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Supplementary information

ctDNA guiding adjuvant immunotherapy in urothelial carcinoma

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STATISTICAL ANALYSIS PLAN

Natera, Inc.
201 Industrial Road, Suite 410,
San Carlos, CA 94070 USA

Personalized circulating tumor DNA analysis for IMvigor 010

STATISTICAL ANALYSIS PLAN

Sponsor Name: Natera, Inc. **Product:** ctDNA

Title: Personalized circulating tumor DNA analysis for IMvigor010

Version: 3.0

Version Date: January 21, 2020

The signatures below indicate approval of the Statistical Analysis plan for this study.	
APPROVAL SIGNATURES	
[redacted]	January 23, 2020 Date
	21 Jan 2020 Date

REVISION HISTORY

SAP Version	Approval Date	Change	Rationale
1.0	Dec 10, 2019	Not Applicable	Original version
2.0	Dec 18, 2019	Added comments based on joint discussion on 12DEC2019	Joint discussion between Natera and GNE teams
3.0	Jan 21, 2020	Final SAP	

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1 INTRODUCTION

This statistical analysis plan (SAP) outlines the data and procedures used to analyze ctDNA data collected for IMvigor010 trial selected samples.

2 STUDY OBJECTIVES

2.1 PRIMARY OBJECTIVES

1. To provide evidence that in the circulating tumor DNA (ctDNA) positive patients at C1D1, atezo provides superior benefit compared to observation arm.
2. (Prognostic question): To provide evidence that the presence of ctDNA in plasma at C1D1 is associated with decreased disease-free survival (DFS)
3. (Prognostic/early surrogate question): To provide evidence that the presence of ctDNA in plasma at C3D1 is associated with decreased disease-free survival (DFS)
4. To provide evidence that the clearance of ctDNA in plasma by C3D1 is associated with increased disease-free survival (DFS), and clearance occurs at a higher rate in atezo arm compared to observation arm.

2.2 SECONDARY OBJECTIVE

1. Repeat the above primary objectives, but as Multivariate analysis (MVA) adjusting for PDL1 status (pos/neg), neoadjuvant chemotherapy (yes/no), lymph node positivity (yes/no), and number of lymph nodes removed (>=10 yes/no). Adjust for each variable independently given that numbers will be too small for combined analysis.
    a. To provide evidence that neoadjuvant therapy affects rates of C1D1 ctDNA positivity.
2. Repeat the above primary objectives, but with ctDNA status defined as a continuous variable (VAF or MTM/mL), and ctDNA change defined as a continuous variable (% change VAF or MTM/mL).
3. Repeat the above primary and secondary objectives, but using overall survival (OS) as endpoint.

3 STUDY DESIGN AND PROCEDURES

Muscle invasive bladder cancer patients from IMvigor010 Tecentriq clinical trial that accrued adjuvant high-risk cisplatin ineligible patients with N = 809. Blood collected within 10 weeks post-cystectomy prior to/at the start of Tecentriq treatment (C1D1 -- baseline), then during treatment (C3D1; 6 weeks from baseline sample), day of disease progression, if occured, (PD), and at the end of treatment (EOT).

4 ANALYSIS SETS

4.1 FULL ANALYSIS SET

Two arms of the study analyzed together and separately. 619 patients out of the full set of 809 are eligible to be evaluated (where matching tumor tissue, PBMC, and at least 1 plasma timepoint are available for Signatera analysis).

