Presence of Circulating Tumor DNA in Surgically Resected Renal Cell Carcinoma is Associated with Advanced Disease and Poor Patient Prognosis

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Background

- Circulating tumor DNA (ctDNA) has emerged as a promising, non-invasive biomarker for preclinical detection and monitoring of various cancers.[1-6]
- The utility of ctDNA assessment in renal cell carcinoma (RCC) is not well established.[2]
- Here we evaluate the potential of a bespoke, multiplex PCR, whole exome sequencing (WES)-based approach for ctDNA detection.

Methods

- A cohort consisted of 45 patients with stage Ib-IV RCC who underwent complete surgical resection.
- ctDNA was measured in plasma samples drawn pre-surgery (n = 37; baseline) and at post-operative time points (n = 44) using bespoke assay targeting patient-specific tumor variants.

Figure 1. Signatera Clinical Protocol

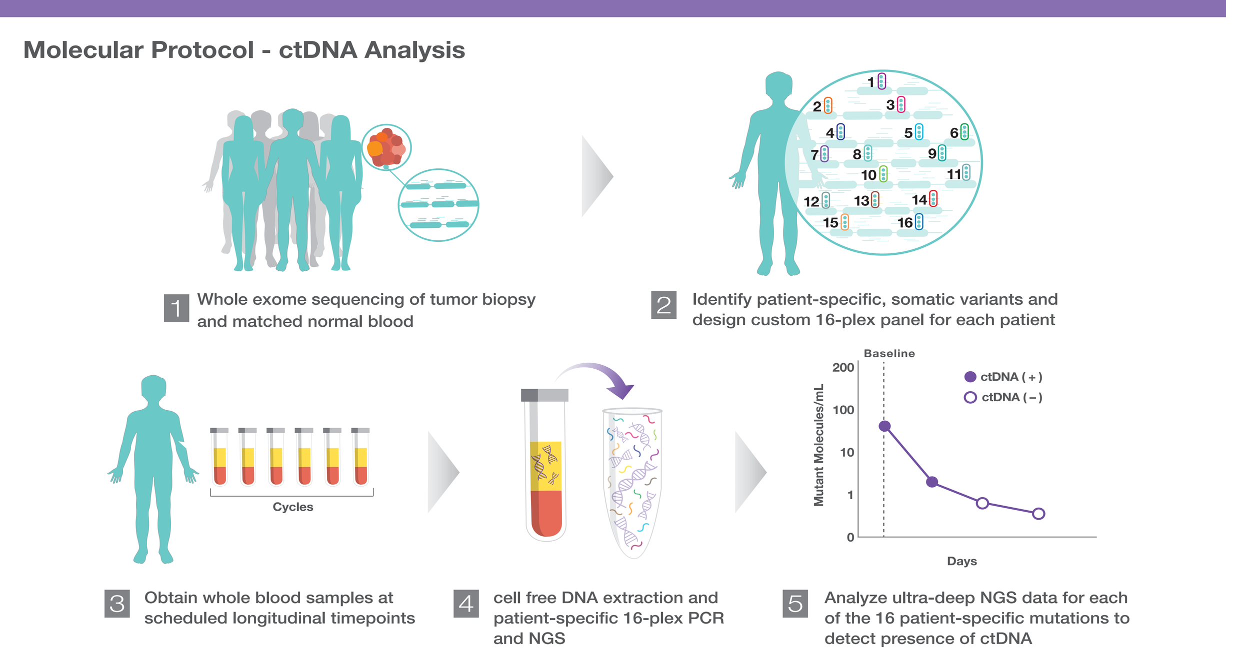

Table 1. Patient Demographics

Characteristics	All Patients (n = 45)	Characteristics	All patients (n = 45)
Sex, n (%)		**Clinical Stage, n (%)**	
Female	6 (13)	I, II	30 (67)
Male	39 (87)	III, IV	10 (22)
Median Age at Diagnosis, yrs	61 (36-73)	Unspecified	5 (11)
Tumor site, n (%)		**Recurrence at Any Site, n (%)**	
Clear Cell	42 (93)	Yes	27 (60)
Papillary	2 (5)	No	18 (40)
Sarcomatoid	1(2)		

