BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-060342
Article Type:	Protocol
Date Submitted by the Author:	21-Dec-2021
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Keywords:	ONCOLOGY, Dermatological tumours < ONCOLOGY, Gastrointestinal tumours < ONCOLOGY, Respiratory tract tumours < THORACIC MEDICINE

BESPOKE study of ctDNA Guided Immunotherapy (BESPOKE IO): A multicenter, prospective observational study evaluating the utility of ctDNA in guiding immunotherapy in patients with advanced solid tumors

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Word Count:

Abstract: 288/300 Words

Main Text: 3651/4000 Words

ABSTRACT

Introduction: Immunotherapy (IO) has transformed the treatment paradigm for a wide variety of solid tumors. However, assessment of response can be challenging with conventional radiologic imaging (e.g., iRECIST), which do not precisely capture the unique response patterns of tumors treated with IO. Emerging data suggest that circulating tumor DNA (ctDNA) can aid in response assessment in patients with solid tumors receiving IO. The short half-life of ctDNA puts it in a unique position for early treatment response monitoring. The BESPOKE IO study is designed to investigate the clinical utility of serial ctDNA testing to assess treatment response using a tumor-informed, bespoke ctDNA assay (Signatera™) and to determine its impact on clinical decision-making with respect to continuation/discontinuation, or escalation/de-escalation of immunotherapy in patients with advanced solid tumors.

Methods and analysis: The BESPOKE IO is a multicenter, prospective, observational study with a goal to enroll over 1500 patients with solid tumors receiving IO in up to 100 U.S. sites. Patients will be followed for up to 2 years with serial ctDNA analysis, timed with every other treatment cycle. The primary endpoint is to determine the percentage of patients who will have their treatment regimen changed as guided by post-treatment bespoke ctDNA results along with standard response assessment tools. The major secondary endpoints include progression-free survival, overall survival, and overall response rate based on the ctDNA dynamics.

Ethics and dissemination: The BESPOKE IO study was approved by the Institutional Review Board [Natera-20-043-NCP BESPOKE Study of ctDNA Guided Immunotherapy (BESPOKE IO)] on February 22, 2021. Data protection and privacy regulations will be strictly observed in the capturing, forwarding, processing, and storing patients' data. Natera will approve the Publication of any study results in accordance with the site-specific contract.

Trial registration number: NCT04761783

STRENGTHS AND LIMITATIONS OF THIS STUDY

- BESPOKE IO is a large, prospective, multicenter, observational study designed to investigate the clinical utility of the personalized, tumor-informed circulating tumor DNA (ctDNA) assay in assessing early treatment response in patients with advanced solid tumors receiving immunotherapy (IO), where currently used radiologic criteria and other immunotherapy biomarkers have significant limitations.
- This clinical study might potentially inform if the pre-treatment ctDNA level can serve as a predictive biomarker for response to IO and prognosis early into treatment course.
- This study might help identify non-responders to IO early based on the ctDNA dynamics that can inform the treating physicians to discontinue, intensify (e.g., addition of CTLA4-inhibitor or chemotherapy in addition to immunotherapy), or switch treatment, thereby avoiding unnecessary treatment-related toxicities and costs.
- This study might inform if ctDNA can distinguish between pseudoprogression and true tumor progression
- One limitation of this study is that it is a strictly observational study. Therapy is physician directed and not dictated by the trial given the non-interventional nature of the study. Another limitation is the fewer tumor types being considered in this study, which may limit the generalizability of ctDNA-based treatment response monitoring in other tumor types. Overall, this study will help generate the relevant data required to allow for future prospective interventional studies.

INTRODUCTION

Immune-checkpoint inhibitors (ICI) targeting programmed cell death-1 (PD-1)/ its ligand-1 (PD-L1) and cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), have transformed treatment paradigms in patients with advanced cancer.[1] A plethora of clinical trials have demonstrated significant antitumor activity with ICIs, often leading to durable and potentially curable responses in a wide variety of solid tumors. ICIs have shown superior survival outcomes compared to conventional chemotherapy in multiple advanced malignancies including melanoma, lung, and subsets of colorectal with mismatch repair deficient tumors, breast, and bladder cancers and have been integrated into the standard treatment algorithms for these tumor types.[1 2] One of the anti-PD1 antibodies (pembrolizumab) hold 2 of the 4 currently approved tissue-agnostic FDA approvals. In addition to the metastatic setting, these drugs are now making their way into the clinic for a number of adjuvant indications.

As ICIs have gained a prominent place in the routine clinical care, response assessment to ICIs has become of paramount importance. The tumor response patterns to ICIs vary widely and are often markedly different from the response pattern observed with cytotoxic chemotherapy, limiting the usefulness of the conventional radiologic studies. Variations e.g., iRECIST and repeat follow up scans are often recommended.[3] Around 10% of patients with solid tumors on ICI experience pseudoprogression, defined as an enlargement of existing tumors or the appearance of a new lesion followed by tumor regression that can be misinterpreted as true progression, leading to the premature discontinuation of a potentially effective treatment[4 5]. Furthermore, the staggering cost of immunotherapy (~ $10,000/ dose) adds significant financial stress on patients and the health system[6], underscoring the importance of identifying non-responders early to avoid the cost and the toxicity burden. Although biomarkers including PD-L1 expression, microsatellite instability-high/deficient

mismatch repair (MSI-H/dMMR) status, and tumor mutational burden (TMB) have shown clinical utility for selecting patients suitable for immunotherapy, the predictive capability of these biomarkers is limited.[7 8] Recently, the use of PD-1 blockade in combination with other therapies was approved e.g., combination immunotherapy with a CTLA-4 inhibitor, or combination immunotherapy in addition to chemotherapy. However, it is unclear who should get PD-1 blockade alone, and who would potentially benefit from the combination approach. Some investigators have described the potential benefit of using CTLA-4 rescue strategy.[9 10] While the combination approaches bring the promise of better response rates and survival, they also incur added risk of severe adverse events (SAEs), as well as financial toxicity. Taken together, the variable treatment efficacy, toxicities, cost, the lack of predictive biomarkers, and difficulty in interpreting radiologic response patterns, underscore the urgent need for a tool that can identify treatment response and disease progression early.

Accumulating data suggest that circulating tumor DNA (ctDNA), a non-invasive, quantitative, and dynamic biomarker, can monitor treatment response in patients with advanced/metastatic cancer.[11 12-16] The kinetics of ctDNA brings in several advantages for early response assessment.[17] Previous studies in patients with advanced solid tumors have demonstrated that a decrease in the ctDNA-level with treatment reflects a response to immunotherapy.[12 16 18-20] Furthermore, undetectable or low ctDNA levels after treatment have been associated with better clinical outcomes with ICIs across multiple advanced stage cancers. [15 19 21-24] Several recent studies in lung cancer have shown that ctDNA dynamics can predict disease progression and response to immunotherapy, weeks to months ahead of conventional radiological imaging.[16 18-20] Several studies have demonstrated that ctDNA can clearly differentiate pseudoprogression from true progression with high sensitivity and

specificity, potentially assisting in interpreting ambiguous imaging findings[24 25]. Despite this, ctDNA is currently not used in clinical practice. Some of the reasons for this include data available from studies with small patient population[12 14 22 26 27] and/or use of static panels focusing on a limited number of somatic variants,[22 23 28] which restrict their applicability and generalizability of findings. This need prompted the development of the BESPOKE IO observational study. Herein, we present a clinical study protocol of a prospective, longitudinal, multicenter observational study to investigate the clinical utility of a personalized, tumor-informed multiplex PCR (mPCR)-NGS ctDNA assay (Signatera™) for treatment response monitoring in patients with advanced solid tumors receiving immunotherapy. The study will also examine the impact of ctDNA-detection on clinical decision-making regarding continuation/discontinuation, or escalation/de-escalation of immunotherapy.

METHODS

Overall study design

The BESPOKE IO (clinicaltrials.gov NCT04761783) is a prospective, longitudinal, multicenter clinical study that utilizes a personalized mPCR-NGS assay (Signatera™), designed to track somatic single nucleotide variants (SNVs) in patients with advanced cancer receiving ICIs. The study started in March 2021 and is actively recruiting. The study is composed of three cohorts representing three unique advanced cancer types: lung, melanoma, and colorectal cancer (dMMR/MSI-H). each cohort has two arms: a prospective arm in which serial ctDNA testing will be performed while patients receive immunotherapy (prospective Signatera arm) and a historical control arm. The data collected from the prospective arm will be compared with the outcomes in the historical control groups to evaluate the role of molecular response reflected by ctDNA levels in the management of patients with advanced cancers receiving IO.

A. Prospective Signatera arm

A total of 1,539 patients with advanced solid tumors (lung, melanoma, and dMMR/MSI-H colorectal cancer) undergoing treatment with IO will be enrolled in up to 100 study sites in the US, and patients will be followed up for up to 2 years with serial blood collection for ctDNA analysis. A whole blood (20 mL) sample will be collected for the Signatera assay at baseline and at subsequent time points and frequency determined by the health care provider (HCP). The sponsor recommends subsequent blood collection for the Signatera testing every 2 cycles, timed according to the immunotherapy treatment regimen (**Table 1)**. Optional blood sample collections for the ctDNA assay between week 2 and week 4 of therapy initiation and 4-6 weeks after the end of treatment/disease progression will be carried out (**Figure 1**). All enrolled patients will be evaluated for immune-related adverse events (iRAEs). written informed consent will be obtained from all patients. Study inclusion/exclusion criteria are detailed in **Table 2**.

B. Historical control arm

Approximately 513 historical control cases will be enrolled retrospectively, at an approximate ratio of 1 patient to every 3 prospective patients who had previously received treatment with an ICI and had minimum 2 years of follow-up data after initiation of immunotherapy or death. Furthermore, the control patients will have to meet all study inclusion criteria as listed in **Table 2**. Data on patients in the control arm will be abstracted retrospectively from the electronic medical records. No written informed consent will be required for the patients in the control arm since they will have completed treatment and/or deceased at the time of enrollment. no biological samples for the study will be collected from the patients in the control arm.

Immunotherapy treatment

Patients scheduled to receive an ICI in the prospective Signatera arm or those who previously received an ICI in the historical control arm will be eligible.

Table 1: Signatera blood draw frequency based on immunotherapy treatment regimen (Prospective Signatera arm)

Immunotherapy Treatment Regimen	Immunotherapy Treatment Dose	Treatment Frequency (every # weeks)	Signatera Blood Draw Frequency[a,b] (every # weeks)
Atezolizumab (Tecentriq)	840 mg	2	8
Atezolizumab (Tecentriq)	1200 mg	3	6
Atezolizumab (Tecentriq)	1680 mg	4	8
Avelumab (Bavencio)	800 mg	2	8
Cemiplimab (Libtayo)	350 mg	3	6
Durvalumab (Imfinzi)	10 mg/kg	2	8
Durvalumab (Imfinzi)	1500 mg	4	8
Durvalumab (Imfinzi)	1500 mg	3	6
Ipilimumab (Yervoy)	3 mg/kg	3	6
Nivolumab (Opdivo)	240 mg	2	8
Nivolumab (Opdivo)	480 mg	4	8
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	1 mg/kg 3 mg/kg	3 3	6
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	360 mg 1mg/kg	3 6	6
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	3 mg/kg 1 mg/kg	3 3	6
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	3 mg/kg 1 mg/kg	2 6	8
Pembrolizumab (Keytruda)	200 mg	3	6
Pembrolizumab (Keytruda)	400 mg	6	6

[a]Signatera blood draw should coincide with every other treatment cycle.
[b]Additional optional SIGNATERA blood draws are recommended on weeks 2-4 of immunotherapy, and 4-6 weeks after the end of treatment or disease progression

Table 2: Eligibility Criteria

Category	Inclusion Criteria	Exclusion Criteria
Demographics	• Male or female patients 18 years of age or older	Female patients that are pregnant
Clinical presentation	• Patients must have measurable disease according to RECIST criteria and at least one lesion that can be accurately measured in at least one dimension as >10 mm. • Any patient with documented metastatic or locally advanced, unresectable cancer of the types within the following cohorts: Melanoma, Non-small cell lung cancer, Colorectal cancer • ECOG Performance status 0,1, or 2	Patients who have initiated Immunotherapy
Medical History	• Patients must be clinically eligible and plan to initiate therapy with an anti-neoplastic agent that works by immune checkpoint blockade, anti-PD-1, anti-CTLA-4, or anti-PD-L1: Pembrolizumab (Keytruda) Nivolumab (Opdivo) Ipilimumab (Yervoy) Durvalumab (Imfinzi) Cemiplimab (Libtayo) Atezolizumab (Tecentriq) Avelumab (Bavencio) • Patients must be able to follow the study visit schedule and be willing to provide up to 20 mL of peripheral blood samples at the indicated time points	Patients with a history of bone marrow or organ transplant, a medical condition that would place the patient at risk as a result of blood donation, such as bleeding disorder, or a serious medical condition that may adversely affect the ability to participate in the study
Provider-Based Criteria	• Selected by their HCP to receive ctDNA assay according to the current evidence-informed schedule as part of their routine of practice	

Study Objectives/Endpoints

Primary endpoint

The primary study objective is to examine the impact of the bespoke ctDNA assay on tumor assessment after initiation of immunotherapy, *i.e.*, the percentage of patients who have their immunotherapy treatment regimen changed due to post-treatment bespoke ctDNA assay result along with standard clinical assessments and care.