1. Flagged vs passing QC for 619 patients
    - i. 4/619 patients failed WES library prep (will be excluded from analysis)
    - ii. 11/619 patients failed WES concordance (will be excluded from analysis)
    - iii. 41/604 patients entered the relaxed primer design parameters (designated as custom assay)
        1. 2/41 patients did not get a 16-plex Signatera assay designed and instead went on to get between 2 and 15 variants designed in the custom assay
    - iv. 590/604 patients had their plasma samples run by 1/17/2020.
        1. Another 14 patients will finish having their plasma samples run by 1/27/2020.
    - v. 50 out of 590 patients had flagged WES QC of less than or equal to 7.6% mean Signatera target VAF in tumor tissue
    - vi. 60 out of 590 patients had less than or equal to 30% pathological tumor purity
    - vii. Plasma Samples QC
        1. Samples that do not meet SNP concordance with any other assayed patients will be excluded from analysis.
            a. 12 processed from 6 patients had swaps due to sample mislabeling upstream of Natera receipt. These will be rerun (available ~1/27)
            b. 4 samples from 3 patients failed SNP concordance (4676, 5412, 6772),
            c. 1 sample failed SNP concordance due to Natera lab swap: 1166 (WES failed lib prep) instead of 1165 had sample run (1165 is being run and will be available ~1/27)
        2. 893/1110 plasma samples had at least 11.25 ng cfDNA extracted, others were flagged
        3. 583/590 patients had plasma samples passing sequencing QC, run by 1/17/2020
        4. 571 out of 583 patients had C1D1 timepoint pass sequencing QC, run by 1/17/2020
        5. 488 out of 583 patients had C3D1 time point pass sequencing QC, run by 1/17/2020
        6. 476 out of 583 patients had both C1D1 and C3D1 timepoints pass sequencing QC, run by 1/17/2020

Analysis population

1. ctDNA C1D1 biomarker evaluable population (BEP): patients who have valid C1D1 ctDNA measure
2. ctDNA C1D1+C3D1 BEP: patients who have valid ctDNA measure at both C1D1 and C3D1
3. ctDNA C3D1 BEP: patients who have valid C3D1 ctDNA measure

The available patients are representative of the full population.

4. BIOMARKERS

4.2 PRIMARY BIOMARKERS

ctDNA in plasma as measured by Natera's Signatera assay based on ctDNA status (positive/negative), mean variant allele frequency (VAF), and mean tumor molecules per mL of plasma (MTM/mL) (see Section 7 for Signatera Assay definitions).

4.3 SECONDARY BIOMARKERS

Below are additional biomarkers which may be collected (not inclusive of all biomarkers we may analyze) which we may analyze in the context of ctDNA metrics.

- Imaging and Radiographic Evaluation Results
- Pathological Complete Response (pCR) or Pathological downstaging at CX
- PD-L1 Status
- Clinical and Pathologic Risk Factors (LVI, PNI, T stage, N stage, nodal sampling, margins, etc)
- If TMB data available, Tumor Mutation Burden (TMB), Prevalence of low vs high-frequency mutations (eg median tumor WES VAF)
- Molecular gene signatures (DDR and cell cycle genes)
- Amount of cfDNA extracted per mL of plasma (ng/mL)

