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Technology Explained

Tumour mutational burden: primary versus metastatic tissue creates systematic bias

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ABSTRACT

Tumour mutational burden (TMB) has emerged as a reproducible biomarker to predict immunotherapy response across multiple cancer types. However, a key aspect of TMB measurement that is often overlooked is the source of tissue sample used, which creates a potential for systematic bias. The predominant source is either primary or metastatic tumour tissue. Primary tumours are more heterogeneous and reflect a longer period of tumour evolution, whereas metastases tend to have a more monoclonal structure and potentially different TMB scores. Studies to date measuring TMB have used a heterogeneous set of primary and metastatic tissues, which may explain some of the variability in predictive TMB values across studies. This paper presents data to show that there is a systematic difference whereby metastatic TMB is biased towards higher values than primary TMB (36% higher, paired Wilcoxon, $P = 0.0008$). However, effectiveness in predicting overall survival during immune checkpoint inhibitor therapy was found to be equivalent between primary and metastatic TMB. We highlight that lower TMB in primary tissue may be important in cases with borderline primary TMB, where assaying metastatic TMB may lead to a different treatment stratification result. As TMB progresses towards clinical implementation, particularly in classically non-immunogenic tumour types, it is important to have better curated trials with either the source of tissue annotated or a prospective study assessing concordance between paired primary and metastatic tissue.

Introduction

Tumour mutational burden (TMB) was first reported as a biomarker for predicting response to immune checkpoint inhibitor (CPI) therapy by Snyder et al. [1] and Van Allen et al. [2] in patients with melanoma treated with CTLA-4 blockade. This was quickly followed by Rizvi et al., in 2015 [3] showing the same association with anti-PD-1 treatment in a cohort of patients with non-small cell lung cancer (NSCLC). The findings were expanded to mismatch repair-deficient cancers independent of histology [4]. In the last 3 years, evidence has accumulated to support TMB as a predictive biomarker in additional histologies such as urothelial carcinoma [5–7], head and neck cancer [8], and in a pan-cancer study of 22 solid tumour types [9]. One of the largest and most recent studies of TMB comprised 1662 patients treated with CPI therapy across 10 cancer types, with TMB assessed using the MSK-IMPACT targeted gene panel assay, approved by the US Food and Drug Administration [10]. Although these studies have used different sequencing assays for the estimation of TMB, they have all shown a consistent association between higher TMB and favourable response to CPI therapy, suggesting a potential utility for TMB as a clinical biomarker to guide patient stratification for immunotherapy.

An important aspect which has been largely overlooked to date is the source of tissue sampled for TMB measurement. TMB can be measured from primary or metastatic tumour samples, each of which may cause systematic bias in TMB values. For example, the clonal structure of the tumour has been shown to vary considerably between primary and metastatic sites, with higher rates of monoclonal structure recorded in metastases due to clonal selection, leading to a reduction in overall genetic diversity ('bottlenecking'). A recent study in renal cell carcinoma, for example, showed that only 32% of all mutations were clonal (i.e. in every cancer cell) in primary tissue, compared with 87% in metastases [11]. Therefore, it is possible that TMB values vary significantly between primary and metastatic tumours within the same patient. An important question is whether there is a systematic pattern, whereby TMB

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measured in each sample type is consistently higher or lower. For example, if metastatic TMB is always higher than primary TMB, this may support differential thresholds for stratification for immunotherapy. This has been poorly studied to date, and very little definitive evidence is available.

Stein et al. [12] compared TMB in unmatched primary and metastatic NSCLC and found that the metastatic samples were significantly more likely to surpass the commonly used TMB threshold of 10 mutations/Mb than primary samples [38% metastases versus 25% primary tissues in lung adenocarcinomas (LUAD) and 41% metastases versus 35% primary tissues in lung squamous cell carcinomas (LUSC)]. In addition, they identified site-specific differences in TMB, with brain and adrenal metastases showing the highest TMB, and bone (LUAD) and liver (LUSC) metastases showing the lowest TMB across all metastatic sites [12]. This study is limited however by unmatched samples, and hence it is important to understand the patterns of TMB change between primary and metastatic sites within the same individuals.