Secondary endpoints

The main secondary endpoints include progression-free survival (PFS) and overall survival (OS) according to change in ctDNA levels from baseline, wherein ctDNA change is defined as: a) 50%

increase or decrease from baseline, b) an analytically significant increase or decrease from baseline, c) ctDNA clearance or no clearance or d) a cut-off as determined in exploratory analysis. Other secondary endpoints include determination of response rate (partial or complete response), response duration, percentage of patients with at least 6 months of durable clinical response, and the impact of Signatera on informing immunotherapy treatment decisions and patient-reported outcomes.

Exploratory endpoints

Exploratory endpoints include evaluating the performance of ctDNA dynamics in detecting pseudoprogression, determining ctDNA cutoffs that predict durable clinical response for 6-12 months in patients who achieve stable disease or partial response, or determining PFS on the subsequent scan. Specifically, the sensitivity, specificity, positive predictive value, negative predictive value, and area under the curve will be analyzed.

Data Collection

Demographic, medical history, disease status, immunotherapy regimen and outcomes, pathological diagnosis including immunotherapy markers, biomarkers, and co-morbidities, and imaging scans will be collected as part of the protocol and recorded (**Tables 3 and 4**). At different time points, questionnaires pertaining to patient-reported outcomes (PROs) and HCP will be completed by patients and HCPs, respectively.

Follow-up data collection

Patients who experience disease progression, complete, or discontinue their immunotherapy treatment will enter the follow-up period of up to 2 years from the date of patient's consent. The following data will be collected:

- Disease status and survival.
- Results of any imaging studies performed since the prior visit (a de-identified copy of the report and images will be provided to the sponsor).
- Immunotherapy treatment discontinuation, change in the treatment regimen, or the initiation of steroids due to side effects.
- Tumor markers (CEA, LDH, CA27-29, CA15-3) laboratory results, if available.
- Description of any procedures performed to treat this cancer, including surgery, additional chemotherapy or immunotherapy, or radiation therapy.
- If additional surgery is performed, results of any pathology testing (a de-identified copy of the report will be provided).

Signatera blood and tissue collection

First blood draw and tissue collection will be done at baseline during the study enrollment period in the prospective Signatera arm. For subsequent time points, up to 20 mL of whole blood will be collected at intervals as determined by the HCP (**Table 3, Figure 1**).

Future research blood collection

Up to 3 optional blood samples for future research may be collected for patients who agree and are enrolled in the Prospective Signatera Arm: at baseline, week 4-8, and at the end of the study (**Figure 1**). Complete instructions for blood collection can be found in the lab manual using research collection kits provided by Natera. Venipuncture will be performed using the standard technique with a collection of up to 20 mL of whole blood. All blood must be de-identified and include a study ID number, and the Signatera case number for each time point must be recorded on the electronic Case Report Form (eCRF).

Table 3: Schedule of Events Prospective Signatera Arm(s):

	Enrollment	Week following Immunotherapy Initiation						
	Baseline Up to 4 weeks prior to Immunotherapy Initiation[a]	**Week** 2-4	**Week** 4-8	**Week** 8-12	**Week** 12-16	**On Treatment Follow-up**	**Post Treatment Follow-up[b]**	**End of study or Early Termination**
Informed Consent	X							
Confirmation of Inclusion/Exclusion Criteria and Enrollment	X							
Optional Future Research Blood Collection (Streck)[c]	X		X					X
	Observational/Data Collection Pieces							
Demographics and Medical History	X							
Height	X							
Weight[d]	X	X	X	X	X	X	X	
Prior and Current Concomitant Medications	X							
Current Cancer Diagnosis Details	X							
Prior and Current Co-morbidities	X							
Laboratory results	X	X	X	X	X	X	X	X
Physician assessment of response (RECIST)[e]		X	X	X	X	X	X	
Radiology	X	X	X	X	X	X	X	
Pathology Results	X	X	X	X	X	X	X	
Immunotherapy treatment regimen[g]	X	X	X	X	X	X	X	
Disease status and Survival		X	X	X	X	X	X	X
Cancer treatment Procedures		X	X	X	X	X	X	X
Adverse Event Reporting	X	X	X	X	X	X	X	X
Patient disposition								X
Patient-Reported								

Outcomes[h]	X		X		X[h]	
HCP Questionnaire[i]	X		X	X		X[i]

[a]Baseline visit may occur the same day as immunotherapy initiation
[b]Patients who experience disease progression and those who complete or discontinue immunotherapy treatment will be followed up to two years from the date of consent. Data will be collected when available in the medical record.
[c]Optional blood collection kit
[d]Collect at baseline. For subsequent treatment visits, the weight will be collected from the patient's medical record, if available
[e] Health Care Provider (HCP) assessment of tumor response based on radiology per RECIST criteria. Performed at an interval determined by HCP
[f]Radiology scans are to be submitted and performed at intervals per standard of care determined by HCP. Reports are collected if available
[g]Collected at every visit and/or if there is a change in treatment or regimen
[h]Patient-Reported outcomes are completed at:
- Baseline
- After second SIGNATERA blood draws (expected week 4-8) and tumor assessment are complete
- Month 12, and every 3 months thereafter until study completion for patients continuing immunotherapy treatment

[i]HCP questionnaires are completed at:
- Baseline
- After second SIGNATERA blood draws (expected week 4-8), imaging and tumor assessment are complete, and all results are discussed with the patient (Tumor assessment 1)
- After the third SIGNATERA blood draw (expected week 8-12), imaging and tumor assessment are complete, and all results are discussed with the patient (Tumor assessment 2)
- Any time there is a change in the treatment regimen, indeterminate image finding, or treatment decision to hold or discontinue treatment due to a suspected side effect of immunotherapy

Table 4: Schedule of Events for Control Arm

	Within two months of cancer diagnosis	**For each clinic visit 1-24 months from time of immunotherapy treatment**
Confirmation of Inclusion/Exclusion Criteria and Enrollment	X	
Demographics and Medical History	X	
Height	X	
Weight	X	X[l]
Prior and Current Concomitant Medications	X	
Current Cancer Diagnosis Details	X	
Prior and Current Co-morbidities	X	
Immunotherapy treatment regimen	X	X
ECOG Performance Status	X	

Cancer treatment procedures		X
Laboratory Results	X	X
Radiology[2]	X	X
Physician assessment of Response (RECIST)		X
Pathology Results	X	X
Patient disposition		X
Disease status	X	X
Side Effects[3]		X

[1]If available in patient's medical record
[2]Radiology scans are to be submitted. Reports are collected if available
[3]Side effects related to immunotherapy treatment

Data Management/Organization

All data will be collected and stored in a secure, Health Insurance Portability and Accountability Act (HIPAA)-compliant database and applicable regulatory requirements appropriate for each clinical site. Before enrollment, signed informed consent will be received from all patients except for the control arm, wherein a consent-waiver will be requested for data collection purposes. Data associated with the samples will be de-identified to maintain patient privacy. Access to the final trial data set will be with Natera; each site will have access to their own site dataset.

Sample Size and Statistical Considerations

The sample size for this study is based on a ±5% margin of error and 95% CI for the percentage of patients with a change in the treatment regimen. The expected percentage of treatment change is unknown and likely to vary by histological indication. Using a normal approximation to the binomial distribution, the worst-case scenario for reducing the width of the CI is when the probability is 0.5. Assuming this value is observed in the study, a minimum of 385 samples are needed to produce a

95% CI ±0.05. Similarly, the minimum number of patients per cohort in each arm (**Table 5**) will be calculated as:

Table 5: Sample size calculations

Assumption	**Prospective Signatera arm**		**Historical control arm**	
Attrition rate	Total number of patients	Minimum number of patients per cohort	Total number of patients	Minimum number of patients per cohort
*25%	1539	513	513	171

*Assumption based on patients lost to follow-up, non-compliance, non-evaluable ctDNA results, etc.

Primary Analysis:

For analysis of the primary endpoint, the point estimate and a 95% Agresti-Coull confidence interval (CI) for the proportion of patients who underwent a change in immunotherapy treatment regimen will be calculated separately for the Lung, Melanoma, and Colorectal cohorts.

General statistical methods:

Dichotomous (e.g., change in postsurgical treatment regimen) and ordinal (e.g., adverse event severity) data will be tabulated by category, expressed as proportions and percentages. The mean, standard deviation, median, maximum, and minimum will be tabulated for continuous data (e.g., age), which may be presented graphically (e.g., box plots). Pairwise comparisons of continuous data will be performed using a *t*-test if the data distribution appears normal; otherwise, a nonparametric rank test will be used. Comparisons of independent binomial data will be performed using Fisher's exact test, and comparisons of dependent binomial data will be performed using McNemar's test. Survival endpoints will be assessed using Kaplan-Meier analysis or Cox Proportional Hazards model; binary endpoints will generally be assessed using logistic regression.

Patient and Public Involvement

The protocol was designed and discussed with the patient advocacy group and academic community (GI oncology). Patients and general public were not involved in the design, conduct, reporting, or dissemination plans of this protocol. Patients will receive ctDNA test results from their provider, according to the current evidence-informed schedule, as part of routine practice.

Ethics and Dissemination

This study will be conducted in accordance with Good Clinical Practice (GCP), International Conference on Harmonization (ICH), the Declaration of Helsinki, and US Food and Drug Administration (FDA) guidelines. Prior to enrollment, written informed consent will be obtained from all patients and compliance with all inclusion and exclusion criteria will be verified and documented. The protocol [Natera-20-043-NCP BESPOKE Study of ctDNA Guided Immunotherapy (BESPOKE IO)] was approved by the Institutional Review Board on February 22, 2021. Publication of any study results in papers, abstracts, posters, or other material presented at scientific meetings or published in professional journals will be approved by Natera in accordance with the site-specific study contract.

DISCUSSION

The BESPOKE IO study is one of the first and large prospective, observational study designed to investigate the utility of ctDNA in guiding treatment response assessment along with standard clinical tools in patients with advanced solid tumors receiving immunotherapy. ctDNA is a highly specific and dynamic blood-based cancer biomarker that provides a real-time snapshot of the tumor burden. It's short half-life of approximately 2 hours puts it in a unique position for assessing early treatment response.[29] Previous studies have demonstrated the ability of ctDNA to detect molecular residual

disease, identify cancer recurrence early, and monitor treatment response across multiple cancers and treatment modalities, including immunotherapy.[11 13 15 21 24 28 30-35]

Using kinetics of ctDNA to predict response to immunotherapy is being described across tumor types and using various assays.[17] Timely identification of non-responders from responders based on the ctDNA status can guide further treatment decisions, wherein non-responders can be switched to alternative treatment and spared of the toxicities associated with IO treatment. Alternatively, it can help inform decisions of escalation to combination immunotherapy e.g., addition of a CTLA-4 inhibitor, or addition of chemotherapy in addition to immunotherapy in malignancies that have these agents approved.[10] Currently there are no dynamic real time biomarkers to help aid in this decision making or early response assessment.

The bespoke tumor-informed (Signatera™) ctDNA assay used in this study tracks tumor-specific somatic, single nucleotide variants (SNVs) in patients' plasma based on the upfront whole-exome sequencing of the patient's tumor tissue and matched normal blood. As described previously,[36] the bespoke ctDNA assay can detect clonal variants with high sensitivity (down to 0.01% tumor fraction) and high specificity (>99.8%), which has been validated across numerous studies.[11 13 15 33 37 38] More importantly, the assay filters out clonal hematopoiesis of indeterminate potential and germline-derived variants from analysis, thereby reducing false-positives.[36]

In this study, ctDNA levels will be evaluated at baseline (immediately before starting treatment) and during treatment with IO, with serial ctDNA analysis planned every 2 cycles during the 2-year long follow-up in all cohorts. Several studies demonstrated that patients with declining ctDNA levels on-treatment had better survival outcomes, suggesting that the decline in the ctDNA level with treatment

reflected a favorable response to IO.[11 12 15 21-23 25 28] In a recent study by Bratman *et al.*, the bespoke ctDNA assay was used in a cohort of 94 patients with 25 different types of solid tumors[11]. In the study, the bespoke assay identified immunotherapy non-responders (e.g., disease progression) with a 98% positive predictive value (PPV). Among patients whose ctDNA levels increased after 6 weeks of treatment, progression-free survival (PFS) at 6 months was only 7.5%, compared to 54.5% in patients whose ctDNA levels decreased at the same time point. In conjunction with increasing tumor volume on a CT scan, bespoke ctDNA assay demonstrated 100% PPV for detecting non-responders. The study also found that complete clearance of ctDNA was associated with exceptionally durable response (100% OS with a median follow-up period of 25.4 months [range, 10.8–29.5]).[11]

By contrast, the OS among patients who did not clear their ctDNA was 42.5% and 17.5% at 12 and 24 months, respectively. These data suggest that ctDNA clearance at any time point during treatment is highly predictive of long-term durable response. This finding is consistent with the results of an independent study in patients with hepatocellular carcinoma (n=48) undergoing treatment with atezolizumab and bevacizumab that used bespoke ctDNA assay and showed longer PFS in patients whose ctDNA level was undetectable with treatment.[39] Not only did ctDNA changes predict the responses, all patients who had their ctDNA cleared were alive till the last date of follow-up. The study by Bratman et al. also demonstrated that 55% of patients experienced molecular progression (ctDNA increase) at 6 weeks, and those patients received on average 2 cycles (6 weeks) of additional immunotherapy guided by radiologic study, which could have been avoided.[11] Thus, bespoke ctDNA assay can enable an earlier switch to an alternative treatment that may have a higher chance of success and lower financial and toxicity burden.