5 ENDPOINTS

5.1.1 Primary Endpoint

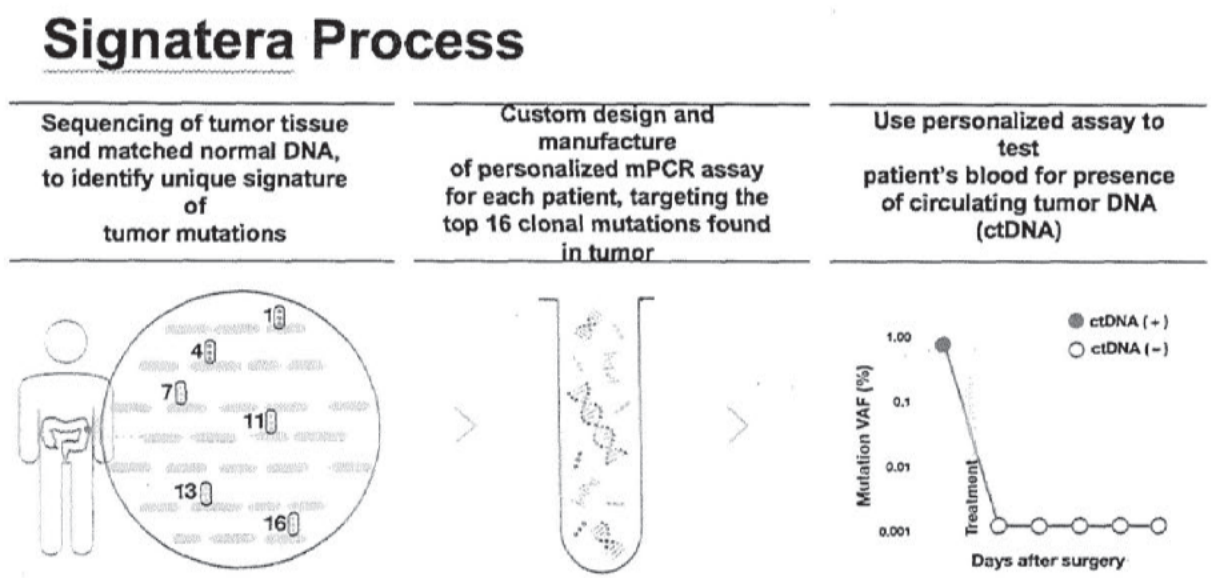
Primary endpoint for the Imvigor010 study is defined as the time from randomization to the time of first occurrence of a DFS event. DFS events include: local (pelvic) recurrence of UC (including soft tissue and regional lymph nodes); urinary tract recurrence of UC (including all pathological stages and grades); distant metastasis of UC; or death from any cause. Tumor assessment will be performed using radiographic evaluations.

5.1.2 Secondary Endpoints

OS: Overall survival for the Imvigor010 is defined as the time from randomization until death due to any cause or last follow-up, upto approx 8 years. Note that OS data will be very immature in 2020.
DSS (Disease-Specific Survival): The time from randomization until the date of death from UC, upto approx 8 years.
DMFS (Distant Metastasis-Free Survival): the time from randomization to the date of diagnosis of distant (that is, non-locoregional) metastases or death from any cause. Tumor assessment will be performed using radiographic evaluations.
NURFS (Non-Urinary Tract Recurrence-Free Survival): the time from randomization to the time of first occurrence of a NURFS event. NURFS events include: local (pelvic) recurrence of UC (including soft tissue and regional lymph nodes); distant metastasis of UC; or death from any cause. Tumor assessment will be performed using radiographic evaluations

6. Signatera Assay Process and Definitions

A) Signatera Assay Process:



B) Signtera Assay Definitions

a) Sample-Level ctDNA Signatera Positive: ≥2 variants must be detected above Signatera calling threshold
b) Mean VAF (Variant Allele Frequency): The average plasma VAF of all Signatera targets that meet QC requirements
   - Tumor variant allele frequency (VAF) for 1 specific variant is reported only if >=2 variants in the sample are detected. VAF for 1 specific variant is calculated as follows: (# mutant reads)/(# total reads)
c) Mean Tumor Molecules per mL of plasma (MTM/mL): The average of tumor molecules across all variants that meet QC requirements per mL of plasma.
   - Tumor Molecules per mL of plasma (for 1 variant) is reported only if >=2 variants in the sample are detected. Calculation for 1 specific

variant is described in the following section (8.1).

7. STATISTICAL METHODS

7.1 GENERAL STATISTICAL CONSIDERATIONS

Continuous data will be summarized using descriptive statistics: N, mean, standard deviation, median, minimum and maximum, unless otherwise noted.

Categorical variables will be summarized using frequency counts and percentages.

ctDNA presence will be assessed in categorical and continuous forms. When assessed categorically, it is referred to as ctDNA status (positive/negative) calculated using Natera's algorithm per plasma time point. When assessed continuously, ctDNA is measured in either dimensionless units of Variant Allele Frequency (VAF) or in terms of Tumor Molecules (TM) per unit of plasma volume having dimensions of counts/mL. The conversion of VAF into TM is done per individual assay according to

$$TM = cfDNA_{extracted}(ng) \times \frac{1000\,pg}{1ng} \times \frac{haploid\ genome\ equivalent}{3.3\,pg\ per\ hGE} \times \frac{VAF}{Plasma\ volume\ for\ extraction\ (mL)}$$

The mean of corresponding variable (VAF or TM), computed over all assays tested for a particular plasma sample, is used as the ctDNA measurement for the time point.