Here we provide an overview of current practices in tissue sourcing for TMB measurement, and an analysis was conducted to assess for systematic biases in the results between primary and metastatic TMB. In addition, this study determined whether the site-specific biases in metastatic TMB reported by Stein et al. [12] can be observed across a wider range of histologies. Lastly, the power of primary and metastatic TMB in predicting overall survival in an immunotherapy-treated cohort was compared. For these analyses, matched primary and metastatic TMB data for 121 patients were used, as well as additional clinical and MSK-IMPACT targeted gene panel data from 1307 samples.

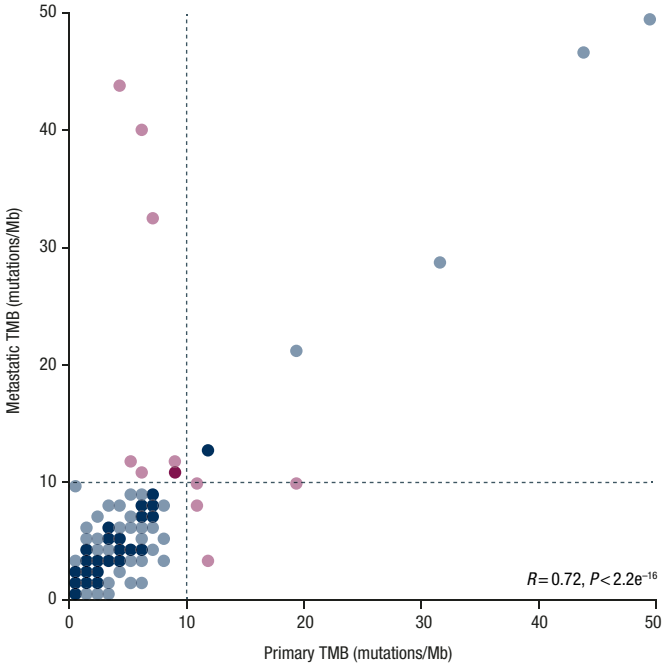

Figure 1. Correlation of tumour mutational burden (TMB) in matched primary and metastatic samples. Correlation of MSK-IMPACT410 panel TMB values (mutations/Mb) from matched primary and metastatic tissue ($n = 121$ patients). Sample pairs with concordant classification as low TMB/high TMB in both tissues are highlighted in blue ($n = 109$ with $n_{high} = 6$, $n_{low} = 103$) and inconsistently classified pairs are highlighted in red ($n = 12$).

Methods

Evaluation of matched primary versus metastatic TMB

Targeted panel sequencing data (MSK-IMPACT assay) from 10 000 patients with advanced-stage cancer published by Zehir et al. [14] were retrieved from cBioportal (https://www.cbioportal.org/study/summary?id=msk_impact_2017; access date 31/10/2019). The dataset was reduced to 121 pairs of matched primary and metastatic paraffin-embedded (FFPE) samples, analysed on the 410 gene panel MSK IMPACT410 (1055.1 kbp) with information on tumour purity and sequencing coverage available. TMB values (mutations/Mb) were calculated by dividing total mutation counts by the panel size. Spearman's rank correlation was used to determine the correlation between matched TMB values. To address potential biases through variability in tumour purity/sequencing coverage, Spearman's rank correlation between TMB and purity/sequencing coverage was assessed. Using a threshold of 10 mutations/Mb, samples were classified as low TMB or high TMB, and the overall misclassification rate was calculated. Matched TMB values were compared with a paired Wilcoxon test on raw TMB values. To enable illustration of the paired TMB data on a $\log_{10}$ scale, zero values were converted to 0.9.

TMB by site of metastasis

To assess site-specific differences in TMB across metastatic sites, targeted panel sequencing data (MSK-IMPACT assay) from 10 000

Table 1
Overview of tissue samples used in tumour mutational burden (TMB) studies