The predictive value of ctDNA was illustrated in a posthoc analysis of IMvigor010 trial, a randomized, phase III study comparing adjuvant atezolizumab to observation after radical cystectomy for urothelial cancer.[15] The study showed that ctDNA detection after radical cystectomy in both arms was associated with reduced disease-free survival (DFS) (atezolizumab arm, HR = 3.36, 95% CI: 2.44–4.62; observation arm, HR= 6.3, 95% CI: 4.45–8.92; P < 0.0001) as well as reduced OS (atezolizumab arm, HR= 3.63, 95% CI: 2.34–5.64; observation arm, HR = 8.0, 95% CI: 4.92–12.99), compared to patients with undetectable postoperative ctDNA. In addition, ctDNA-positive patients in the adjuvant atezolizumab arm had an improved OS (HR =0.59, 95%CI: 0.41–0.86; median DFS 25.8 *vs.* 15.8 months in the observation arm), while ctDNA-negative patients showed no difference in survival if they received adjuvant atezolizumab. Furthermore, patients who cleared ctDNA with adjuvant atezolizumab had dramatically better survival outcomes compared to those who did not clear ctDNA (DFS, HR = 0.26, 95% CI: 0.12−0.56; P = 0.0014; median DFS: 5.7 months versus not reached; and OS, HR = 0.41, 95% CI: 0.1–1.70.[15] Overall, this study demonstrated that post-operative ctDNA could predict benefit from adjuvant immunotherapy in resected urothelial cancer patients. Furthermore, patients can be stratified based on the presence/absence of ctDNA after resection, and the ctDNA-negative patients may be spared of adjuvant immunotherapy.[15]

Pseudoprogression poses a unique challenge in solid tumor patients receiving immunotherapy as validated methods that differentiate between true progression and pseudoprogression are lacking. Limited studies have shown the potential of ctDNA in distinguishing pseudoprogression from true progression.[11 24 26] In the study reported by Bratman *et al.*, 7 patients showed pseudoprogression (tumor progression on scans but decreasing ctDNA level at 6 weeks). Of these, 4 patients exhibited a better OS >18 months (range, 19-27) when compared with patients who showed true progression (n=30, increasing ctDNA and progressive disease on scan).[11] Further, the bespoke ctDNA assay was

able to detect pseudoprogression 5 months earlier than the imaging studies.[11] In the present study, as one of the exploratory endpoints, we plan to evaluate the association of ctDNA dynamics with pseudoprogression. ctDNA clearance or decline in such patients could help differentiate and direct patients with true progression to alternative treatment.

Taken together, the studies described above provide preliminary evidence that ctDNA can help in immunotherapy response monitoring. however, most of these studies included a small patient population. [14 18 20 22 23 26] Additionally, several of these studies have utilized targeted panels to select the variants and tracked the variants with droplet digital polymerase chain reaction (ddPCR). However, the use of a targeted gene panel can result in suboptimal variant selection and decreased ctDNA sensitivity (43% - 73%).[12 23 25 40] By contrast, the bespoke ctDNA assay selects clonal variants from a whole-exome analysis of the tumor (approximately 20,000 genes), minimizing suboptimal variant selection potential.

The predictive role of ctDNA is currently being studied in several ongoing clinical trials investigating the role of immunotherapy across multiple cancer types (NCT03512847, NCT04636047, NCT04053725, NCT03712566, NCT04589845, NCT04853017, NCT03409848, NCT03178552). Although Most of these trials are designed to include small to moderate sample sizes and employ variable assay designs, these trials would be instrumental in establishing ctDNA's role as a surrogate endpoint for immunotherapy treatment efficacy.

The limitation of our study is that it is purely observational. Therapy is physician directed and not dictated by the trial given the non-interventional nature of the study. However, the prospective design

of the study, a large sample size, and the 2-year long follow-up period will allow us to compare the sensitivity, specificity, PPV, NPV, and clinical utility within as well as among different study cohorts. Of note, our study design includes a retrospectively enrolled control group for adequate comparisons, which will further help in determining the clinical utility of the personalized, tumor-informed ctDNA assay in guiding treatment monitoring in patients receiving immunotherapy. Another limitation is the fewer tumor types being considered in this clinical study, which may limit the generalizability of ctDNA-based treatment response monitoring in patients with other tumor types getting IO therapy.

We believe, this study will also help generate the relevant data required to allow for future prospective interventional studies. We expect that our study will help establish the real-world evidence of ctDNA's utility in monitoring treatment response to immunotherapy in patients with solid tumors and support its integration into clinical practice and guidelines, leading to meaningful improvements in patient outcomes and quality of life.

AUTHOR'S CONTRIBUTIONS

- Study coordinator: SS
- Site Identification: MK, SS
- Design and writing of the protocol: AA, SS, MK, PMK, JE, AR, SE
- Data collection: SS, SE, MK
- Data analysis: JE
- Data interpretation: JE, MK, AA, AR, SS
- Writing of the manuscript: MM, MM, PMK, SC
- Statistical setting of the study design and data analysis: JE

- All authors reviewed and approved the final manuscript: PMK, SC, SS, MK, AP, GA, MM[#], MM*, JE, LG, ZE, SE, PRB, AR, AA.

[#]Minu Maninder; *Meenakshi Malhotra

FUNDING STATEMENT

This study was supported by Natera, Inc. This study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

COMPETING INTERESTS

PMK acknowledges role as a consultant/advisor for Taiho Oncology, Ipsen, Natera, Foundation Medicine, Research/Trial Support (to institution): BMS, Celgene, AstraZeneca, BTG, Advanced Accelerator Applications, Array Biopharma.

SC acknowledges membership of the Natera's speakers' bureau

AP: acknowledges membership of the Bristol Myers Squibb speakers' bureau, role as a consultant for Novartis, participatory role in a Natera Ad Board, and role as a speaker for Natera and Grail.

GA: Nothing to disclose

LG is a federal employee and reports no conflict of interest.

ZE: Research Support: Pfizer, Novartis; Consultancy: Pfizer, Eisai, Natera Inc., OncoSec, Genentech.

All other authors are employees of Natera, Inc. with stock/options to own stock on the company.

This study is being sponsored by Natera, Inc.

ACKNOWLEDGMENTS

Authors would like to acknowledge the clinical project management and data management support provided by Worldwide Clinical Trials.

References:

1. Borcoman E, Kanjanapan Y, Champiat S, et al. Novel patterns of response under immunotherapy. *Ann Oncol* 2019;30(3):385-96. doi: 10.1093/annonc/mdz003 [published Online First: 2019/01/19]

2. Akinleye A, Rasool Z. Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. *J Hematol Oncol* 2019;12(1):92. doi: 10.1186/s13045-019-0779-5 [published Online First: 2019/09/07]

3. Seymour L, Bogaerts J, Perrone A, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *The Lancet Oncology* 2017;18(3):e143-e52. doi: 10.1016/S1470-2045(17)30074-8 [published Online First: 2017/03/02]

4. Borcoman E, Nandikolla A, Long G, et al. Patterns of Response and Progression to Immunotherapy. *Am Soc Clin Oncol Educ Book* 2018;38:169-78. doi: 10.1200/edbk_200643 [published Online First: 2018/09/21]

5. Thomas R, Somarouthu B, Alessandrino F, et al. Atypical Response Patterns in Patients Treated With Nivolumab. *AJR Am J Roentgenol* 2019:1-5. doi: 10.2214/AJR.18.20938 [published Online First: 2019/03/28]

6. Monthly and Median Costs of Cancer Drugs at the Time of FDA Approval 1965-2016. *J Natl Cancer Inst* 2017;109(8) doi: 10.1093/jnci/djx173 [published Online First: 2017/11/09]

7. Gjoerup O, Brown CA, Ross JS, et al. Identification and Utilization of Biomarkers to Predict Response to Immune Checkpoint Inhibitors. *Aaps j* 2020;22(6):132. doi: 10.1208/s12248-020-00514-4 [published Online First: 2020/10/16]

8. Wang Y, Tong Z, Zhang W, et al. FDA-Approved and Emerging Next Generation Predictive Biomarkers for Immune Checkpoint Inhibitors in Cancer Patients. *Front Oncol* 2021;11:683419. doi: 10.3389/fonc.2021.683419 [published Online First: 2021/06/25]

9. Kasi P, Chan C. 23 Circulating tumor DNA (ctDNA) serial analysis during progression on PD-1 blockade and later CTLA4 rescue in patients with mismatch repair deficient metastatic colorectal cancer. *Journal for immunotherapy of cancer* 2020;8(Suppl 3):A12-A13. doi: 10.1136/jitc-2020-SITC2020.0023

10. Kooshkaki O, Derakhshani A, Hosseinkhani N, et al. Combination of Ipilimumab and Nivolumab in Cancers: From Clinical Practice to Ongoing Clinical Trials. *International journal of molecular sciences* 2020;21(12):4427. doi: 10.3390/ijms21124427

11. Bratman SV, Yang SYC, Iafolla MAJ, et al. Personalized circulating tumor DNA analysis as a predictive biomarker in solid tumor patients treated with pembrolizumab. *Nature Cancer* 2020;1(9):873-81. doi: 10.1038/s43018-020-0096-5

12. Cabel L, Riva F, Servois V, et al. Circulating tumor DNA changes for early monitoring of anti-PD1 immunotherapy: a proof-of-concept study. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2017;28(8):1996-2001. doi: 10.1093/annonc/mdx212 [published Online First: 2017/05/02]

13. Christensen E, Birkenkamp-Demtröder K, Sethi H, et al. Early Detection of Metastatic Relapse and Monitoring of Therapeutic Efficacy by Ultra-Deep Sequencing of Plasma Cell-Free DNA in Patients With Urothelial Bladder Carcinoma. *J Clin Oncol* 2019;37(18):1547-57. doi: 10.1200/jco.18.02052 [published Online First: 2019/05/07]

14. Giroux Leprieur E, Herbretau G, Dumenil C, et al. Circulating tumor DNA evaluated by Next-Generation Sequencing is predictive of tumor response and prolonged clinical

benefit with nivolumab in advanced non-small cell lung cancer. *Oncoimmunology* 2018;7(5):e1424675. doi: 10.1080/2162402x.2018.1424675 [published Online First: 2018/05/04]

15. Powles T, Assaf ZJ, Davarpanah N, et al. ctDNA guiding adjuvant immunotherapy in urothelial carcinoma. *Nature* 2021;595(7867):432-37. doi: 10.1038/s41586-021-03642-9 [published Online First: 2021/06/18]
16. Ricciuti B, Jones G, Severgnini M, et al. Early plasma circulating tumor DNA (ctDNA) changes predict response to first-line pembrolizumab-based therapy in non-small cell lung cancer (NSCLC). *J Immunother Cancer* 2021;9(3) doi: 10.1136/jitc-2020-001504 [published Online First: 2021/03/28]
17. PM K. Kinetics of Liquid Biopsies in Predicting Response to Immunotherapy: ASCO Daily News; Oct 1, 2020 [Available from: https://dailynews.ascopubs.org/do/10.1200/ADN.20.200338/full/ accessed December 14, 2021.
18. Anagnostou V, Forde PM, White JR, et al. Dynamics of Tumor and Immune Responses during Immune Checkpoint Blockade in Non-Small Cell Lung Cancer. *Cancer Res* 2019;79(6):1214-25. doi: 10.1158/0008-5472.Can-18-1127 [published Online First: 2018/12/14]
19. Chaudhuri AA, Chabon JJ, Lovejoy AF, et al. Early Detection of Molecular Residual Disease in Localized Lung Cancer by Circulating Tumor DNA Profiling. *Cancer Discov* 2017;7(12):1394-403. doi: 10.1158/2159-8290.Cd-17-0716 [published Online First: 2017/09/14]