Table 2. ctDNA Detection and its Association with Clinicopathological Characteristics

Detection of ctDNA in Plasma of Patients with Renal Cell Carcinoma			
ctDNA Detection	**Total, n (%)**	**ctDNA-negative, n (%)**	**ctDNA-postive, n (%)**
Pre-operative	37 (46)	19 (51)	18 (49)
Post-operative	44 (54)	32 (73)	12(27)
Association of Pre-surgical ctDNA Status with Clinicopathological Status			
Grade (n = 35)			
Low Grade (II)	11 (30)	8 (73)	3 (27)
High Grade (III & IV)	26 (70)	11 (42)	15 (58)
Average Tumor Size, cm (range)	**8 (2.9-17)**	**6.9 (2.9-12)**	**9.3 (4-17)**

Figure 2. Genetic Variants Most Frequently Observed in the Patient Cohort with Renal Cell Carcinoma

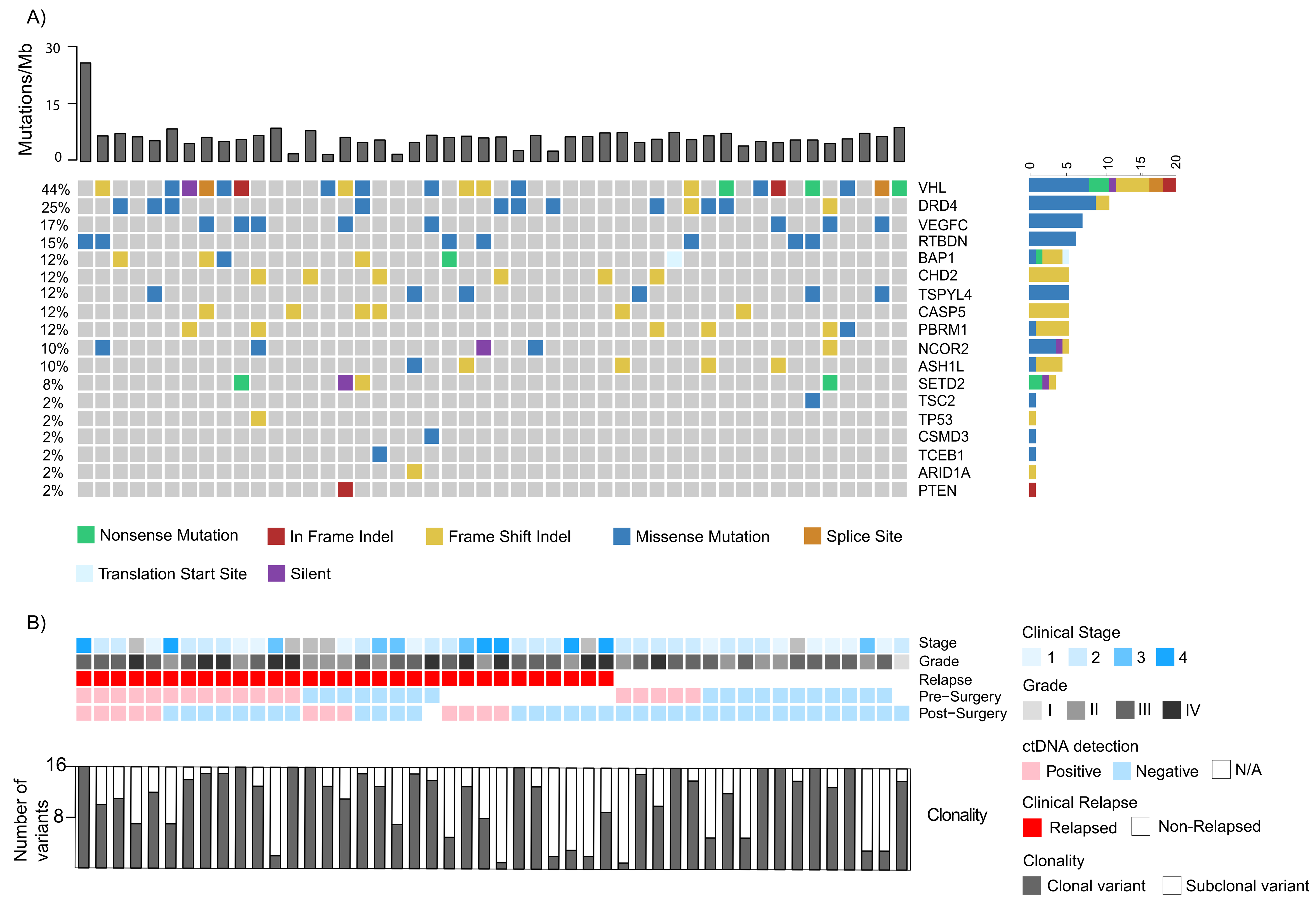

Figure 2. (A) Distribution of genetic alterations, tumor mutation burden; (B) Clinical characteristics, predicted clonality and ctDNA detection for each patient in the cohort with renal cell carcinoma

Figure 4. ctDNA Status and Clinicopathological Characteristics

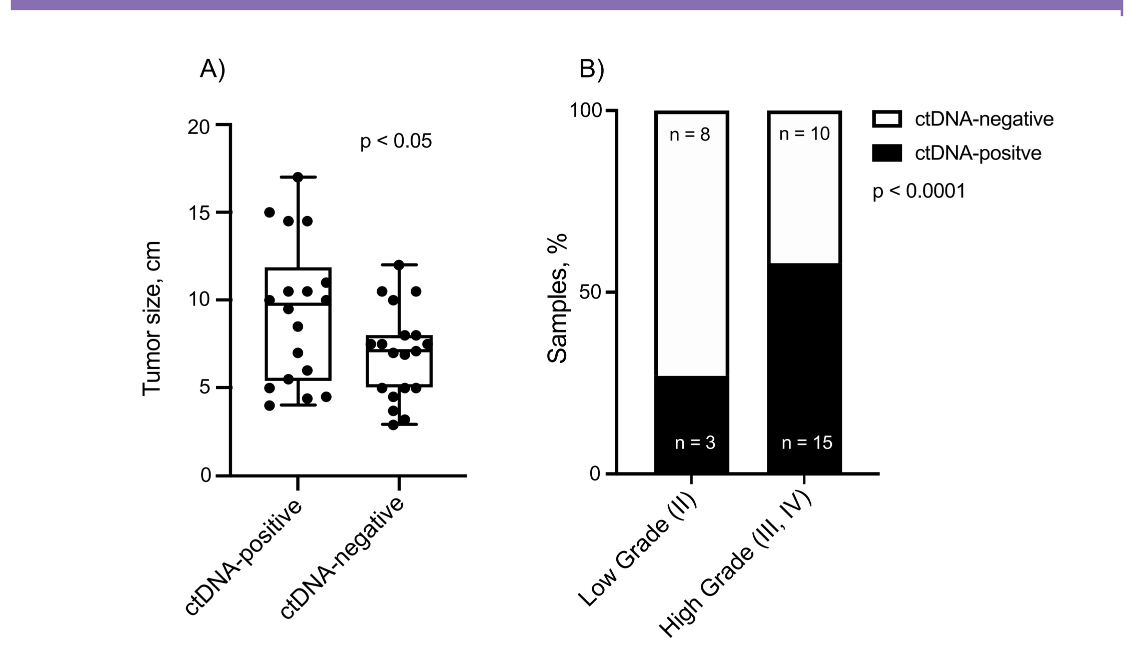

Figure 4. Presence of ctDNA in pre-operative plasma is significantly associated with increased tumor size (mean 9.3 vs. 7 cms, $p < 0.05$) and poorly differentiated tumors (grade III-IV vs. II, $p < 0.0001$)