Reference	Cancer type	Treatment	Tissue for TMB measurement	Timepoint of tissue collection relative to treatment(s)
Snyder et al., 2014 [1]	Melanoma	Ipilimumab/tremelimumab	Not specified	Pre- and post-treatment (Il-2, cytotoxic chemotherapy)
Rizvi et al., 2015 [3]	NSCLC	Pembrolizumab	Primaries and metastases	Pre- and post-treatment (pembrolizumab[a])
Le et al., 2015 [4]	Multiple tumour types	Pembrolizumab	Primary	Pre-treatment
Rosenberg et al., 2016 [5]	Urothelial carcinoma	Atezolizumab	Primaries and metastases	Not specified
Balar et al., 2017 [6]	Urothelial carcinoma	Atezolizumab	Primaries and metastases	Pre-treatment
Carbone et al., 2017 [18]	NSCLC	Nivolumab	Not specified	Pre-treatment
Riaz et al., 2017 [16]	Melanoma	Nivolumab ± ipilimumab	Not specified	Pre- and post-treatment (ipilimumab)
Cristescu et al., 2018 [9]	Multiple tumour types	Pembrolizumab	Not specified	Pre-treatment
Hellmann et al., 2018 [15]	SCLC	Nivolumab ± ipilimumab	Primaries and metastases	Not specified
Hellmann et al., 2018 [17]	NSCLC	Nivolumab + ipilimumab	Not specified	Not specified
Hellmann et al., 2018 [19]	NSCLC	Nivolumab + ipilimumab	Not specified	Pre- and post-treatment (nivolumab + ipilimumab[a])
Powles et al., 2018 [7]	Urothelial carcinoma	Atezolizumab	Primaries and metastases	Not specified
Seiwert et al., 2018 [8]	HNSCC	Pembrolizumab	Not specified	Not specified
Samstein et al., 2019 [10]	Multiple tumour types	Diverse CPI	Primaries and metastases	Pre- and post-treatment (CPI; not further specified)

HNSCC, head and neck cancer; Il-2, interleukin-2; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; CPI, checkpoint inhibitor.
[a] Post-treatment tissue was used for one non-responder.

