TMB-H and non–TMB-H were 10 mut/Mb for TSO500 and FoundationOneCDx and 175 mut/exome for WES.
Table 1: Number of participants with evaluable TMB scores by respective TMB assays in the included clinical trials

Table lists the clinical study protocols and the major tumor types (defined as with ≥5 participants per study) of the 294 participants whose archival tumor samples were analyzed by TSO500. Of the 294 participants included, TMB scores were evaluable for 294 of 294 by TSO500, 269 of 270 by FoundationOneCDx, and 293 of 294 by WES.

<table>
<thead>
<tr>
<th>Study</th>
<th>Tumor type</th>
<th>No. of participants with evaluable TMB scores /No. of participants analyzed by Respective TMB Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TSO500</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>294/294</td>
</tr>
<tr>
<td>KEYNOTE-001</td>
<td>NSCLC</td>
<td>19/19</td>
</tr>
<tr>
<td>(NCT01295827)</td>
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<tr>
<td>KEYNOTE-012</td>
<td>Bladder</td>
<td>5/5</td>
</tr>
<tr>
<td>(NCT01848834)</td>
<td>HNSCC</td>
<td>62/62</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>2/2</td>
</tr>
<tr>
<td>KEYNOTE-028</td>
<td>Anal</td>
<td>5/5</td>
</tr>
<tr>
<td>(NCT02054806)</td>
<td>Carcinoid</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Mesothelioma</td>
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</tr>
<tr>
<td></td>
<td>Salivary gland</td>
<td>8/8</td>
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<tr>
<td></td>
<td>Thyroid</td>
<td>7/7</td>
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<tr>
<td>Other</td>
<td></td>
<td>26/26</td>
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<tr>
<td>KEYNOTE-055</td>
<td>HNSCC</td>
<td>29/29</td>
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<tr>
<td>(NCT02255097)</td>
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<td></td>
</tr>
<tr>
<td>Study</td>
<td>Tumor Type</td>
<td>TMB (40/40)</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>KEYNOTE-061</td>
<td>Gastric</td>
<td>24/24</td>
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<td>(NCT02370498)</td>
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<tr>
<td>KEYNOTE-086</td>
<td>TNBC</td>
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<td>(NCT02447003)</td>
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<tr>
<td>KEYNOTE-100</td>
<td>Ovarian</td>
<td>48/48</td>
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<tr>
<td>(NCT02674061)</td>
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<td>KEYNOTE-199</td>
<td>Prostate</td>
<td>9/9</td>
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<tr>
<td>(NCT02787005)</td>
<td></td>
<td></td>
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</tbody>
</table>

*Identifier numbers can be retrieved from [https://clinicaltrials.gov/](https://clinicaltrials.gov/).

†Samples from KEYNOTE-061 were not analyzed by FoundationOneCDx.

HNSCC, head and neck squamous cell carcinoma; NSCLC, non–small cell lung cancer; TMB, tumor mutational burden; TNBC, triple-negative breast cancer; WES, whole exome sequencing.
**Table 2: Correlation of TSO500 TMB with FoundationOneCDx TMB and WES TMB**

Pearson correlation coefficient \( r \) and Spearman's rank correlation coefficient \( \rho \) and the associated 95% confidence interval (CI) were calculated for the correlation of TSO500 TMB with FoundationOneCDx TMB and WES TMB, respectively. Correlation analysis was performed separately for the whole data set, for the TMB-H subset, and for the non-TMB-H subset (TMB-H and non-TMB-H were determined by the reference method’s cut-point).

<table>
<thead>
<tr>
<th>Reference data set</th>
<th>( r^* [95% \text{ CI}] )</th>
<th>( \rho [95% \text{ CI}] )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole data set</td>
<td>TMB-H subset</td>
</tr>
<tr>
<td>FoundationOneCDx TMB(^\d)</td>
<td>0.98 [0.98, 0.99]</td>
<td>0.99 [0.97, 0.99]</td>
</tr>
<tr>
<td>(N(^\d)=269)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WES TMB(^\d)</td>
<td>0.96 [0.96, 0.97]</td>
<td>0.97 [0.95, 0.98]</td>
</tr>
<tr>
<td>(N=293)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^\d\)Pearson correlation coefficients were calculated in the linear scale.

\(^\d\)TMB-H subset, samples with FoundationOneCDx TMB \( \geq 10 \) mut/Mb; non–TMB-H subset, samples with TMB <10 mut/Mb.

\(^\d\)N: the number of patients whose samples had evaluable TMB scores by both TSO500 and the respective reference method.

\(^\d\)TMB-H subset, samples with WES TMB \( \geq 175 \) mut/exome; non–TMB-H subset, samples with TMB <175/exome.
Table 3: Clinical utility metrics for the selected TSO500 TMB cut-point compared to reference methods

Comparison of clinical utility metrics and associated 95% confidence intervals between TSO500 and FoundationOneCDx at respective TMB cut-points for 269 patient samples with evaluable TMB data from both assays, and the comparison between TSO500 and WES at respective TMB cut-points for 293 patient samples with evaluable TMB data from both assays.

<table>
<thead>
<tr>
<th></th>
<th>TSO500 vs FoundationOneCDx (N*=269)</th>
<th>TSO500 vs WES (N*=293)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSO500†</td>
<td>FoundationOneCDx‡</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.425 [0.275, 0.575]</td>
<td>0.350 [0.200, 0.500]</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.838 [0.789, 0.886]</td>
<td>0.890 [0.851, 0.930]</td>
</tr>
<tr>
<td>PPV¶</td>
<td>0.315 [0.218, 0.419]</td>
<td>0.359 [0.227, 0.500]</td>
</tr>
<tr>
<td>NPVǁ</td>
<td>0.888 [0.867, 0.918]</td>
<td>0.883 [0.864, 0.911]</td>
</tr>
<tr>
<td>Prevalence of TMB-H</td>
<td>20.0% [15.5%, 25.4%]</td>
<td>14.5% [10.5%, 19.3%]</td>
</tr>
</tbody>
</table>

*N: the number of patients whose samples had evaluable TMB scores by both TSO500 and the corresponding reference method.
†TSO500 TMB cut-point of 10 mut/Mb corresponds to the Youden index of 10.17 rounded to the nearest whole number.

‡FoundationOneCDx TMB cut-point of 10 mut/Mb is the roundup of 10.09 mut/Mb to the nearest whole number.

§WES TMB cut-point of 175 mut/exome.

¶ORR for participants with TMB-H = PPV × 100.

ǁORR for participants with non-TMB-H = (1 – NPV) × 100.

**Enrichment = PPV / (1 – NPV).

mut/Mb, mutations per mega base pairs; ORR, objective response rate; NPV, negative predictive value; PPV, positive predictive value; TMB-H, tumor mutational burden–high; WES, whole exome sequencing.