Figure 5. ctDNA Status and Patient Survival

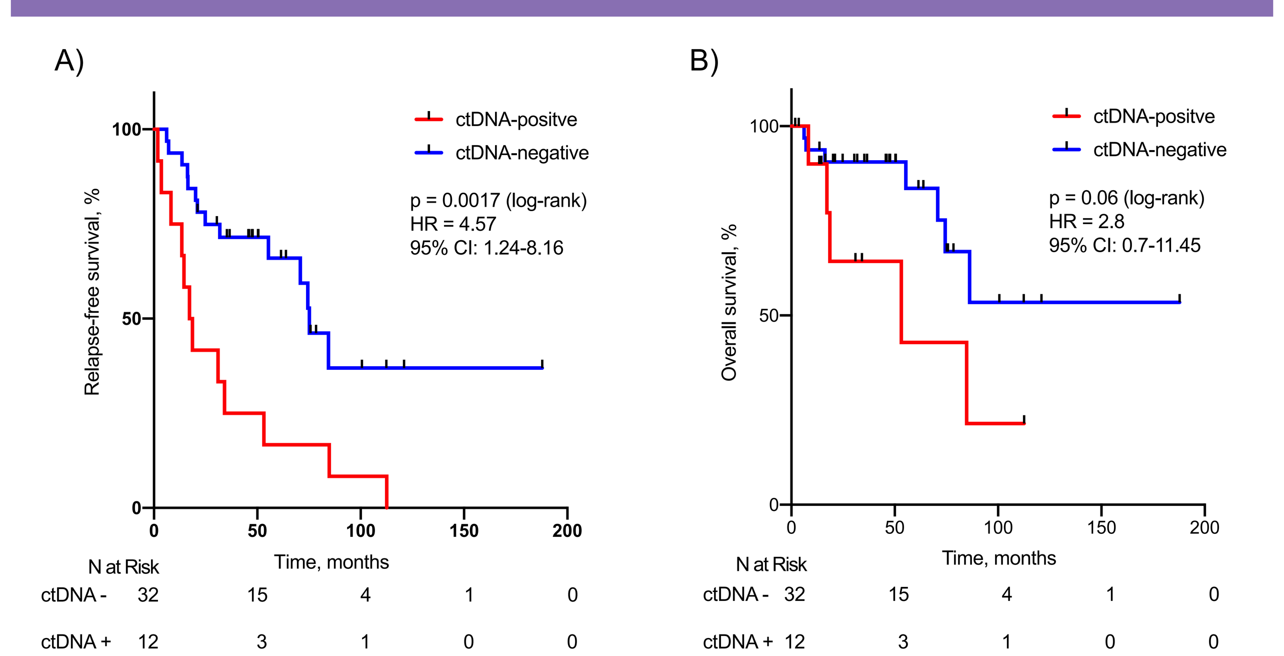

Figure 5. Presence of ctDNA in post-operative plasma sample is associated with reduced relapse free survival ($p < 0.0017$, HR = 4.57, 95% CI: 1.24 - 8.16). while overall survival was not observed to be not statistically significant ($p = 0.06$, HR = 2.8, 95% CI: 0.7 - 11.45).