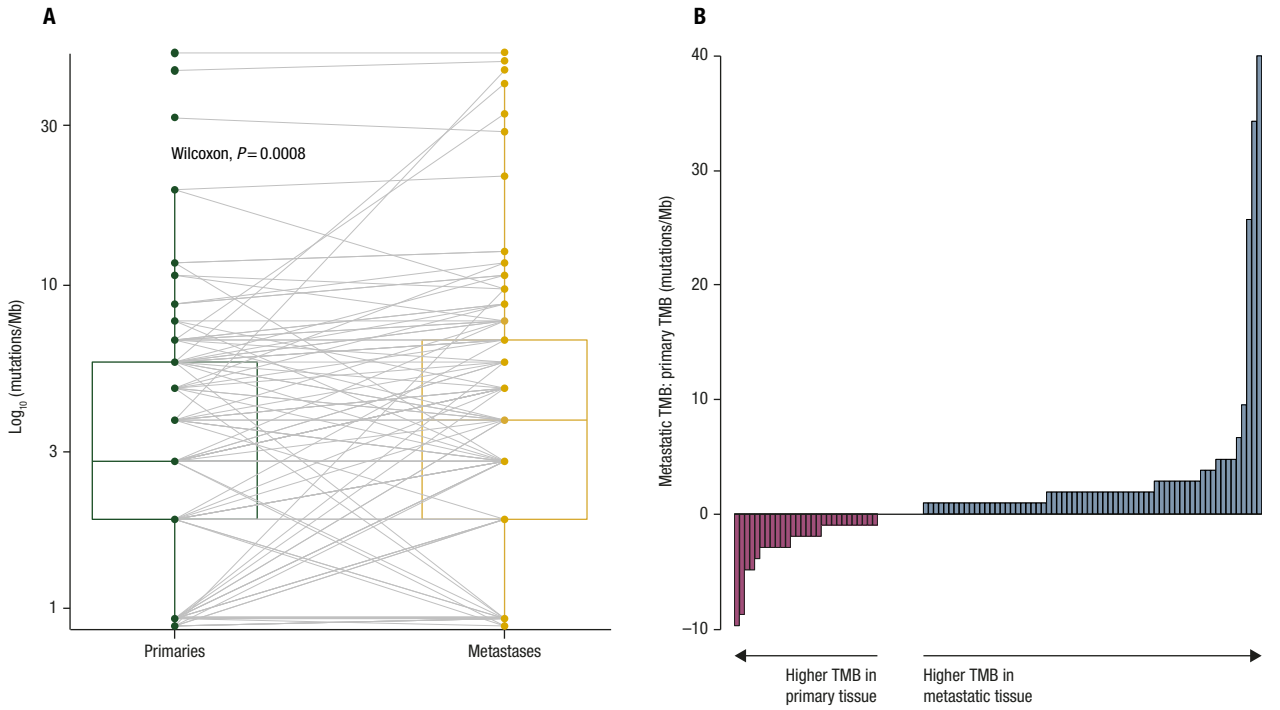

Figure 2. (A) Tumour mutational burden (TMB) scores in matched primary and metastatic tissues. MSK-IMPACT410 panel TMB scores of matched primary (median TMB = 2.8) and metastatic tissues (median TMB = 3.8) from 121 patients. (B) Difference in TMB in matched primary and metastatic tissues. Difference between MSK-IMPACT410 panel TMB values from matched primary and metastatic tissues (n = 121 patients).

patients with advanced-stage cancer published by Zehir et al. [14] were retrieved from cBioportal (https://www.cbioportal.org/study/summary?id=msk_impact_2017; access date 31/10/2019). The dataset was reduced to 3688 metastatic samples with at least 50 samples per metastatic site and primary cancer type, and 4733 primary samples from the same cancer types. Distant and regional lymph node metastases were summarized as 'lymph node' metastases. TMB values (mutations/Mb) were calculated by dividing total mutation counts by the panel size. To enable illustration on a $\log_{10}$ scale, zero values were converted to 0.9. Samples were classified as low TMB or high TMB, with a threshold of 10 mutations/Mb, and the proportion of high TMB samples in each metastatic site was calculated.

Primary versus metastatic TMB: impact on overall survival prediction

To compare survival prediction from primary and metastatic TMB, targeted panel sequencing (MSK-IMPACT assay) and survival data from 1662 patients with advanced-stage cancer treated with CPI therapy published by [10] were retrieved from cBioportal (https://www.cbioportal.org/study/summary?id=tmb_mskcc_2018; access date 31/10/2019). The cohort was split into two subcohorts with either TMB from primary or metastatic tissue available. TMB scores were used as provided in the dataset. Patients were classified as low TMB or high TMB based on a threshold of 10 mutations/Mb, and overall survival between TMB groups was compared in a multivariate Cox proportional hazard model (including tumour type, panel type, sequencing coverage and tumour purity). To test for a potential bias in survival between the subcohorts, overall survival was compared in a multivariate Cox proportional hazard model (including tumour type, panel type, sequencing coverage and tumour purity).

Results

To understand whether historic practices to date have relied upon primary or metastatic tissue for TMB testing, an overview of all tissue sources used in published TMB studies was established (Table 1). The majority of studies included a mixture of primary and metastatic samples [1,3,5–7,10,15] or did not specify their source of tissue [8,9,16–19]. Only one study reported the use of primary tumour tissue as their only source for TMB measurement [4]. Furthermore, the timepoint of tissue collection relative to treatment(s) varied within and across studies. In four studies, a subset of patients received CPI treatment prior to tissue collection [3,10,16,19], while another study included samples taken after treatment with interleukin-2 and/or cytotoxic chemotherapy [1]. Only four studies specified that their analysis was restricted to pre-treatment samples [4,6,9,18].

Next, it was evaluated if a systematic difference in TMB scores existed between matched primary and metastatic samples. First, a positive, but not perfect, correlation was found between matched primary and metastatic TMB (Spearman rank correlation, $r = 0.72$, $P < 2.2 \times 10^{-16}$, Figure 1). Further, for 9.9% (12/121) of patients, the classification as low TMB or high TMB was inconsistent between the matched tissues (Figure 1). In terms of systematic patterns, the analysis shows, on a matched basis, that metastatic TMB is significantly higher than primary TMB (36% higher, paired Wilcoxon, $P = 0.0008$; median primary TMB = 2.8, median metastatic TMB = 3.8; Figure 2). To control for potential confounding factors reported in previous literature [20,21], correlation between TMB and sequencing coverage, as well as tumour purity, was tested; no significant correlation was observed between these measures (Figure S1, see online supplementary material). Overviews on tumour types and sites of metastasis of these samples are provided in Tables S1 and S2 (see online supplementary material).