Wherever a significance test is appropriate, significance assessment will be made at a level of p-value < 0.05.

7.2 STUDY HYPOTHESES

7.2.1 Primary and Secondary

- The presence of ctDNA is associated with decreased DFS and worse outcome with other measures like OS, DMFS, DSS, NURFS.
- ctDNA status is prognostic and predictive of treatment response.
- Lower ctDNA levels at baseline/pretreatment are associated with better clinical outcomes and higher rates of treatment response.
- ctDNA negative post-Cystectomy status is associated with improved outcomes, stratified by neoadjuvant treatment.

7.2.2 Exploratory

- The presence of ctDNA corresponds to tumor burden, and therefore ctDNA detection will be associated with stage of disease
- ctDNA levels at baseline (C1D1) correlate with clearance rates after treatment in the atezo treated group.
- ctDNA levels at baseline (C1D1) and C3D1 correlate with radiological recurrence in the observation arm

- ctDNA detection at baseline (C1D1) correlates with the number of days from radical cystectomy and neoadjuvant treatment.
- ctDNA will remain an independent risk factor for DFS in multivariate analysis in the observation arm.

7.3 DETERMINATION OF EFFECT SIZE, SAMPLE SIZE, AND POWER

In the cohort described above, we are able to detect the hazard ratio (HR) of 1.5 with required statistical significance and power of 80% at the expected rate of recurrence of 40-50%. This estimate is obtained by using standard approach in survival analysis, see, for example, ref. 3. The sample size relation to significance, power, and effect size is given by $n = \frac{(z_{\alpha/2} + z_{\theta})^2}{\beta_0^2 p(1-p)P(\Delta=1)}$ where z denotes the corresponding upper percentile of the standard normal distribution for significance and power, β determines the effect size (hazard ratio), p is the group ratio, and P is the censoring distribution. A variety of packages exist to calculate this number. See, ref 4, for example.

7.4 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ) FOR CONTINUOUS VARIABLES

[redacted]

9. STATISTICAL ANALYSES

9.1 PATIENT DISPOSITION

Patients were censored after their last follow-up.

9.2 DEMOGRAPHIC AND BASELINE CHARACTERISTICS

9.2.1 Demographic and Baseline Characteristics

Sex and primary site of cancer will be summarized categorically. Age will be summarized as a continuous variable.

9.2.2 Medical History

Medical history will include age at diagnosis, date of surgery, and histological diagnosis. The latter is further detailed as follows: tumor size (in cm) for each tumor, histological subtype, post-operative TNM, clinical M-status (pre-surgical radiological M-staging), number of nodes examined, and number of positive vs negative nodes.

9.2.3 OTHER CLINICAL INFORMATION

Tumor sequencing information and method of testing, outcome (relapse and no-relapse), neoadjuvant treatment (yes/no), Atezo vs. observation in adjuvant setting and follow-up post-treatment will be summarized. The primary outcome measure is DFS.

10. ANALYSIS

10.1 Primary Analyses

10.1.1 Comparing the intent-to-treat (ITT) versus biomarker evaluable populations (BEP)

A) To assess consistencies between different populations, by-arm demographics tables will be generated to compare following pair of populations
   a) ITT vs C1D1 BEP
   b) ITT vs C1D1+C3D1 BEP
   c) ITT vs C3D1 BEP
   d) C1D1 BEP vs C1D1+C3D1 BEP

B) by-arm KM plots and median survival estimates comparing the population pairs in A)