20. Goldberg SB, Narayan A, Kole AJ, et al. Early Assessment of Lung Cancer Immunotherapy Response via Circulating Tumor DNA. *Clin Cancer Res* 2018;24(8):1872-80. doi: 10.1158/1078-0432.Ccr-17-1341 [published Online First: 2018/01/14]

21. Cabel L, Proudhon C, Romano E, et al. Clinical potential of circulating tumour DNA in patients receiving anticancer immunotherapy. *Nature reviews Clinical oncology* 2018;15(10):639-50. doi: 10.1038/s41571-018-0074-3 [published Online First: 2018/07/28]

22. Gray ES, Rizos H, Reid AL, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. *Oncotarget* 2015;6(39):42008-18. doi: 10.18632/oncotarget.5788 [published Online First: 2015/11/03]

23. Herbreteau G, Vallée A, Knol AC, et al. Quantitative monitoring of circulating tumor DNA predicts response of cutaneous metastatic melanoma to anti-PD1 immunotherapy. *Oncotarget* 2018;9(38):25265-76. doi: 10.18632/oncotarget.25404 [published Online First: 2018/06/05]

24. Lee JH, Long GV, Menzies AM, et al. Association Between Circulating Tumor DNA and Pseudoprogression in Patients With Metastatic Melanoma Treated With Anti-Programmed Cell Death 1 Antibodies. *JAMA Oncol* 2018;4(5):717-21. doi: 10.1001/jamaoncol.2017.5332 [published Online First: 2018/02/10]

25. Lee JH, Long GV, Boyd S, et al. Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2017;28(5):1130-36. doi: 10.1093/annonc/mdx026 [published Online First: 2017/03/23]

26. Guibert N, Mazieres J, Delaunay M, et al. Monitoring of KRAS-mutated ctDNA to discriminate pseudo-progression from true progression during anti-PD-1 treatment of lung adenocarcinoma. *Oncotarget* 2017;8(23):38056-60. doi: 10.18632/oncotarget.16935

27. Raja R, Kuziora M, Brohawn PZ, et al. Early Reduction in ctDNA Predicts Survival in Patients with Lung and Bladder Cancer Treated with Durvalumab. *Clin Cancer Res* 2018;24(24):6212-22. doi: 10.1158/1078-0432.Ccr-18-0386 [published Online First: 2018/08/11]

28. Zhang Q, Luo J, Wu S, et al. Prognostic and Predictive Impact of Circulating Tumor DNA in Patients with Advanced Cancers Treated with Immune Checkpoint Blockade. *Cancer Discov* 2020;10(12):1842-53. doi: 10.1158/2159-8290.Cd-20-0047 [published Online First: 2020/08/21]

29. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nature Medicine* 2008;14(9):985-90. doi: 10.1038/nm.1789

30. Jia N, Sun Z, Gao X, et al. Serial Monitoring of Circulating Tumor DNA in Patients With Metastatic Colorectal Cancer to Predict the Therapeutic Response. *Front Genet* 2019;10:470. doi: 10.3389/fgene.2019.00470 [published Online First: 2019/06/06]

31. Parikh AR, Mojtahed A, Schneider JL, et al. Serial ctDNA Monitoring to Predict Response to Systemic Therapy in Metastatic Gastrointestinal Cancers. *Clin Cancer Res* 2020;26(8):1877-85. doi: 10.1158/1078-0432.Ccr-19-3467 [published Online First: 2020/01/17]

32. Pereira E, Camacho-Vanegas O, Anand S, et al. Personalized Circulating Tumor DNA Biomarkers Dynamically Predict Treatment Response and Survival In Gynecologic Cancers. *PLoS One* 2015;10(12):e0145754. doi: 10.1371/journal.pone.0145754

33. Coombes RC, Page K, Salari R, et al. Personalized Detection of Circulating Tumor DNA Antedates Breast Cancer Metastatic Recurrence. *Clinical Cancer Research* 2019;25(14):4255-63. doi: 10.1158/1078-0432.Ccr-18-3663

34. Goodall J, Mateo J, Yuan W, et al. Circulating Cell-Free DNA to Guide Prostate Cancer Treatment with PARP Inhibition. *Cancer Discov* 2017;7(9):1006-17. doi: 10.1158/2159-8290.Cd-17-0261 [published Online First: 2017/04/30]

35. Moding EJ, Liu Y, Nabet BY, et al. Circulating tumor DNA dynamics predict benefit from consolidation immunotherapy in locally advanced non-small-cell lung cancer. *Nature Cancer* 2020;1(2):176-83. doi: 10.1038/s43018-019-0011-0

36. Kasi PM, Sawyer S, Guilford J, et al. BESPOKE study protocol: a multicentre, prospective observational study to evaluate the impact of circulating tumour DNA guided therapy on patients with colorectal cancer. *BMJ Open* 2021;11(9):e047831. doi: 10.1136/bmjopen-2020-047831

37. Magbanua MJM, Swigart LB, Wu HT, et al. Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2021;32(2):229-39. doi: 10.1016/j.annonc.2020.11.007 [published Online First: 2020/11/25]

38. Reinert T, Henriksen TV, Christensen E, et al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. *JAMA Oncology* 2019;5(8):1124-31. doi: 10.1001/jamaoncol.2019.0528

39. Hsu C-H, Lu S, Abbas A, et al. Longitudinal and personalized detection of circulating tumor DNA (ctDNA) for monitoring efficacy of atezolizumab plus bevacizumab in patients

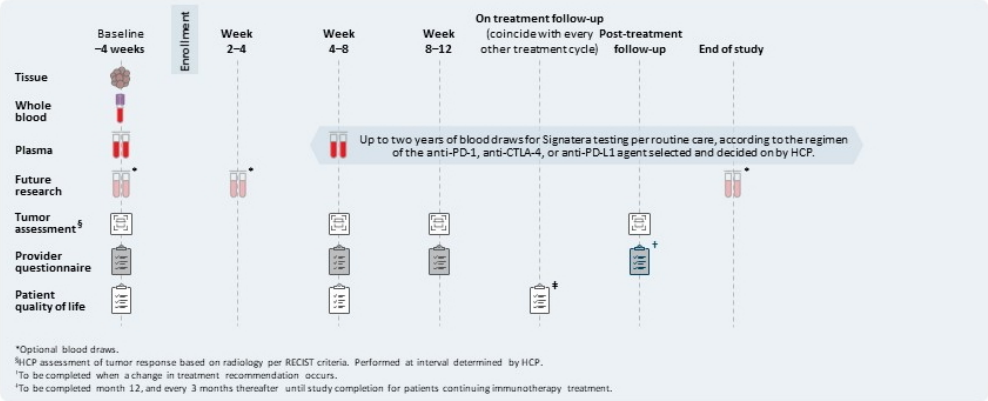
with unresectable hepatocellular carcinoma (HCC). *Journal of Clinical Oncology* 2020;38(15_suppl):3531-31. doi: 10.1200/JCO.2020.38.15_suppl.3531

40. Keller L, Guibert N, Casanova A, et al. Early Circulating Tumour DNA Variations Predict Tumour Response in Melanoma Patients Treated with Immunotherapy. *Acta Derm Venereol* 2019;99(2):206-10. doi: 10.2340/00015555-3080 [published Online First: 2018/11/06]

Figure Legend:

Figure 1: BESPOKE immunotherapy study design overview

Overview of the BESPOKE IO study design. Samples (whole blood, FFPE tissue, plasma) will be collected, and questionnaires (physician assessment, quality of life (QoL), will be completed at the indicated times (weeks/months).



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225x93mm (96 x 96 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Author's Annotation
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	See manuscript page 1, title
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	See manuscript page 2
	2b	All items from the World Health Organization Trial Registration Data Set	N/A; this is a registry study
Protocol version	3	Date and version identifier	Jan 25, 2021 Version 1.0 Clinical study protocol, page 2
Funding	4	Sources and types of financial, material, and other support	See manuscript page 23, funding.
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	See manuscript page 23, authors contributions.
	5b	Name and contact information for the trial sponsor	Trial sponsored by Natera, Inc. Contact

			corresponding author; see manuscript page 1 for corresponding author contact information.
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	See manuscript page 23, authors contributions.
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	See manuscript page 23, authors contributions, where applicable.
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	See abstract, manuscript page 2; see manuscript, background, page 4-6.
	6b	Explanation for choice of comparators	N/A; this is an observational trial
Objectives	7	Specific objectives or hypotheses	See manuscript page 10, study objectives/endpoints.
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	See manuscript page 6-7, overall study design.

Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	See manuscript page 2; design; page 7, study design
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	See manuscript page 9, Table 2.
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	This is an observational study. Immunotherapy treatment regimen is listed in Table 1, page 8
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A; this is an observational study.
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	N/A; this is an observational study.
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A; this is an observational study.
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation	See manuscript page 10, study objectives/endpoints.

		of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	See manuscript page 13, Table 3, schedule of events; manuscript page 7, figure 1.
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	See manuscript page 16, sample size and statistical considerations and Table 5
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	To achieve adequate participant enrollment to reach the target sample size, we are recruiting at a large number of participating sites (up to 100 sites) to reach our enrollment target.
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	N/A; this is an observational study.

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Combination of site ID and sequentially numbered
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Site will generate the number
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	N/A; this is an observational study.
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A; this is an observational study.
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Manuscript page 11-15, including Table 3 and 4.
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	N/A; this is an observational study.

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Manuscript Page 12. Data will be entered using an electronic data capture and will be monitored either remotely or on-site on a bi-annual basis
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Overview of primary and secondary endpoints are on page 10 of the manuscript. Statistical methods are detailed on page 16-17. Detailed statistical analysis is available in the IRB approved Clinical protocol statistical considerations.
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Results from the additional analyses will be summarized. Please see exploratory analysis, page 10.
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	No imputation of missing data will be made. Data will be analyzed as is and patients with missing data will be excluded from final analysis.
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its	N/A; its an observational study.

		charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	No formal interim analysis is planned. However, results from analyses performed may be reported from time to time over the course of the study. Applicable study team members will have access to the data. Termination: N/A, this is an observational study.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	See manuscript, page 11, data collection.
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A.
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	IRB approved protocol. See manuscript page 2; Please also see uploaded WCG IRB approval letter

Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	No amendments have been made till date.
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Manuscript page 7 and Page 15. PI will obtain informed consent from the patient. No assent or authorised legal reps may provide consent
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	See manuscript page 17, ethics and dissemination
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial – **may need a statement**	See manuscript page 15, data management and organization.
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	See manuscript page 23, competing interests/disclosures
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Natera has access to the final trial data set and each site will have access to their own site dataset.
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A. This is an observational study.

Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Publication of any study results in papers, abstracts, posters or other material presented at scientific meetings or published in professional journals must be approved by Natera in accordance with the site-specific study contract. .
	31b	Authorship eligibility guidelines and any intended use of professional writers	Authorship is based on the author contributions as outlined on page 23 of the manuscript
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	We have uploaded a model consent form for your review as part of the submission materials.
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	See manuscript page 15, data management/organization .

BMJ Open

BESPOKE IO Protocol: A multicenter, prospective observational study evaluating the utility of ctDNA in guiding immunotherapy in patients with advanced solid tumors

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-060342.R1
Article Type:	Protocol
Date Submitted by the Author:	26-Apr-2022
Complete List of Authors:	Kasi, Pashtoon ; Weill Cornell Medicine Chakrabarti, Sakti; Medical College of Wisconsin Sawyer, Sarah; Natera Inc Krainock, Michael; Natera Inc Poklepovic, Andrew; VCU Health Annstas, George; Washington University in St Louis Maninder, Minu; Natera Inc Malhotra, Meenakshi; Natera Inc Ensor, Joe; Natera Inc Gao, Ling; VA Long Beach Healthcare System; University of California Irvine Eroglu, Zeynep; Moffitt Cancer Center Ellers, Sascha; Natera Inc Billings, Paul; Natera Inc Rodriguez, Angel; Natera Inc Aleshin, Alexey; Natera Inc
Primary Subject Heading:	Oncology
Secondary Subject Heading:	Diagnostics
Keywords:	ONCOLOGY, Dermatological tumours < ONCOLOGY, Gastrointestinal tumours < ONCOLOGY, Respiratory tract tumours < THORACIC MEDICINE

BESPOKE IO Protocol: A multicenter, prospective observational study evaluating the utility of ctDNA in guiding immunotherapy in patients with advanced solid tumors

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Word Count:

Abstract: 289/300 Words

Main Text: 3715/4000 Words

ABSTRACT

Introduction: Immunotherapy (IO) has transformed the treatment paradigm for a wide variety of solid tumors. However, assessment of response can be challenging with conventional radiologic imaging (e.g., iRECIST), which do not precisely capture the unique response patterns of tumors treated with IO. Emerging data suggest that circulating tumor DNA (ctDNA) can aid in response assessment in patients with solid tumors receiving IO. The short half-life of ctDNA puts it in a unique position for early treatment response monitoring. The BESPOKE IO study is designed to investigate the clinical utility of serial ctDNA testing to assess treatment response using a tumor-informed, bespoke ctDNA assay (Signatera™) and to determine its impact on clinical decision-making with respect to continuation/discontinuation, or escalation/de-escalation of immunotherapy in patients with advanced solid tumors.