To determine whether the site-specific difference in TMB reported by Stein et al. [12] can be observed independently of cancer-type, targeted panel sequencing data were obtained from 3688 metastatic samples across 18 different cancer types and 15 metastatic sites. In agreement with Stein et al. [12], this study found that brain (39%) and adrenal (37%) metastases were most likely to surpass a TMB threshold of 10 mutations/Mb, whereas bone (13%) and liver (9%) metastases were less frequently classified as high TMB (Figure 3). An overview of the cancer types included in this analysis is provided in Table S3 (see online supplementary material).

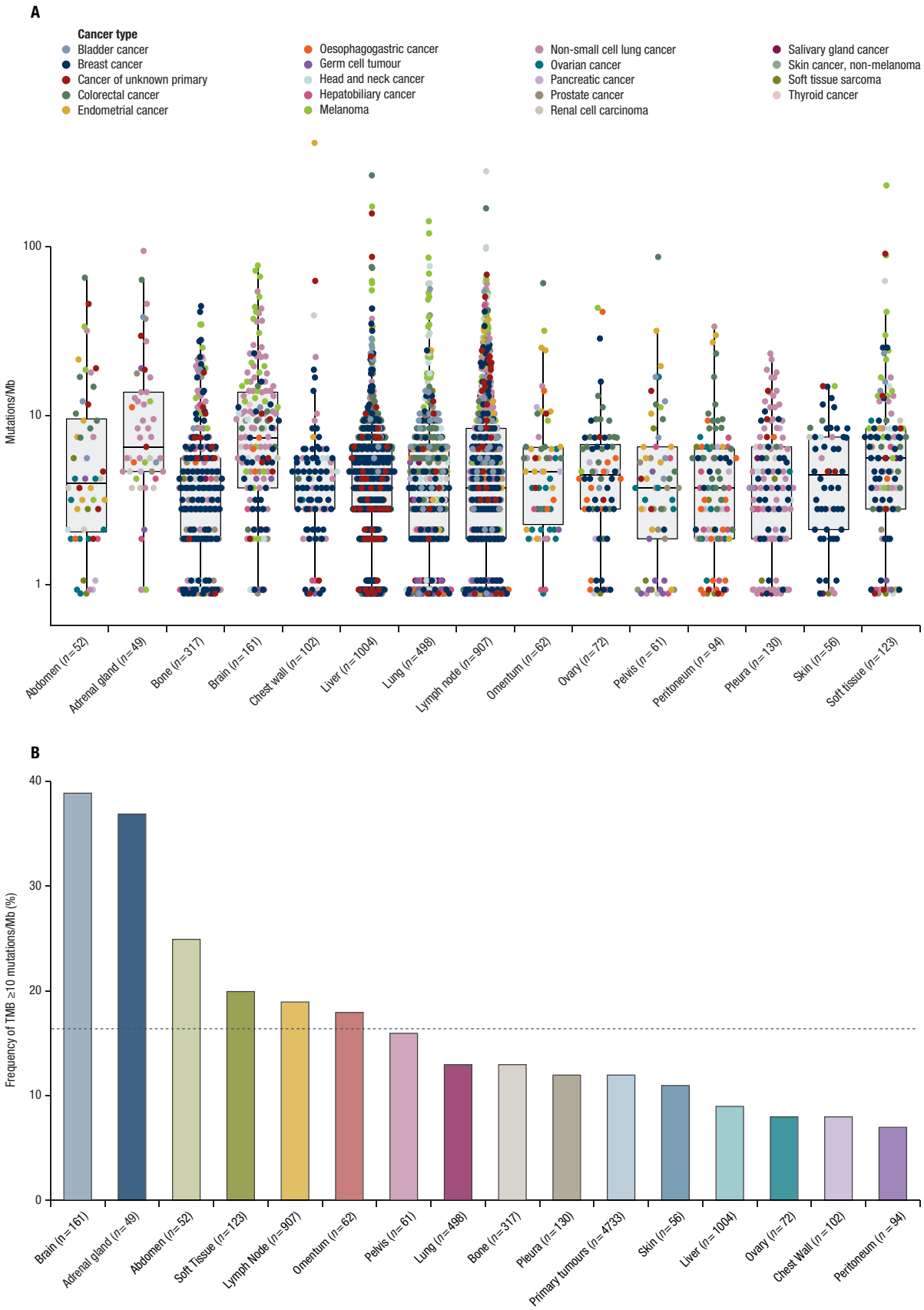

Figure 3. Metastatic tumour mutational burden (TMB) by site of metastasis. (A) Range of TMB scores across 15 metastatic sites. MSK-IMPACT panel TMB scores (mutations/Mb) across 15 metastatic sites with colours indicating the cancer type. (B) TMB classification across 15 metastatic sites and primary tumours. Proportion of samples classified as high TMB across 15 metastatic sites and in comparison with primary tumours. The horizontal line at 14.8% indicates the proportion of samples classified as high TMB across all metastatic samples.