Figure 3. Disease Recurrence and Survival of Patients with Detected ctDNA

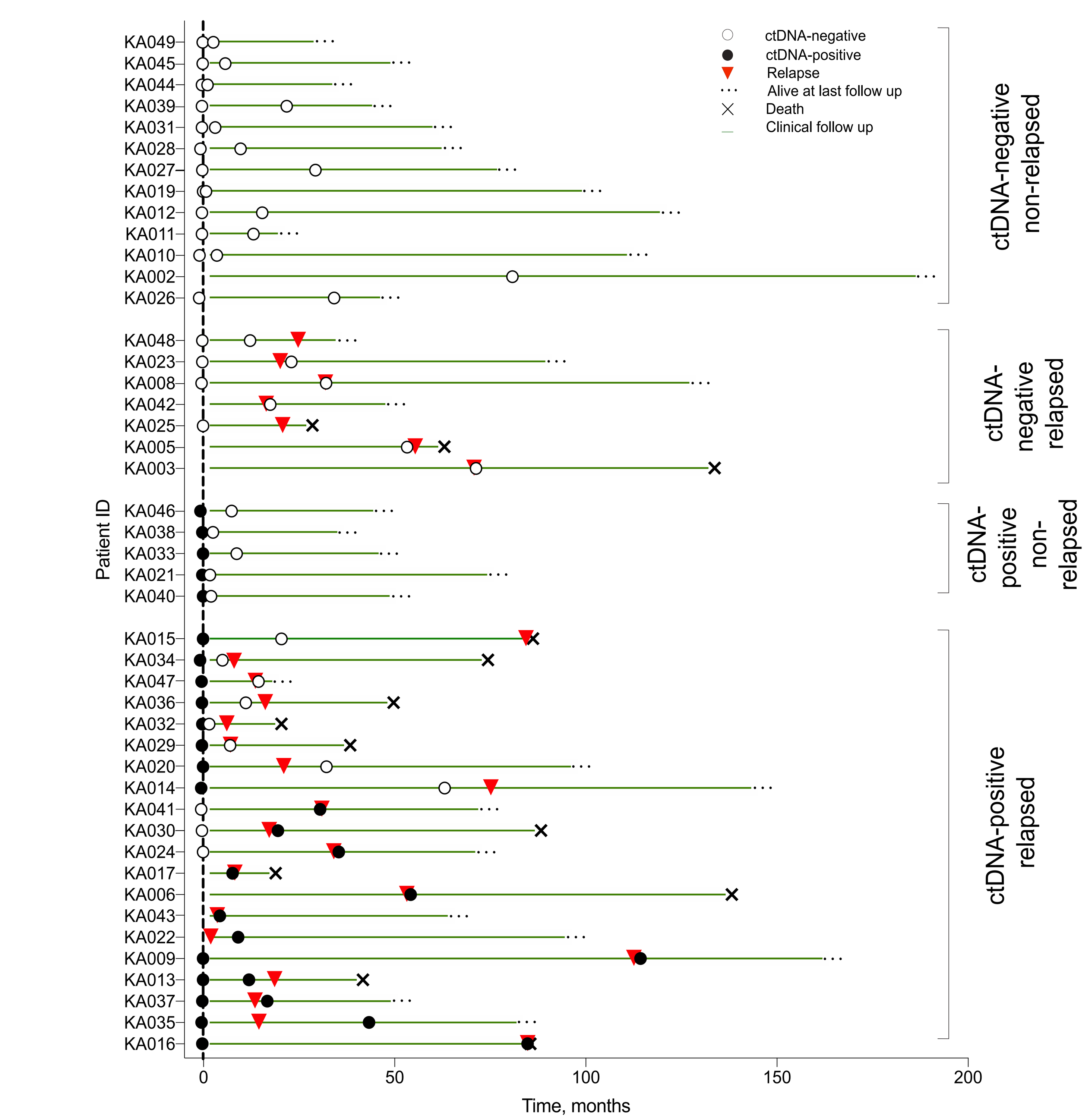

Figure 3. Baseline ctDNA was detected in 49% (18/37) of patients. In the post-operative setting, 100% (12/12) ctDNA-postive patients relapsed, while 47% (15/32) ctDNA-negative patients relapsed. None of the post-surgical samples from 18 non-relapsing patients were ctDNA-positive (specificity of 100%; median follow-up of 64 months).