Methods and analysis: The BESPOKE IO is a multicenter, prospective, observational study with a goal to enroll over 1500 patients with solid tumors receiving IO in up to 100 U.S. sites. Patients will be followed for up to 2 years with serial ctDNA analysis, timed with every other treatment cycle. The primary endpoint is to determine the percentage of patients who will have their treatment regimen changed as guided by post-treatment bespoke ctDNA results along with standard response assessment tools. The major secondary endpoints include progression-free survival, overall survival, and overall response rate based on the ctDNA dynamics.

Ethics and dissemination: The BESPOKE IO study was approved by the WCG Institutional Review Board [Natera-20-043-NCP BESPOKE Study of ctDNA Guided Immunotherapy (BESPOKE IO)] on February 22, 2021. Data protection and privacy regulations will be strictly observed in the capturing, forwarding, processing, and storing patients' data. Natera will approve the publication of any study results in accordance with the site-specific contract.

Trial registration number: NCT04761783

STRENGTHS AND LIMITATIONS OF THIS STUDY

- BESPOKE IO is a large, prospective, multicenter, observational study designed to investigate the clinical utility of the personalized, tumor-informed circulating tumor DNA (ctDNA) assay in assessing early treatment response in patients with advanced solid tumors receiving immunotherapy (IO)
- This clinical study might potentially inform if the pre-treatment ctDNA level can serve as a predictive biomarker for response to IO and prognosis early into treatment course.
- This study might help with early identification of non-responders to IO based on the ctDNA dynamics that can inform the treating physicians to discontinue, intensify, or switch treatment, thereby avoiding unnecessary treatment-related toxicities and costs.
- This study might inform if ctDNA can distinguish between pseudoprogression and true tumor progression
- Given the non-interventional nature of the study, therapy is physician directed and not dictated by the trial.

INTRODUCTION

Immune-checkpoint inhibitors (ICI) targeting programmed cell death-1 (PD-1)/ its ligand-1 (PD-L1) and cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), have transformed treatment paradigms in patients with advanced cancer.[1] A plethora of clinical trials have demonstrated significant antitumor activity with ICIs, often leading to durable and potentially curable responses in a wide variety of solid tumors. ICIs have shown superior survival outcomes compared to conventional chemotherapy in multiple advanced malignancies including melanoma, lung, and subsets of colorectal with mismatch repair deficient tumors, breast, and bladder cancers and have been integrated into the standard treatment algorithms for these tumor types.[1 2] One of the anti-PD1 antibodies (pembrolizumab) hold 2 of the 4 currently approved tissue-agnostic FDA approvals. In addition to the metastatic setting, these drugs are now making their way into the clinic for a number of adjuvant indications.

As ICIs have gained a prominent place in the routine clinical care, response assessment to ICIs has become of paramount importance. The tumor response patterns to ICIs vary widely and are often markedly different from the response pattern observed with cytotoxic chemotherapy, limiting the usefulness of the conventional radiologic studies. Variations e.g., iRECIST and repeat follow up scans are often recommended.[3] Around 10% of patients with solid tumors on ICI experience pseudoprogression, defined as an enlargement of existing tumors or the appearance of a new lesion followed by tumor regression that can be misinterpreted as true progression, leading to the premature discontinuation of a potentially effective treatment[4 5]. Furthermore, the staggering cost of immunotherapy (~ $10,000/ dose) adds significant financial stress on patients and the health system[6], underscoring the importance of identifying non-responders early to avoid the cost and the toxicity burden. Although biomarkers including PD-L1 expression, microsatellite instability-high/deficient

mismatch repair (MSI-H/dMMR) status, and tumor mutational burden (TMB) have shown clinical utility for selecting patients suitable for immunotherapy, the predictive capability of these biomarkers is limited.[7 8] Recently, the use of PD-1 blockade in combination with other therapies was approved e.g., combination immunotherapy with a CTLA-4 inhibitor, or combination immunotherapy in addition to chemotherapy. However, it is unclear who should get PD-1 blockade alone, and who would potentially benefit from the combination approach. Some investigators have described the potential benefit of using CTLA-4 rescue strategy.[9 10] While the combination approaches bring the promise of better response rates and survival, they also incur added risk of severe adverse events (SAEs), as well as financial toxicity. Taken together, the variable treatment efficacy, toxicities, cost, the lack of predictive biomarkers, and difficulty in interpreting radiologic response patterns, underscore the urgent need for a tool that can identify treatment response and disease progression early.

Accumulating data suggest that circulating tumor DNA (ctDNA), a non-invasive, quantitative, and dynamic biomarker, can monitor treatment response in patients with advanced/metastatic cancer.[11 12-16] The kinetics of ctDNA brings in several advantages for early response assessment.[17] Previous studies in patients with advanced solid tumors have demonstrated that a decrease in the ctDNA-level with treatment reflects a response to immunotherapy.[12 16 18-20] Furthermore, undetectable or low ctDNA levels after treatment have been associated with better clinical outcomes with ICIs across multiple advanced stage cancers. [15 19 21-24] Several recent studies in lung cancer have shown that ctDNA dynamics can predict disease progression and response to immunotherapy, weeks to months ahead of conventional radiological imaging.[16 18-20] Existing evidence in literature supports that ctDNA can clearly differentiate pseudoprogression from true progression with high sensitivity and

specificity, potentially assisting in interpreting ambiguous imaging findings[24 25]. Despite this, ctDNA is currently not used in clinical practice. Some of the reasons for this include, data available from studies with small patient population[12 14 22 26 27] and/or use of static panels focusing on a limited number of somatic variants,[22 23 28] which restrict the applicability and generalizability of their findings. This need prompted the development of the BESPOKE IO observational study. Herein, we present a clinical study protocol of a prospective, longitudinal, multicenter observational study to investigate the clinical utility of a personalized, tumor-informed multiplex PCR (mPCR)-NGS ctDNA assay (Signatera™) for treatment response monitoring in patients with advanced solid tumors receiving immunotherapy. The study will also examine the impact of ctDNA-detection on clinical decision-making regarding continuation/discontinuation, or escalation/de-escalation of immunotherapy.

METHODS

Overall study design

The BESPOKE IO (clinicaltrials.gov NCT04761783) is a prospective, longitudinal, multicenter clinical study that utilizes a personalized mPCR-NGS assay (Signatera™), designed to track somatic single nucleotide variants (SNVs) in patients with advanced cancer receiving ICIs. The study started in March 2021 and is actively recruiting. The study is composed of three cohorts representing three unique advanced cancer types: lung, melanoma, and colorectal cancer (dMMR/MSI-H). each cohort has two arms: a prospective arm in which serial ctDNA testing will be performed while patients receive immunotherapy (prospective Signatera arm) and a historical control arm. The data collected from the prospective arm will be compared with the outcomes in the historical control groups to

evaluate the role of molecular response reflected by ctDNA levels in the management of patients with advanced cancers receiving IO.

A. Prospective Signatera arm

A total of 1,539 patients with advanced solid tumors (lung, melanoma, and dMMR/MSI-H colorectal cancer) undergoing treatment with IO will be enrolled in up to 100 study sites in the US, and patients will be followed up for up to 2 years with serial blood collection for ctDNA analysis. A whole blood (20 mL) sample will be collected for the Signatera assay at baseline and at subsequent time points and frequency determined by the health care provider (HCP). The sponsor recommends subsequent blood collection for the Signatera testing every 2 cycles, timed according to the immunotherapy treatment regimen (**Table 1)**. Optional blood sample collections for the ctDNA assay between week 2 and week 4 of therapy initiation and 4-6 weeks after the end of treatment/disease progression will be carried out (**Figure 1**). All enrolled patients will be evaluated for immune-related adverse events (iRAEs). written informed consent will be obtained from all patients. Study inclusion/exclusion criteria are detailed in **Table 2**.

B. Historical control arm

Approximately 513 historical control cases will be enrolled retrospectively, at an approximate ratio of 1 patient to every 3 prospective patients who had previously received treatment with an ICI and had minimum 2 years of follow-up data after initiation of immunotherapy or death. Furthermore, the control patients will have to meet all study inclusion criteria as listed in **Table 2**. Data on patients in the control arm will be abstracted retrospectively from the electronic medical records. No written informed consent will be required for the patients in the control arm since they will have completed treatment and/or deceased at the time of enrollment. no biological samples for the study will be collected from the patients in the control arm.

Immunotherapy treatment

Patients scheduled to receive an ICI in the prospective Signatera arm or those who previously received an ICI in the historical control arm will be eligible.

Table 1: Signatera blood draw frequency based on immunotherapy treatment regimen (Prospective Signatera arm)

Immunotherapy Treatment Regimen	Immunotherapy Treatment Dose	Treatment Frequency (every # weeks)	Signatera Blood Draw Frequency[a,b] (every # weeks)
Atezolizumab (Tecentriq)	840 mg	2	8
Atezolizumab (Tecentriq)	1200 mg	3	6
Atezolizumab (Tecentriq)	1680 mg	4	8
Avelumab (Bavencio)	800 mg	2	8
Cemiplimab (Libtayo)	350 mg	3	6
Durvalumab (Imfinzi)	10 mg/kg	2	8
Durvalumab (Imfinzi)	1500 mg	4	8
Durvalumab (Imfinzi)	1500 mg	3	6
Ipilimumab (Yervoy)	3 mg/kg	3	6
Nivolumab (Opdivo)	240 mg	2	8
Nivolumab (Opdivo)	480 mg	4	8
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	1 mg/kg 3 mg/kg	3 3	6
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	360 mg 1mg/kg	3 6	6
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	3 mg/kg 1 mg/kg	3 3	6
Nivolumab (Opdivo) and Ipilimumab (Yervoy)	3 mg/kg 1 mg/kg	2 6	8
Pembrolizumab (Keytruda)	200 mg	3	6
Pembrolizumab (Keytruda)	400 mg	6	6

[a]Signatera blood draw should coincide with every other treatment cycle.
[b]Additional optional SIGNATERA blood draws are recommended on weeks 2-4 of immunotherapy, and 4-6 weeks after the end of treatment or disease progression

Table 2: Eligibility Criteria

Category	Inclusion Criteria	Exclusion Criteria
Demographics	• Male or female patients 18 years of age or older	Female patients that are pregnant
Clinical presentation	• Patients must have measurable disease according to RECIST criteria and at least one lesion that can be accurately measured in at least one dimension as >10 mm. • Any patient with documented metastatic or locally advanced, unresectable cancer of the types within the following cohorts: Melanoma, Non-small cell lung cancer, Colorectal cancer • ECOG Performance status 0,1, or 2	Patients who have initiated Immunotherapy
Medical History	• Patients must be clinically eligible and plan to initiate therapy with an anti-neoplastic agent that works by immune checkpoint blockade, anti-PD-1, anti-CTLA-4, or anti-PD-L1: Pembrolizumab (Keytruda) Nivolumab (Opdivo) Ipilimumab (Yervoy) Durvalumab (Imfinzi) Cemiplimab (Libtayo) Atezolizumab (Tecentriq) Avelumab (Bavencio) • Patients must be able to follow the study visit schedule and be willing to provide up to 20 mL of peripheral blood samples at the indicated time points	Patients with a history of bone marrow or organ transplant, a medical condition that would place the patient at risk as a result of blood donation, such as bleeding disorder, or a serious medical condition that may adversely affect the ability to participate in the study
Provider-Based Criteria	• Selected by their HCP to receive ctDNA assay according to the current evidence-informed schedule as part of their routine of practice	

Study Objectives/Endpoints

Primary endpoint

The primary study objective is to examine the impact of the bespoke ctDNA assay on tumor assessment after initiation of immunotherapy, *i.e.*, the percentage of patients who have their immunotherapy treatment regimen changed due to post-treatment bespoke ctDNA assay result along with standard clinical assessments and care.

Secondary endpoints

The main secondary endpoints include progression-free survival (PFS) and overall survival (OS) according to change in ctDNA levels from baseline, wherein ctDNA change is defined as: a) 50% increase or decrease from baseline, b) an analytically significant increase or decrease from baseline, c) ctDNA clearance or no clearance or d) a cut-off as determined in exploratory analysis. Other secondary endpoints include determination of response rate (partial or complete response), response duration, percentage of patients with at least 6 months of durable clinical response, and the impact of Signatera on informing immunotherapy treatment decisions and patient-reported outcomes.