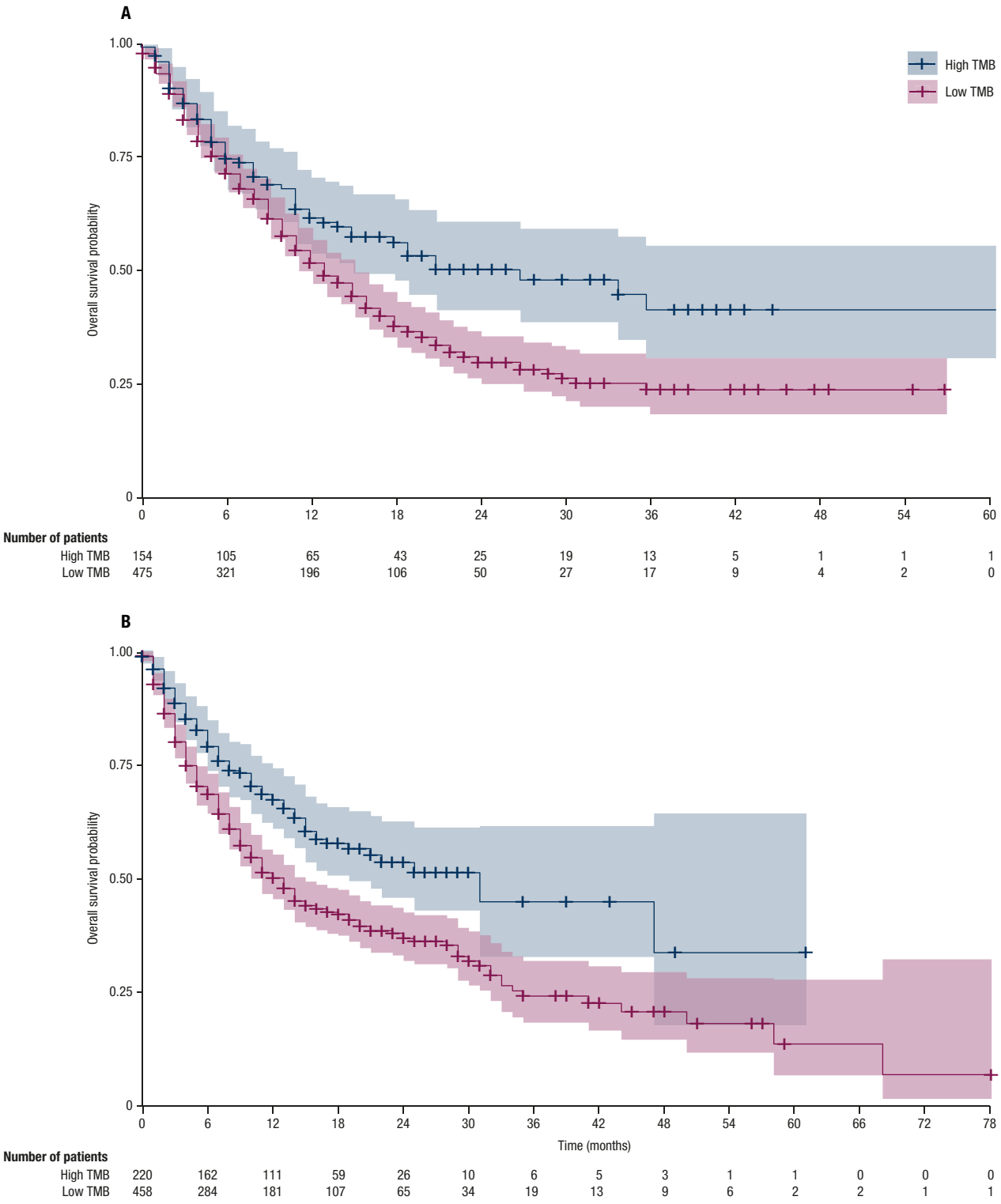

Figure 4. Kaplan–Meier plot of overall survival for high versus low tumour mutational burden (TMB) (10 mutations/Mb threshold). (A) TMB measured from primary tissues ($n = 629$). Hazard ratio (HR) 0.61 [95% confidence interval (CI) 0.45–0.82, $P = 1.1 \times 10^{-3}$]. (B) TMB measured from metastatic tissues ($n = 678$). HR 0.59 (95% CI 0.45–0.76, $P = 6.0 \times 10^{-5}$). HR for high TMB group calculated from a multivariate Cox regression including tumour type, panel type, sequencing coverage and tumour purity.

Next, it was established whether TMB from primary or metastatic tissue would better predict overall survival outcome during immune checkpoint inhibitor therapy. In an unmatched dataset including 629 primary and 678 metastatic samples from patients treated with anti-PD-1 or anti-PDL-1 therapy, it was found that TMB from either tissue site could successfully predict overall survival with hazard ratios (HR) of nearly identical values [primary TMB: HR 0.61, 95% confidence interval (CI) 0.45–0.82, $P = 1.1 \times 10^{-3}$; metastatic TMB: HR 0.59, 95% CI 0.45–0.76, $P = 6.0 \times 10^{-5}$; Figure 4. As shown in Figure S2 (see online supplementary material), there is no bias in survival between the two subcohorts (HR 1.04, 95% CI 0.81–1.15, $P = 0.69$ for patients with primary versus metastatic tissue samples), and consistent with the paired analysis,

there is no significant correlation between TMB and tumour purity/sequencing coverage (Figure S3, see online supplementary material). Table S4 (see online supplementary material) provides an overview of the cancer types included in this analysis, and detailed results of the multivariate Cox proportional hazard model (including tumour type, panel type, sequencing coverage and tumour purity) are shown in Table S5 (see online supplementary material).

Discussion

This study has shown that the current evidence supporting TMB as a biomarker for immunotherapy response comes from a heterogeneous mix of sample types, including (un)treated primary tissue as well as metastatic tissue. Given the lack of consistency in tissue sampling practices across previous studies, this study sought to assess whether the choice of tissue source would systematically bias TMB measurements, and whether primary or metastatic TMB could better predict survival outcome.