Conclusions

- Presence of pre-surgical ctDNA strongly correlates with advanced grade RCC and increased tumor volume. Despite low plasma volumes, the bespoke assay detected ctDNA in 49% of baseline samples.
- Post-operative ctDNA presence is correlated with clinical recurrence. However, absence of ctDNA does not preclude recurrence as RCC is known to shed limited amounts of ctDNA.
- Higher sample volumes and multi-region tumor biopsies could enhance detection rates. This personalized approach has the potential to be used for ctDNA-based detection of recurrence in patients with advanced stage RCC.

References

1. Corcoran RB, et al. *N Engl J Med.* 2018; 379(18):1754-1765.
2. Maia MC, et al. *Kidney Cancer.* 2017; 1(1): 65-70.
3. Abbosh C, et al. *Nature*. 2017;545(7655):446-451.
4. Christensen E, et al. *J Clin Oncol.* 2019. 37(18):1547-1557.
5. Reinert T, et al. *JAMA Oncol.* 2019; 5(8):1124-1131.
6. Coombes RC, et al. *Clin Cancer Res.* 2019; 25(14):4255-4263.

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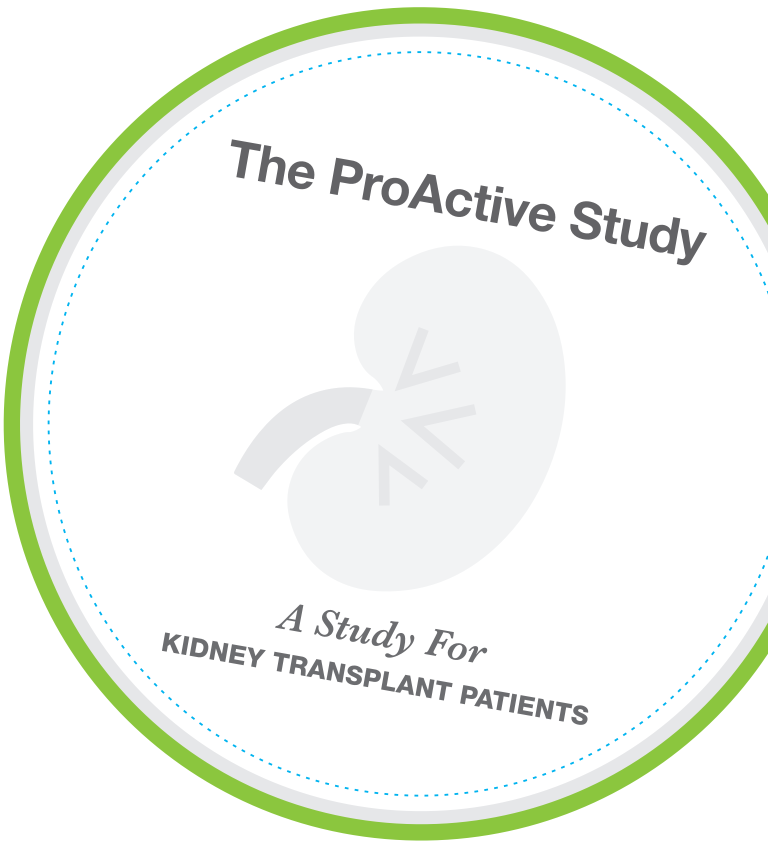
Partner with us to help your patients thrive and prosper

A kidney transplant can feel like getting another chance at life. That's why taking care of a patient's new kidney is of utmost importance.

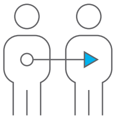


The ProActive Study For Kidney Transplant Patients

Prospera for Active Rejection

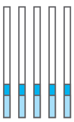
We can advance innovation in organ transplant care

Together with you and other partners in the ProActive Study, we will generate insights that enable earlier, more precise assessment of allograft rejection. Over time, these insights hold the promise to improve the lives of renal transplant patients everywhere.