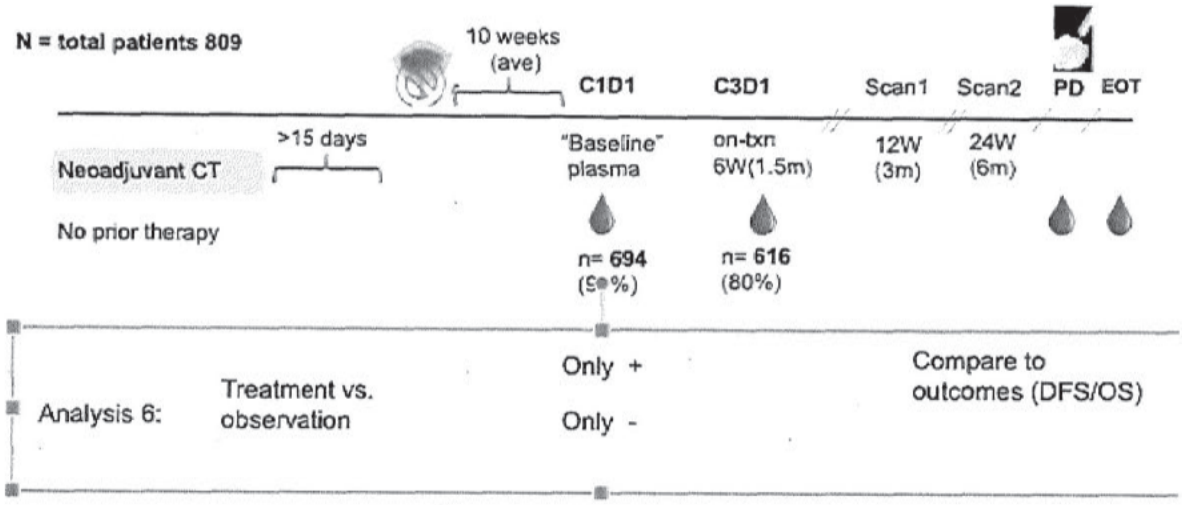
10.1.2 Univariate analysis for categorical ctDNA

A univariate Cox proportional hazard model will be fit to calculate a hazard ratio (HR) and the 95% confidence interval to estimate the relative risk of recurrence/event in the ctDNA-positive compared to the ctDNA-negative group. Proportional hazards assumption will be tested to evaluate the application of Cox modelling, log-rank test will be used when Cox regression is not applicable. It will be concluded that ctDNA is prognostic and predictive for DFS if the stated significance level is achieved.

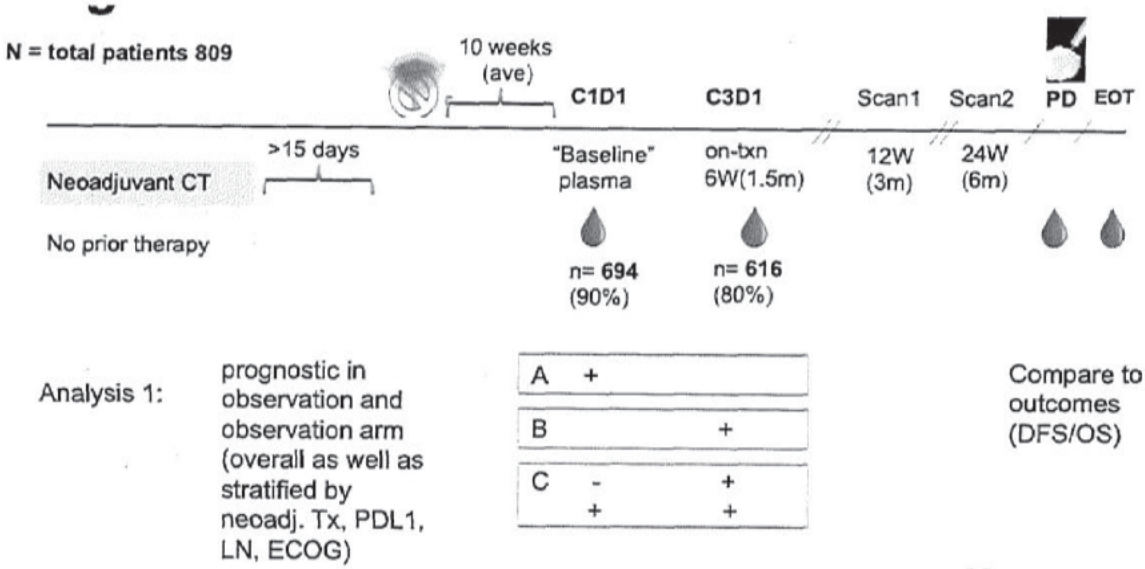
Patient stratification into comparison groups will be performed by the following approaches:

1. Comparison of DFS by ctDNA status in the treatment arm with the observation arm (Primary Objective #1)
   a) ctDNA positive at baseline and DFS in the treatment vs observation arm
   b) ctDNA negative at baseline and DFS in the treatment vs observation arm

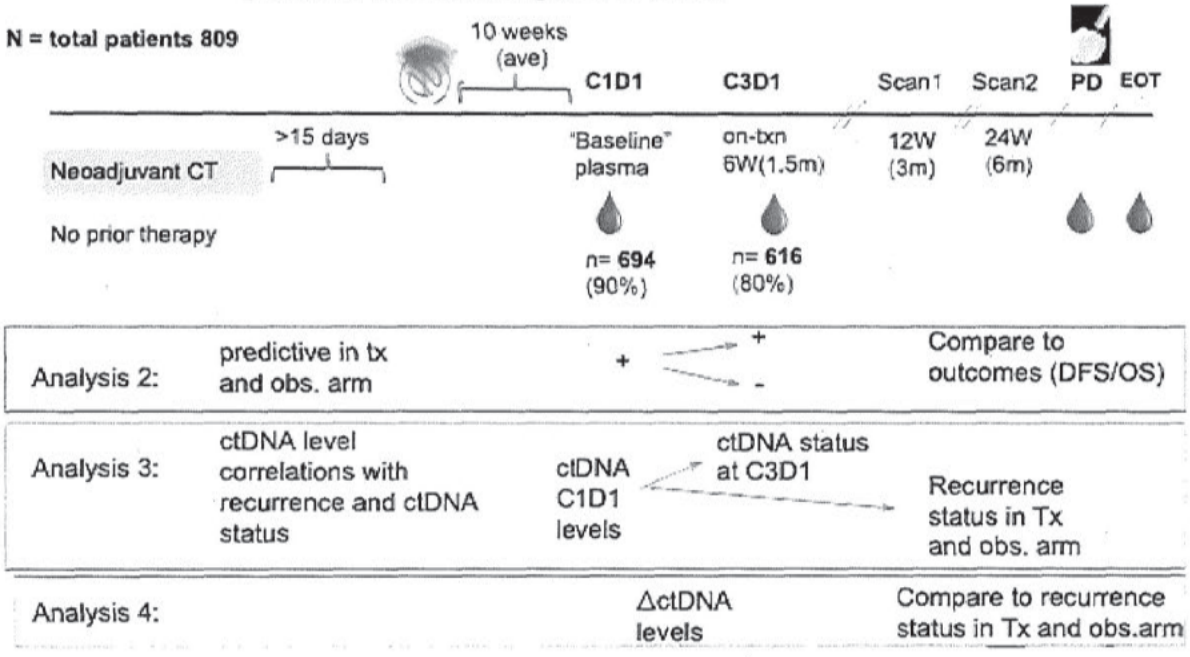
c) Analysis population: C1D1 BEP



2. (Prognostic question) Comparison of DFS by ctDNA status in the observation arm, the treatment arm (Primary Objectives #2 & #3)
    a) At the baseline time point (C1D1), compare ctDNA positive population vs ctDNA negative population
        - Analysis population: C1D1 BEP in the observation and treatment arm separately
    b) At C3D1, compare ctDNA positive population vs ctDNA negative population (
        - Analysis population: C3D1 BEP in the observation and treatment arm separately
    c) Combined ctDNA status of baseline and C3D1. ctDNA positive population will be defined as patients who have positive measure at C1D1 or C3D1.
        - Analysis population: C1D1+C3D1 BEP in the observation and treatment arm separately
    d) Combined ctDNA status of baseline and any available during treatment time point (including PD, EOT). During treatment ctDNA status is determined to be positive if one of the following occurs: i) all timepoints are positive (AND); ii) the latest time point is positive; iii) at least one time point is positive (OR).
    e) Also conduct the above analysis (1a, 1b, 1c) stratified by neoadjuvant treatment (yes/no), PDL1 status (pos/neg), lymph node positivity (yes/no), and ECOG (low/high).