The present data, together with previous reports, suggest that TMB in metastatic tissues is systematically higher than in primary tissues. Biologically, the reason for elevated TMB in metastases may be due to bottlenecking, whereby the private mutational burden from a small group of metastasizing cells may increase to become clonal in the metastatic site and detectable by sequencing assays, hence elevating the overall TMB. The notion of a small number of cells seeding metastases is supported by recent modelling data, which estimates that 10–150 cells seed each metastasis [22].

Another potential reason for the highly elevated TMB values in some of the metastases might be treatment with alkylating agents (e.g. dacarbazine, temozolomide), platinum-based therapies (e.g. cisplatin, carboplatin) or radiotherapy, which all may increase TMB by inducing novel subclonal mutations [23–26]. The concept behind TMB as a biomarker for immunotherapy response is that a higher burden of mutations increases the probability of generating immunogenic neoantigens which can trigger a T-cell-mediated antitumour response. An important question therefore is whether the subclonal nature of these mutations makes them less likely to stimulate a sustained immune response [25].

In our pan-cancer analysis, we observed further evidence to support metastasis-site specific biases in TMB scores, confirming the results in previous reports [12]. In keeping with the previous study, the present study showed that, across 15 metastatic sites, brain and adrenal metastases were the most likely to be classified as high TMB. This raises the question of whether there is a mechanism by which cells with a higher TMB preferentially drive metastases to the brain, or if the observed phenomenon could represent a passenger effect whereby smaller populations of cells achieve metastasis to the brain, causing their private TMB to become clonally detectable. A caveat of this pan-cancer analysis however is a potential bias through organ tropisms, for example cancer types with typically high-TMB such as lung cancer and melanoma are the most likely to cause brain metastases [32]. Therefore, larger single-histology studies across a range cancer types are needed.