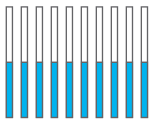


~20,000
Kidney transplants performed each year in the U.S.[1]

~190,000
People living with a kidney transplant in the U.S.[2]



~20–30%
Kidney transplant failures within 5 years[3]



~50%
Kidney transplant failures within 10 years[4]

ORGAN REJECTION IS A PROBLEM

Many kidney transplant failures occur within the first five to ten years[3,4] because organ rejection isn't caught early enough and treated effectively. As a result, the patient needs a new organ and must start the waitlist process again.

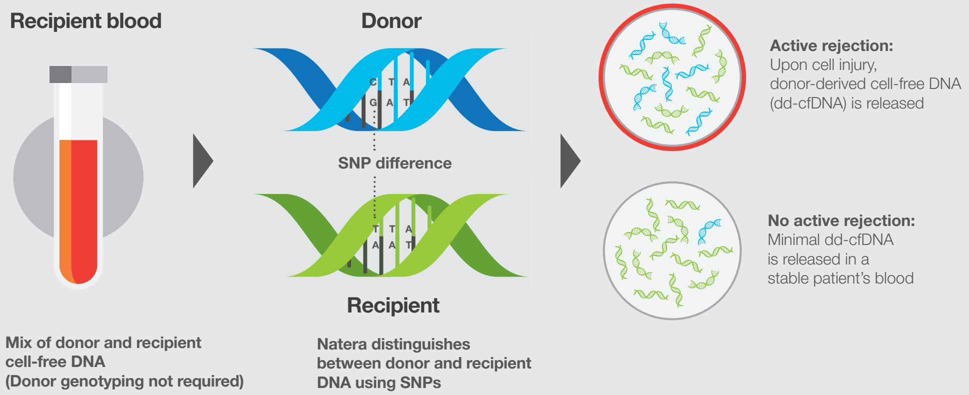
Let's improve the management of transplant patients

Prospera™ and the ProActive Study have the power and potential to change transplant patient management for good. The study will follow approximately 3,000 renal transplant patients over three years to evaluate the impact of the Prospera transplant assessment test across three critical measures:

1. **Management through effective use of biopsy**
2. **Outcomes including improved graft survival**
3. **Ability to identify subclinical and clinical rejection compared to serum creatinine**

Our core technology

Prospera is designed to assess kidney transplant damage by evaluating the percentage of donor-derived cell-free DNA (dd-cfDNA) in a transplant recipient's blood. Too much donor-derived dd-cfDNA in the recipient's blood is an early indication of potential organ rejection.



Supplementing the current standards of monitoring (i.e., regular biopsies, evaluation of serum creatinine), Prospera is intended to evaluate kidney rejection status to help you:

- **Eliminate unnecessary biopsies resulting from inaccurate tests**
- **Make treatment more timely**
- **Personalize immunosuppression therapy**

The Prospera transplant assessment test is:

Simpler and less invasive than biopsy; no donor genotyping required

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Suitable for most renal allograft patients across ethnicities

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More sensitive and specific for clinical and subclinical active rejection than serum creatinine[5]

PROACTIVE STUDY ELIGIBILITY

Inclusion Criteria	Exclusion Criteria
Must be willing to provide informed consent	Other non-kidney transplanted organ(s)
Must be a kidney transplant recipient	Pregnant
	Genetically identical donor organs

Together, we can help patients thrive and prosper

We need your help. Natera is applying its experience of performing over 1.5 million cell-free DNA tests in the prenatal setting to the complex world of transplantation. Thought leaders like you are essential to successfully advancing the management of post-kidney transplantation through innovation. Your participation in the ProActive Study contributes to the exploration of cell-free DNA's potential to assess the health of a transplanted organ.