3. Comparison of DFS by ctDNA clearance in the observation arm, the treatment arm, and . Hypothesis is that clearance occurs at a higher rate in atezo arm versus observation arm. (Primary Objective #4)
    - Clearance is defined as going from positive at C1D1 to negative at C3D1.
    - Analysis population: within C1D1+C3D1 BEP and patients C1D1 status must be positive.
    - Clearance rate is defined as the proportion of patients with C1D1 positivity which are negative at C3D1.



10.1.3 Univariate analysis for continuous ctDNA:

- Repeat the univariate analyses listed above for categorical ctDNA, but using continuous ctDNA (VAF and MTM/mL).
- C1D1 ctDNA level correlation with C3D1 clearance of ctDNA in the treatment arm: ctDNA levels considered as dependent variable and ctDNA status at C3D1 as independent variable.
    - Analysis population: C1D1 + C3D1 BEP in the treatment arm

B. C1D1 ctDNA level correlation with recurrence status in the treatment arm. ctDNA levels considered as dependent variable and recurrence status as independent variable.
    - Analysis population: C1D1 BEP

C. C1D1 ctDNA level correlation with radiological recurrence in the observation arm: ctDNA levels considered as dependent variable and recurrence status as independent variable.
    - Analysis population: C1D1 + C3D1 BEP; patients who have no C3D1 radiological assessment will be classified as PD

10.1.4 Multivariate analysis:

1. Multivariable Cox analysis including ctDNA as well as known clinical and pathological risk factors associated with DFS and OS.

10.2 Secondary Analyses

The secondary analysis is identical to the primary for the categorical ctDNA univariate analysis, except for OS instead of DFS.

10.2.1 Exploratory analyses

- Calculate the molecular lead time (MLT) as the difference between the dates of the first ctDNA positive status and the date of DFS event as determined by standard methods (radiological or biochemical).
- ctDNA status correlation with the PDL1 status
- Compare other molecular and clinical features with ctDNA status
    - Include prior neoadjuvant therapy and ctDNA status at C1D1

- Comparison of rates of ctDNA clearance/decrease among patients with and without adverse events (AEs), including but not limited to: AEs, Serious Adverse Events (SAEs), and immune related AEs (irAEs) (Note these types of AEs may overlap).
- Amount of cfDNA extracted per mL of plasma (ng/mL) for C1D1 time point compared to expected amount of 5-7 ng/mL and association with number of days post-cystectomy and ctDNA detection rates
- Amount of cfDNA extracted per mL of plasma (ng/mL) at any time point and association with ctDNA detection rates
- Type of tumor tissue sample used in WES for variant selection and correlation with ctDNA detection rates

- Pathological tumor purity for tumor tissue sample that underwent WES and correlation with ctDNA detection rates
- Somatic allele frequency of Signatera or custom assay variants and correlation with ctDNA detection rates

11. REFERENCES

1. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). Guidance for industry: clinical trial endpoints for the approval of cancer drugs and biologics. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/clinical-trial-endpoints-approval-cancer-drugs-and-biologics. Published December 2018. Accessed August 21, 2019.
2. Strimbu K, Tavel JA. What are biomarkers? *Curr Opin HIV AIDS*. 2010 Nov;5(6):463-6. doi: 10.1097/COH.0b013e32833ed177. PMID: 20978388; PMCID: PMC3078627.
3. SongfengWang, JiajiaZhang, WenbinLu. Sample size calculation for the proportional hazards model with a time-dependent covariate. *Computational Statistics and Data Analysis* 74 (2014) 217–227.
4. Survival analysis sample size calculator. http://www.sample-size.net/sample-size-survival-analysis/