Next, the prediction of survival from primary and metastatic TMB measurements was investigated. Surprisingly, near equivalence in the predictive utility of both assays was found. Thus, in a clinical context, the findings do not currently advocate the preferential use of either primary or metastatic tissue for TMB testing. Instead, the results support equivalence of TMB biomarker effectiveness in the prediction of overall survival during immune CPI therapy. However, the main implication from the work presented here is that primary and metastatic TMB scores are systematically different, with primary TMB assays less likely to return a high TMB score than metastatic assays. In particular, metastatic TMB was found to be 36% higher on a matched basis, and a 9.9% discordance rate was observed in low TMB versus high TMB results between matched primary and metastatic samples. These results warrant particular consideration in borderline cases where TMB in a primary site is moderate but TMB in metastases may be high. Future work should aim to establish whether this should be factored into clinical decision-making. In addition, future research will need to assess how metastatic and primary TMB relates back to intratumour heterogeneity, building on previous work demonstrating that heterogeneity of a tumour affects on immunotherapy response [25,27]. The consideration here is that polyclonal tumours may bias TMB scores. This has been emphasized by previous studies which compared TMB values across multiple regions from the same tumour. In 12.5% (3/24) of pulmonary adenocarcinomas [28], 20% (20/100) of NSCLC and 52% (12/23) of urothelial carcinomas [29], the TMB classification was inconsistent across multiple regions. The current study was not powered for adequate subclonality analysis due to its single biopsy sampling approach and panel dataset alone; therefore, subclonal deconstruction is not feasible. Future studies that are powered with multiregion sampling in patients treated with immunotherapy will be of significant interest to understand if the heterogeneity within the primary tumour has additional prognostic relevance for response. While the degree of intratumour heterogeneity and the clonality of mutations are best determined with multiregion sequencing, this is generally not feasible in clinical practice. Alternative sequencing approaches, such as the use of homogenized residual tumour material, could therefore help to enhance the utility of TMB as a predictor for immunotherapy response [29].

Finally, it is worth noting that in a clinical setting, the source for TMB measurement is usually based on tissue availability. In most cases, FFPE primary tumour or loco-regional disease is available for patients who were treated surgically with curative intent. For patients who present with de-novo metastatic disease, a metastatic biopsy rather than resection material is used, again usually as FFPE. It is appreciated that sourcing an exact tissue type [i.e. primary or metastatic (and which metastasis)] may not be clinically feasible in all settings. In summary, CPIs have provided breakthrough efficacy in the treatment of multiple solid tumour types, with TMB emerging as a reproducible biomarker associated with response. The strength of TMB as a biomarker may be undermined, however, by inconsistent sources of tissue sample, causing underappreciated bias in TMB values. Given the exceptional duration of response experienced in some patients, the effectiveness of immunotherapy in additional tumour types, such as breast cancer and prostate cancer, is now under investigation as an urgent priority. Preliminary data from early-phase trials in these histologies have shown that the response rate can be as low as 5% [30,31]. Hence a biomarker-led trial design is essential for these tumour types, and TMB may play an important role. Thus, further larger studies are required to confirm equivalence of TMB from primary and metastatic tissue, and to determine the mechanistic reasons for systematic differences observed between tissue types and sites.

Ethics information

For this study, publically available data from Zehir et al. [14] and Samstein et al. [10] was used. Ethics information is provided in these publications.

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Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

ST has received speaking fees from Roche and Astra Zeneca. ST has the following patents filed: Indel mutations as a therapeutic target and predictive biomarker PCTGB2018/051892 and PCTGB2018/051893 and Clear Cell Renal Cell Carcinoma Biomarkers P113326GB. K.L. reports speaker fees from Roche Tissue Diagnostics, and patents pending on indel burden as a predictor of checkpoint inhibitor response and targeting of frameshift neoantigens for personalised immunotherapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.iotech.2019.11.003.

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