Exploratory endpoints

Exploratory endpoints include evaluating the performance of ctDNA dynamics in detecting pseudoprogression, determining ctDNA cutoffs that predict durable clinical response for 6-12 months in patients who achieve stable disease or partial response, or determining PFS on the subsequent scan. Specifically, the sensitivity, specificity, positive predictive value, negative predictive value, and area under the curve will be analyzed.

Data Collection

Demographic, medical history, disease status, immunotherapy regimen and outcomes, pathological diagnosis including immunotherapy markers, biomarkers, and co-morbidities, and imaging scans will be collected as part of the protocol and recorded (**Tables 3 and 4**). At different time points, questionnaires pertaining to patient-reported outcomes (PROs) and HCP will be completed by patients and HCPs, respectively.

Follow-up data collection

Patients who experience disease progression, complete, or discontinue their immunotherapy treatment will enter the follow-up period of up to 2 years from the date of patient's consent. The following data will be collected:

- Disease status and survival.
- Results of any imaging studies performed since the prior visit (a de-identified copy of the report and images will be provided to the sponsor).
- Immunotherapy treatment discontinuation, change in the treatment regimen, or the initiation of steroids due to side effects.
- Tumor markers (CEA, LDH, CA27-29, CA15-3) laboratory results, if available.
- Description of any procedures performed to treat this cancer, including surgery, additional chemotherapy or immunotherapy, or radiation therapy.
- If additional surgery is performed, results of any pathology testing (a de-identified copy of the report will be provided).

Signatera blood and tissue collection

First blood draw and tissue collection will be done at baseline during the study enrollment period in the prospective Signatera arm. For subsequent time points, up to 20 mL of whole blood will be collected at intervals as determined by the HCP (**Table 3, Figure 1**).

Future research blood collection

Up to 3 optional blood samples for future research may be collected for patients who agree and are enrolled in the Prospective Signatera Arm: at baseline, week 4-8, and at the end of the study (**Figure 1**). Complete instructions for blood collection can be found in the lab manual using research collection kits provided by Natera. Venipuncture will be performed using the standard

technique with a collection of up to 20 mL of whole blood. All blood must be de-identified and include a study ID number, and the Signatera case number for each time point must be recorded on the electronic Case Report Form (eCRF).

Table 3: Schedule of Events Prospective Signatera Arm(s):

	Enrollment	Week following Immunotherapy Initiation						
	Baseline Up to 4 weeks prior to Immunotherapy Initiation[a]	Week 2-4	Week 4-8	Week 8-12	Week 12-16	On Treatment Follow-up	Post Treatment Follow-up[b]	End of study or Early Termination
Informed Consent	X							
Confirmation of Inclusion/Exclusion Criteria and Enrollment	X							
Optional Future Research Blood Collection (Streck)[c]	X		X					X
	Observational/Data Collection Pieces							
Demographics and Medical History	X							
Height	X							
Weight[d]	X	X	X	X	X	X	X	
Prior and Current Concomitant Medications	X							
Current Cancer Diagnosis Details	X							
Prior and Current Co-morbidities	X							
Laboratory results	X	X	X	X	X	X	X	X
Physician assessment of response (RECIST)[e]		X	X	X	X	X	X	
Radiology	X	X	X	X	X	X	X	
Pathology Results	X	X	X	X	X	X	X	
Immunotherapy treatment regimen[g]	X	X	X	X	X	X	X	
Disease status and Survival		X	X	X	X	X	X	X
Cancer treatment Procedures		X	X	X	X	X	X	X
Adverse Event Reporting	X	X	X	X	X	X	X	X
Patient disposition								X
Patient-Reported								

Outcomes[h]	X		X		X[h]	
HCP Questionnaire[i]	X		X	X		X[i]

[a]Baseline visit may occur the same day as immunotherapy initiation
[b]Patients who experience disease progression and those who complete or discontinue immunotherapy treatment will be followed up to two years from the date of consent. Data will be collected when available in the medical record.
[c]Optional blood collection kit
[d]Collect at baseline. For subsequent treatment visits, the weight will be collected from the patient's medical record, if available
[e] Health Care Provider (HCP) assessment of tumor response based on radiology per RECIST criteria. Performed at an interval determined by HCP
[f]Radiology scans are to be submitted and performed at intervals per standard of care determined by HCP. Reports are collected if available
[g]Collected at every visit and/or if there is a change in treatment or regimen
[h]Patient-Reported outcomes are completed at:
- Baseline
- After second SIGNATERA blood draws (expected week 4-8) and tumor assessment are complete
- Month 12, and every 3 months thereafter until study completion for patients continuing immunotherapy treatment

[i]HCP questionnaires are completed at:
- Baseline
- After second SIGNATERA blood draws (expected week 4-8), imaging and tumor assessment are complete, and all results are discussed with the patient (Tumor assessment 1)
- After the third SIGNATERA blood draw (expected week 8-12), imaging and tumor assessment are complete, and all results are discussed with the patient (Tumor assessment 2)
- Any time there is a change in the treatment regimen, indeterminate image finding, or treatment decision to hold or discontinue treatment due to a suspected side effect of immunotherapy

Table 4: Schedule of Events for Control Arm

	Within two months of cancer diagnosis	For each clinic visit 1-24 months from time of immunotherapy treatment
Confirmation of Inclusion/Exclusion Criteria and Enrollment	X	
Demographics and Medical History	X	
Height	X	
Weight	X	X[1]
Prior and Current Concomitant Medications	X	
Current Cancer Diagnosis Details	X	
Prior and Current Co-morbidities	X	
Immunotherapy treatment regimen	X	X
ECOG Performance Status	X	

Cancer treatment procedures		X
Laboratory Results	X	X
Radiology[2]	X	X
Physician assessment of Response (RECIST)		X
Pathology Results	X	X
Patient disposition		X
Disease status	X	X
Side Effects[3]		X

[1]If available in patient's medical record
[2]Radiology scans are to be submitted. Reports are collected if available
[3]Side effects related to immunotherapy treatment

Data Management/Organization

All data will be collected and stored in a secure, Health Insurance Portability and Accountability Act (HIPAA)-compliant database and applicable regulatory requirements appropriate for each clinical site. Before enrollment, signed informed consent will be received from all patients except for the control arm, wherein a consent-waiver will be requested for data collection purposes. Data associated with the samples will be de-identified to maintain patient privacy. Access to the final trial data set will be with Natera; each site will have access to their own site dataset.

Sample Size and Statistical Considerations

The sample size for this study is based on a ±5% margin of error and 95% CI for the percentage of patients with a change in the treatment regimen. The expected percentage of treatment change is unknown and likely to vary by histological indication. Using a normal approximation to the binomial distribution, the worst-case scenario for reducing the width of the CI is when the probability is 0.5. Assuming this value is observed in the study, a minimum of 385 samples are needed to produce a

95% CI ±0.05. Similarly, the minimum number of patients per cohort in each arm (**Table 5**) will be calculated as:

Table 5: Sample size calculations

Assumption	**Prospective Signatera arm**			**Historical control arm**	
Attrition rate	Total number of patients	Minimum number of patients per cohort		Total number of patients	Minimum number of patients per cohort
*25%	1539	513		513	171

*Assumption based on patients lost to follow-up, non-compliance, non-evaluable ctDNA results, etc.

Primary Analysis:

For analysis of the primary endpoint, the point estimate and a 95% Agresti-Coull confidence interval (CI) for the proportion of patients who underwent a change in immunotherapy treatment regimen will be calculated separately for the Lung, Melanoma, and Colorectal cohorts.

General statistical methods:

Dichotomous (e.g., change in postsurgical treatment regimen) and ordinal (e.g., adverse event severity) data will be tabulated by category, expressed as proportions and percentages. The mean, standard deviation, median, maximum, and minimum will be tabulated for continuous data (e.g., age), which may be presented graphically (e.g., box plots). Pairwise comparisons of continuous data will be performed using a *t*-test if the data distribution appears normal; otherwise, a nonparametric rank test will be used. Comparisons of independent binomial data will be performed using Fisher's exact test, and comparisons of dependent binomial data will be performed using McNemar's test. Survival endpoints will be assessed using Kaplan-Meier analysis or Cox Proportional Hazards model; binary endpoints will generally be assessed using logistic regression.

Patient and Public Involvement

The protocol was designed and discussed with the patient advocacy group and academic community (GI oncology). Patients and general public were not involved in the design, conduct, reporting, or dissemination plans of this protocol. Patients will receive ctDNA test results from their provider, according to the current evidence-informed schedule, as part of routine practice.

Ethics and Dissemination

This study will be conducted in accordance with Good Clinical Practice (GCP), International Conference on Harmonization (ICH), the Declaration of Helsinki, and US Food and Drug Administration (FDA) guidelines. Prior to enrollment, written informed consent will be obtained from all patients and compliance with all inclusion and exclusion criteria will be verified and documented. The protocol [Natera-20-043-NCP BESPOKE Study of ctDNA Guided Immunotherapy (BESPOKE IO)] was approved by the WCG Institutional Review Board on February 22, 2021. Publication of any study results in papers, abstracts, posters, or other material presented at scientific meetings or published in professional journals will be approved by Natera in accordance with the site-specific study contract.

DISCUSSION

The BESPOKE IO study is one of the first and large prospective, observational study designed to investigate the utility of ctDNA in guiding treatment response assessment along with standard clinical tools in patients with advanced solid tumors receiving immunotherapy. ctDNA is a highly specific and dynamic blood-based cancer biomarker that provides a real-time snapshot of the tumor burden. It's short half-life of approximately 2 hours puts it in a unique position for assessing early treatment response.[29] Previous studies have demonstrated the ability of ctDNA to detect molecular residual

disease, identify cancer recurrence early, and monitor treatment response across multiple cancers and treatment modalities, including immunotherapy.[11 13 15 21 24 28 30-35]

The use ctDNA kinetics to predict response to immunotherapy has been described across tumor types, using various assays.[17] Timely identification of non-responders from responders based on the ctDNA status can guide further treatment decisions, wherein non-responders can be switched to alternative treatment and spared of the toxicities associated with IO treatment. Alternatively, it can help inform decisions of escalation to combination immunotherapy e.g., addition of a CTLA-4 inhibitor, or addition of chemotherapy in addition to immunotherapy in malignancies that have these agents approved.[10] Currently there are no dynamic real time biomarkers to help aid in this decision making or early response assessment. The commonly used biomarkers used in the IO setting include, PD-L1,[25 36-38] TMB,[39 40] and MSI[41]. Although these biomarkers may help select patients who are most likely to respond ICI, most of these patients may still never respond to treatment. Thus, these biomarkers have limited predictive accuracy and specificity and are unsuitable for early response assessment **(Table 6)**.

Table 6: Limitations with existing predictive biomarkers

Predictive Biomarkers	Limitations
PD-L1 expression – IHC assay[25 36-38]	• Across 45 primary drug approval studies from 2011-April 2019, PD-L1 was predictive in only 28.9% of cases • Low specificity (62-72% across trials) • Heterogeneous marker (expression variability both intratumorally and temporally) • Different assays have different scoring criteria and positivity thresholds
Tissue-based TMB[39 40]	• High TMB did not predict improved overall survival after treatment with ICI • Lack of standardization: the cut-off for positivity varies between ≥7.4 to ≥20 mut/Mb for different tests
MSI[41]	• Across 5 different clinical trials, only 39.6% of MSI-high patients responded to ICI

The bespoke tumor-informed (Signatera™) ctDNA assay used in this study tracks tumor-specific somatic, single nucleotide variants (SNVs) in patients' plasma based on the upfront whole-exome

sequencing of the patient's tumor tissue and matched normal blood. As described previously,[42] the bespoke ctDNA assay can detect clonal variants with high sensitivity (down to 0.01% tumor fraction) and high specificity (>99.8%), which has been validated across numerous studies.[11 13 15 33 43 44] More importantly, the assay filters out clonal hematopoiesis of indeterminate potential and germline-derived variants from analysis, thereby reducing false-positives.[42]

In this study, ctDNA levels will be evaluated at baseline (immediately before starting treatment) and during treatment with IO, with serial ctDNA analysis planned every 2 cycles during the 2-year long follow-up in all cohorts. Several studies demonstrated that patients with declining ctDNA levels on-treatment had better survival outcomes, suggesting that the decline in the ctDNA level with treatment reflected a favorable response to IO.[11 12 15 21-23 25 28] In a recent study by Bratman *et al.*, the bespoke ctDNA assay was used in a cohort of 94 patients with 25 different types of solid tumors[11]. In the study, the bespoke assay identified immunotherapy non-responders (e.g., disease progression) with a 98% positive predictive value (PPV). Among patients whose ctDNA levels increased after 6 weeks of treatment, progression-free survival (PFS) at 6 months was only 7.5%, compared to 54.5% in patients whose ctDNA levels decreased at the same time point. In conjunction with increasing tumor volume on a CT scan, bespoke ctDNA assay demonstrated 100% PPV for detecting non-responders. The study also found that complete clearance of ctDNA was associated with exceptionally durable response (100% OS with a median follow-up period of 25.4 months [range, 10.8–29.5]).[11]

By contrast, the OS among patients who did not clear their ctDNA was 42.5% and 17.5% at 12 and 24 months, respectively. These data suggest that ctDNA clearance at any time point during treatment is highly predictive of long-term durable response. This finding is consistent with the results of an independent study in patients with hepatocellular carcinoma (n=48) undergoing treatment with

atezolizumab and bevacizumab that used bespoke ctDNA assay and showed longer PFS in patients whose ctDNA level was undetectable with treatment.[45] Not only did ctDNA changes predict the responses, all patients who had their ctDNA cleared were alive till the last date of follow-up. The study by Bratman et al. also demonstrated that 55% of patients experienced molecular progression (ctDNA increase) at 6 weeks, and those patients received on average 2 cycles (6 weeks) of additional immunotherapy guided by radiologic study, which could have been avoided.[11] Thus, bespoke ctDNA assay can enable an earlier switch to an alternative treatment that may have a higher chance of success and lower financial and toxicity burden.

The predictive value of ctDNA was illustrated in a posthoc analysis of IMvigor010 trial, a randomized, phase III study comparing adjuvant atezolizumab to observation after radical cystectomy for urothelial cancer.[15] The study showed that ctDNA detection after radical cystectomy in both arms was associated with reduced disease-free survival (DFS) (atezolizumab arm, HR = 3.36, 95% CI: 2.44–4.62; observation arm, HR = 6.3, 95% CI: 4.45–8.92; P < 0.0001) as well as reduced OS (atezolizumab arm, HR = 3.63, 95% CI: 2.34–5.64; observation arm, HR = 8.0, 95% CI: 4.92–12.99), compared to patients with undetectable postoperative ctDNA. In addition, ctDNA-positive patients in the adjuvant atezolizumab arm had an improved OS (HR = 0.59, 95%CI: 0.41–0.86; median DFS 25.8 *vs.* 15.8 months in the observation arm), while ctDNA-negative patients showed no difference in survival if they received adjuvant atezolizumab. Furthermore, patients who cleared ctDNA with adjuvant atezolizumab had dramatically better survival outcomes compared to those who did not clear ctDNA (DFS, HR = 0.26, 95% CI: 0.12−0.56; P = 0.0014; median DFS: 5.7 months versus not reached; and OS, HR = 0.41, 95% CI: 0.1–1.70.[15] Overall, this study demonstrated that post-operative ctDNA could predict benefit from adjuvant immunotherapy in resected urothelial

cancer patients. Furthermore, patients can be stratified based on the presence/absence of ctDNA after resection, and the ctDNA-negative patients may be spared of adjuvant immunotherapy.[15]

Pseudoprogression poses a unique challenge in solid tumor patients receiving immunotherapy as validated methods that differentiate between true progression and pseudoprogression are lacking. Limited studies have shown the potential of ctDNA in distinguishing pseudoprogression from true progression.[11 24 26] In the study reported by Bratman *et al.*, 7 patients showed pseudoprogression (tumor progression on scans but decreasing ctDNA level at 6 weeks). Of these, 4 patients exhibited a better OS >18 months (range, 19-27) when compared with patients who showed true progression (n=30, increasing ctDNA and progressive disease on scan).[11] Further, the bespoke ctDNA assay was able to detect pseudoprogression 5 months earlier than the imaging studies.[11] In the present study, as one of the exploratory endpoints, we plan to evaluate the association of ctDNA dynamics with pseudoprogression. ctDNA clearance or decline in such patients could help differentiate and direct patients with true progression to alternative treatment.

Taken together, the studies described above provide preliminary evidence that ctDNA can help in immunotherapy response monitoring. however, most of these studies included a small patient population. [14 18 20 22 23 26] Additionally, several of these studies have utilized targeted panels to select the variants and tracked the variants with droplet digital polymerase chain reaction (ddPCR). However, the use of a targeted gene panel can result in suboptimal variant selection and decreased ctDNA sensitivity (43% - 73%).[12 23 25 46] By contrast, the bespoke ctDNA assay selects clonal variants from a whole-exome analysis of the tumor (approximately 20,000 genes), minimizing suboptimal variant selection potential.

The predictive role of ctDNA is currently being studied in several ongoing clinical trials investigating the role of immunotherapy across multiple cancer types (NCT03512847, NCT04636047, NCT04053725, NCT03712566, NCT04589845, NCT04853017, NCT03409848, NCT03178552). Although Most of these trials are designed to include small to moderate sample sizes and employ variable assay designs, these trials would be instrumental in establishing ctDNA's role as a surrogate endpoint for immunotherapy treatment efficacy.

The limitation of our study is that it is purely observational. Therapy is physician directed and not dictated by the trial given the non-interventional nature of the study. However, the prospective design of the study, a large sample size, and the 2-year long follow-up period will allow us to compare the sensitivity, specificity, PPV, NPV, and clinical utility within as well as among different study cohorts. Of note, our study design includes a retrospectively enrolled control group for adequate comparisons, which will further help in determining the clinical utility of the personalized, tumor-informed ctDNA assay in guiding treatment monitoring in patients receiving immunotherapy. Another limitation is the fewer tumor types being considered in this clinical study, which may limit the generalizability of ctDNA-based treatment response monitoring in patients with other tumor types getting IO therapy.

We believe this study will also help generate the relevant data required to allow for future prospective interventional studies. We expect that our study will help establish the real-world evidence of ctDNA's utility in monitoring treatment response to immunotherapy in patients with solid tumors and support its integration into clinical practice and guidelines, leading to meaningful improvements in patient outcomes and quality of life.

AUTHOR'S CONTRIBUTIONS

- Study coordinator: SS
- Site Identification: MK, SS
- Design and writing of the protocol: AA, SS, MK, PMK, JE, AR, SE
- Data collection: SS, SE, MK
- Data analysis: JE
- Data interpretation: JE, MK, AA, AR, SS
- Writing of the manuscript: MM, MM, PMK, SC
- Statistical setting of the study design and data analysis: JE
- All authors reviewed and approved the final manuscript: PMK, SC, SS, MK, AP, GA, MM[#], MM*, JE, LG, ZE, SE, PRB, AR, AA.

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FUNDING STATEMENT

This study was supported by Natera, Inc. This study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

COMPETING INTERESTS

PMK acknowledges role as a consultant/advisor for Taiho Oncology, Ipsen, Natera, Foundation Medicine, Research/Trial Support (to institution): BMS, Celgene, AstraZeneca, BTG, Advanced Accelerator Applications, Array Biopharma.

SC acknowledges membership of the Natera's speakers' bureau

AP: acknowledges membership of the Bristol Myers Squibb speakers' bureau, role as a consultant for Novartis, participatory role in a Natera Ad Board, and role as a speaker for Natera and Grail.

GA: Nothing to disclose

LG is a federal employee and reports no conflict of interest.

ZE: Research Support: Pfizer, Novartis; Consultancy: Pfizer, Eisai, Natera Inc., OncoSec, Genentech.

All other authors are employees of Natera, Inc. with stock/options to own stock on the company.

This study is being sponsored by Natera, Inc.

ACKNOWLEDGMENTS

Authors would like to acknowledge the clinical project management and data management support provided by Worldwide Clinical Trials.

FIGURE LEGEND

Figure 1: Overview of the BESPOKE IO study design: Samples (whole blood, FFPE tissue, plasma) will be collected, and questionnaires (physician assessment, quality of life (QoL), will be completed at the indicated times (weeks/months).

References:

1. Borcoman E, Kanjanapan Y, Champiat S, et al. Novel patterns of response under immunotherapy. *Ann Oncol* 2019;30(3):385-96. doi: 10.1093/annonc/mdz003 [published Online First: 2019/01/19]

2. Akinleye A, Rasool Z. Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. *J Hematol Oncol* 2019;12(1):92. doi: 10.1186/s13045-019-0779-5 [published Online First: 2019/09/07]

3. Seymour L, Bogaerts J, Perrone A, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *The Lancet Oncology* 2017;18(3):e143-e52. doi: 10.1016/S1470-2045(17)30074-8 [published Online First: 2017/03/02]

4. Borcoman E, Nandikolla A, Long G, et al. Patterns of Response and Progression to Immunotherapy. *Am Soc Clin Oncol Educ Book* 2018;38:169-78. doi: 10.1200/edbk_200643 [published Online First: 2018/09/21]

5. Thomas R, Somarouthu B, Alessandrino F, et al. Atypical Response Patterns in Patients Treated With Nivolumab. *AJR Am J Roentgenol* 2019:1-5. doi: 10.2214/AJR.18.20938 [published Online First: 2019/03/28]

6. Monthly and Median Costs of Cancer Drugs at the Time of FDA Approval 1965-2016. *J Natl Cancer Inst* 2017;109(8) doi: 10.1093/jnci/djx173 [published Online First: 2017/11/09]

7. Gjoerup O, Brown CA, Ross JS, et al. Identification and Utilization of Biomarkers to Predict Response to Immune Checkpoint Inhibitors. *Aaps j* 2020;22(6):132. doi: 10.1208/s12248-020-00514-4 [published Online First: 2020/10/16]

8. Wang Y, Tong Z, Zhang W, et al. FDA-Approved and Emerging Next Generation Predictive Biomarkers for Immune Checkpoint Inhibitors in Cancer Patients. *Front Oncol* 2021;11:683419. doi: 10.3389/fonc.2021.683419 [published Online First: 2021/06/25]

9. Kasi P, Chan C. 23 Circulating tumor DNA (ctDNA) serial analysis during progression on PD-1 blockade and later CTLA4 rescue in patients with mismatch repair deficient metastatic colorectal cancer. *Journal for immunotherapy of cancer* 2020;8(Suppl 3):A12-A13. doi: 10.1136/jitc-2020-SITC2020.0023

10. Kooshkaki O, Derakhshani A, Hosseinkhani N, et al. Combination of Ipilimumab and Nivolumab in Cancers: From Clinical Practice to Ongoing Clinical Trials. *International journal of molecular sciences* 2020;21(12):4427. doi: 10.3390/ijms21124427

11. Bratman SV, Yang SYC, Iafolla MAJ, et al. Personalized circulating tumor DNA analysis as a predictive biomarker in solid tumor patients treated with pembrolizumab. *Nature Cancer* 2020;1(9):873-81. doi: 10.1038/s43018-020-0096-5

12. Cabel L, Riva F, Servois V, et al. Circulating tumor DNA changes for early monitoring of anti-PD1 immunotherapy: a proof-of-concept study. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2017;28(8):1996-2001. doi: 10.1093/annonc/mdx212 [published Online First: 2017/05/02]

13. Christensen E, Birkenkamp-Demtröder K, Sethi H, et al. Early Detection of Metastatic Relapse and Monitoring of Therapeutic Efficacy by Ultra-Deep Sequencing of Plasma Cell-Free DNA in Patients With Urothelial Bladder Carcinoma. *J Clin Oncol* 2019;37(18):1547-57. doi: 10.1200/jco.18.02052 [published Online First: 2019/05/07]

14. Giroux Leprieur E, Herbretau G, Dumenil C, et al. Circulating tumor DNA evaluated by Next-Generation Sequencing is predictive of tumor response and prolonged clinical

benefit with nivolumab in advanced non-small cell lung cancer. *Oncoimmunology* 2018;7(5):e1424675. doi: 10.1080/2162402x.2018.1424675 [published Online First: 2018/05/04]

15. Powles T, Assaf ZJ, Davarpanah N, et al. ctDNA guiding adjuvant immunotherapy in urothelial carcinoma. *Nature* 2021;595(7867):432-37. doi: 10.1038/s41586-021-03642-9 [published Online First: 2021/06/18]
16. Ricciuti B, Jones G, Severgnini M, et al. Early plasma circulating tumor DNA (ctDNA) changes predict response to first-line pembrolizumab-based therapy in non-small cell lung cancer (NSCLC). *J Immunother Cancer* 2021;9(3) doi: 10.1136/jitc-2020-001504 [published Online First: 2021/03/28]
17. PM K. Kinetics of Liquid Biopsies in Predicting Response to Immunotherapy: ASCO Daily News; Oct 1, 2020 [Available from: https://dailynews.ascopubs.org/do/10.1200/ADN.20.200338/full/ accessed December 14, 2021.
18. Anagnostou V, Forde PM, White JR, et al. Dynamics of Tumor and Immune Responses during Immune Checkpoint Blockade in Non-Small Cell Lung Cancer. *Cancer Res* 2019;79(6):1214-25. doi: 10.1158/0008-5472.Can-18-1127 [published Online First: 2018/12/14]
19. Chaudhuri AA, Chabon JJ, Lovejoy AF, et al. Early Detection of Molecular Residual Disease in Localized Lung Cancer by Circulating Tumor DNA Profiling. *Cancer Discov* 2017;7(12):1394-403. doi: 10.1158/2159-8290.Cd-17-0716 [published Online First: 2017/09/14]

20. Goldberg SB, Narayan A, Kole AJ, et al. Early Assessment of Lung Cancer Immunotherapy Response via Circulating Tumor DNA. *Clin Cancer Res* 2018;24(8):1872-80. doi: 10.1158/1078-0432.Ccr-17-1341 [published Online First: 2018/01/14]

21. Cabel L, Proudhon C, Romano E, et al. Clinical potential of circulating tumour DNA in patients receiving anticancer immunotherapy. *Nature reviews Clinical oncology* 2018;15(10):639-50. doi: 10.1038/s41571-018-0074-3 [published Online First: 2018/07/28]

22. Gray ES, Rizos H, Reid AL, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. *Oncotarget* 2015;6(39):42008-18. doi: 10.18632/oncotarget.5788 [published Online First: 2015/11/03]

23. Herbreteau G, Vallée A, Knol AC, et al. Quantitative monitoring of circulating tumor DNA predicts response of cutaneous metastatic melanoma to anti-PD1 immunotherapy. *Oncotarget* 2018;9(38):25265-76. doi: 10.18632/oncotarget.25404 [published Online First: 2018/06/05]

24. Lee JH, Long GV, Menzies AM, et al. Association Between Circulating Tumor DNA and Pseudoprogression in Patients With Metastatic Melanoma Treated With Anti-Programmed Cell Death 1 Antibodies. *JAMA Oncol* 2018;4(5):717-21. doi: 10.1001/jamaoncol.2017.5332 [published Online First: 2018/02/10]

25. Lee JH, Long GV, Boyd S, et al. Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2017;28(5):1130-36. doi: 10.1093/annonc/mdx026

26. Guibert N, Mazieres J, Delaunay M, et al. Monitoring of KRAS-mutated ctDNA to discriminate pseudo-progression from true progression during anti-PD-1 treatment of lung adenocarcinoma. *Oncotarget* 2017;8(23):38056-60. doi: 10.18632/oncotarget.16935

27. Raja R, Kuziora M, Brohawn PZ, et al. Early Reduction in ctDNA Predicts Survival in Patients with Lung and Bladder Cancer Treated with Durvalumab. *Clin Cancer Res* 2018;24(24):6212-22. doi: 10.1158/1078-0432.Ccr-18-0386 [published Online First: 2018/08/11]

28. Zhang Q, Luo J, Wu S, et al. Prognostic and Predictive Impact of Circulating Tumor DNA in Patients with Advanced Cancers Treated with Immune Checkpoint Blockade. *Cancer Discov* 2020;10(12):1842-53. doi: 10.1158/2159-8290.Cd-20-0047 [published Online First: 2020/08/21]

29. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nature Medicine* 2008;14(9):985-90. doi: 10.1038/nm.1789

30. Jia N, Sun Z, Gao X, et al. Serial Monitoring of Circulating Tumor DNA in Patients With Metastatic Colorectal Cancer to Predict the Therapeutic Response. *Front Genet* 2019;10:470. doi: 10.3389/fgene.2019.00470 [published Online First: 2019/06/06]

31. Parikh AR, Mojtahed A, Schneider JL, et al. Serial ctDNA Monitoring to Predict Response to Systemic Therapy in Metastatic Gastrointestinal Cancers. *Clin Cancer Res* 2020;26(8):1877-85. doi: 10.1158/1078-0432.Ccr-19-3467 [published Online First: 2020/01/17]

32. Pereira E, Camacho-Vanegas O, Anand S, et al. Personalized Circulating Tumor DNA Biomarkers Dynamically Predict Treatment Response and Survival In Gynecologic Cancers. *PLoS One* 2015;10(12):e0145754. doi: 10.1371/journal.pone.0145754

33. Coombes RC, Page K, Salari R, et al. Personalized Detection of Circulating Tumor DNA Antedates Breast Cancer Metastatic Recurrence. *Clinical Cancer Research* 2019;25(14):4255-63. doi: 10.1158/1078-0432.Ccr-18-3663

34. Goodall J, Mateo J, Yuan W, et al. Circulating Cell-Free DNA to Guide Prostate Cancer Treatment with PARP Inhibition. *Cancer Discov* 2017;7(9):1006-17. doi: 10.1158/2159-8290.Cd-17-0261 [published Online First: 2017/04/30]

35. Moding EJ, Liu Y, Nabet BY, et al. Circulating tumor DNA dynamics predict benefit from consolidation immunotherapy in locally advanced non-small-cell lung cancer. *Nature Cancer* 2020;1(2):176-83. doi: 10.1038/s43018-019-0011-0

36. Büttner R, Gosney JR, Skov BG, et al. Programmed Death-Ligand 1 Immunohistochemistry Testing: A Review of Analytical Assays and Clinical Implementation in Non-Small-Cell Lung Cancer. *J Clin Oncol* 2017;35(34):3867-76. doi: 10.1200/jco.2017.74.7642 [published Online First: 20171020]

37. Davis AA, Patel VG. The role of PD-L1 expression as a predictive biomarker: an analysis of all US Food and Drug Administration (FDA) approvals of immune checkpoint inhibitors. *Journal for immunotherapy of cancer* 2019;7(1):278. doi: 10.1186/s40425-019-0768-9 [published Online First: 20191026]

38. Kaderbhaï C, Tharin Z, Ghiringhelli F. The Role of Molecular Profiling to Predict the Response to Immune Checkpoint Inhibitors in Lung Cancer. *Cancers* 2019;11(2):201. doi: 10.3390/cancers11020201

39. Meléndez B, Van Campenhout C, Rorive S, et al. Methods of measurement for tumor mutational burden in tumor tissue. *Translational lung cancer research* 2018;7(6):661-67. doi: 10.21037/tlcr.2018.08.02

40. Rousseau B, Foote MB, Maron SB, et al. The Spectrum of Benefit from Checkpoint Blockade in Hypermutated Tumors. *New England Journal of Medicine* 2021;384(12):1168-70. doi: 10.1056/NEJMc2031965

41. Marcus L, Lemery SJ, Keegan P, et al. FDA Approval Summary: Pembrolizumab for the Treatment of Microsatellite Instability-High Solid Tumors. *Clin Cancer Res* 2019;25(13):3753-58. doi: 10.1158/1078-0432.Ccr-18-4070 [published Online First: 20190220]

42. Kasi PM, Sawyer S, Guilford J, et al. BESPOKE study protocol: a multicentre, prospective observational study to evaluate the impact of circulating tumour DNA guided therapy on patients with colorectal cancer. *BMJ Open* 2021;11(9):e047831. doi: 10.1136/bmjopen-2020-047831

43. Magbanua MJM, Swigart LB, Wu HT, et al. Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2021;32(2):229-39. doi: 10.1016/j.annonc.2020.11.007 [published Online First: 2020/11/25]

44. Reinert T, Henriksen TV, Christensen E, et al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. *JAMA Oncology* 2019;5(8):1124-31. doi: 10.1001/jamaoncol.2019.0528

45. Hsu C-H, Lu S, Abbas A, et al. Longitudinal and personalized detection of circulating tumor DNA (ctDNA) for monitoring efficacy of atezolizumab plus bevacizumab in patients with unresectable hepatocellular carcinoma (HCC). *Journal of Clinical Oncology* 2020;38(15_suppl):3531-31. doi: 10.1200/JCO.2020.38.15_suppl.3531

46. Keller L, Guibert N, Casanova A, et al. Early Circulating Tumour DNA Variations Predict Tumour Response in Melanoma Patients Treated with Immunotherapy. *Acta Derm Venereol* 2019;99(2):206-10. doi: 10.2340/00015555-3080 [published Online First: 2018/11/06]

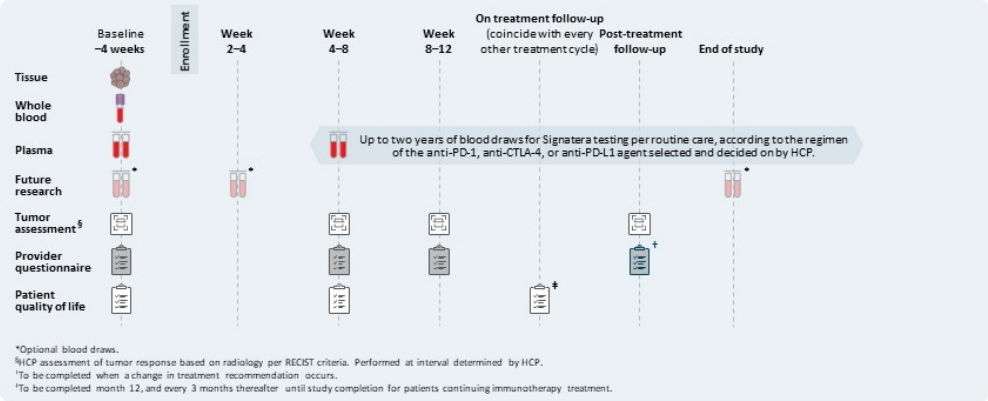

Figure 1: BESPOKE immunotherapy study design overview
Overview of the BESPOKE IO study design. Samples (whole blood, FFPE tissue, plasma) will be collected, and questionnaires (physician assessment, quality of life (QoL), will be completed at the indicated times (weeks/months).

225x93mm (96 x 96 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Author's Annotation
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	See manuscript page 1, title
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	See manuscript page 2
	2b	All items from the World Health Organization Trial Registration Data Set	N/A; this is a registry study
Protocol version	3	Date and version identifier	Jan 25, 2021 Version 1.0 Clinical study protocol, page 2
Funding	4	Sources and types of financial, material, and other support	See manuscript page 23, funding.
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	See manuscript page 23, authors contributions.
	5b	Name and contact information for the trial sponsor	Trial sponsored by Natera, Inc. Contact

			corresponding author; see manuscript page 1 for corresponding author contact information.
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	See manuscript page 23, authors contributions.
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	See manuscript page 23, authors contributions, where applicable.
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	See abstract, manuscript page 2; see manuscript, background, page 4-6.
	6b	Explanation for choice of comparators	N/A; this is an observational trial
Objectives	7	Specific objectives or hypotheses	See manuscript page 10, study objectives/endpoints.
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	See manuscript page 6-7, overall study design.

Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	See manuscript page 2; design; page 7, study design
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	See manuscript page 9, Table 2.
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	This is an observational study. Immunotherapy treatment regimen is listed in Table 1, page 8
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A; this is an observational study.
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	N/A; this is an observational study.
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A; this is an observational study.
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation	See manuscript page 10, study objectives/endpoints.

		of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	See manuscript page 13, Table 3, schedule of events; manuscript page 7, figure 1.
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	See manuscript page 16, sample size and statistical considerations and Table 5
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	To achieve adequate participant enrollment to reach the target sample size, we are recruiting at a large number of participating sites (up to 100 sites) to reach our enrollment target.
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	N/A; this is an observational study.

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Combination of site ID and sequentially numbered
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Site will generate the number
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	N/A; this is an observational study.
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A; this is an observational study.
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Manuscript page 11-15, including Table 3 and 4.
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	N/A; this is an observational study.

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Manuscript Page 12. Data will be entered using an electronic data capture and will be monitored either remotely or on-site on a bi-annual basis
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Overview of primary and secondary endpoints are on page 10 of the manuscript. Statistical methods are detailed on page 16-17. Detailed statistical analysis is available in the IRB approved Clinical protocol statistical considerations.
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Results from the additional analyses will be summarized. Please see exploratory analysis, page 10.
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	No imputation of missing data will be made. Data will be analyzed as is and patients with missing data will be excluded from final analysis.
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its	N/A; its an observational study.

		charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	No formal interim analysis is planned. However, results from analyses performed may be reported from time to time over the course of the study. Applicable study team members will have access to the data. Termination: N/A, this is an observational study.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	See manuscript, page 11, data collection.
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A.
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	IRB approved protocol. See manuscript page 2; Please also see uploaded WCG IRB approval letter

Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	No amendments have been made till date.
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Manuscript page 7 and Page 15. PI will obtain informed consent from the patient. No assent or authorised legal reps may provide consent
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	See manuscript page 17, ethics and dissemination
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial – **may need a statement**	See manuscript page 15, data management and organization.
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	See manuscript page 23, competing interests/disclosures
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Natera has access to the final trial data set and each site will have access to their own site dataset.
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A. This is an observational study.

Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Publication of any study results in papers, abstracts, posters or other material presented at scientific meetings or published in professional journals must be approved by Natera in accordance with the site-specific study contract. .
	31b	Authorship eligibility guidelines and any intended use of professional writers	Authorship is based on the author contributions as outlined on page 23 of the manuscript
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	We have uploaded a model consent form for your review as part of the submission materials.
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	See manuscript page 15, data management/organization .