1 Organ Donation Statistics. U.S. Department of Health and Human Services. U.S. Government Information on Organ Donation and Transplantation. https://www.organdonor.gov/statistics-stories/statistics.html. Published March 31, 2016.
2 Kidney Disease Statistics for the United States. National Institute of Diabetes and Digestive and Kidney Diseases. https://www.niddk.nih.gov/health-information/health-statistics/kidney-disease. Published Dec. 1, 2016.
3 Stegall et al,Through a Glass Darkly: Seeking Clarity in Preventing Late Kidney Transplant Failure, *J Am Soc Nephrol*. 2015; 26 (1):20-9
4 Lamb et al, Long-term renal allograft survival in the United States: a critical reappraisal, *Am J of Transplantation*. 2011; Mar;11(3):450-62.5. Altug, et al.
5 Sigdel TK, et al. *J. Clin. Med*. 2019, 8, 19.

This test was developed by Natera, Inc., a laboratory certified under the Clinical Laboratory Improvement Amendments (CLIA). This test has not been cleared or approved by the US Food and Drug Administration (FDA). Although FDA does not currently clear or approve laboratory-developed tests in the US, certification of the laboratory is required under CLIA to ensure the quality and validity of the tests.

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The ProActive Study For Kidney Transplant Patients

Prospera for Active Rejection

IMAGINE

a future where your kidney transplant patients live longer, healthier lives thanks to more timely actions to prevent kidney graft failure. Imagine relying on just a simple blood test for an earlier, more comprehensive picture of transplanted kidney health.

TOGETHER

we can provide important benefits for your transplant patients: truly meaningful hope for greater longevity and the best quality of life possible.

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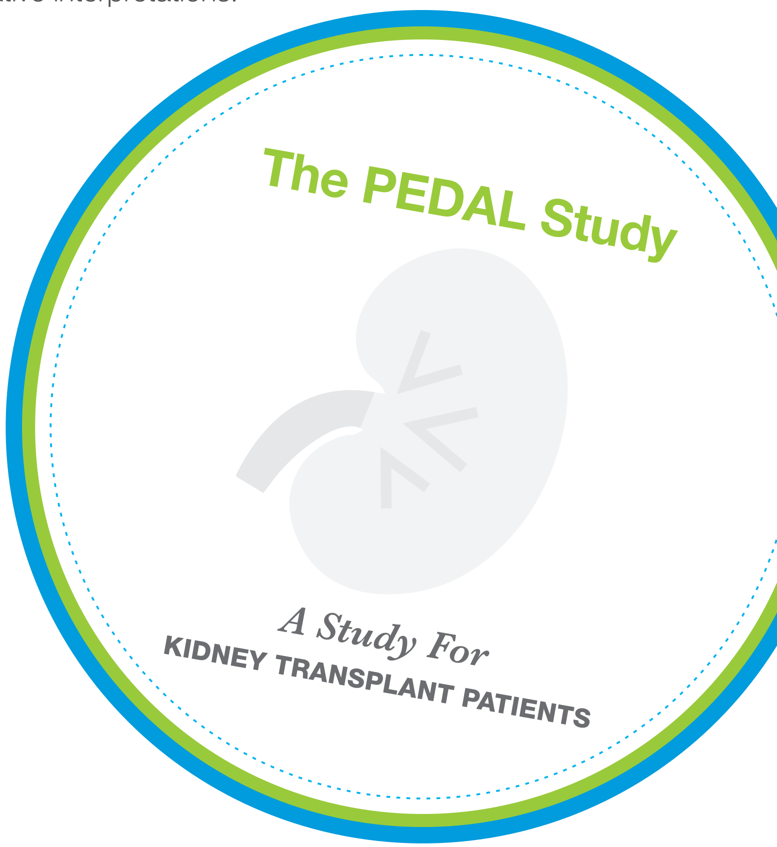
Explore a new technology for more-informed rejection assessment

Join us in understanding how the quantification of background cell-free DNA (cfDNA) may facilitate more precise rejection assessment and flag patients at high risk for false-negative interpretations.



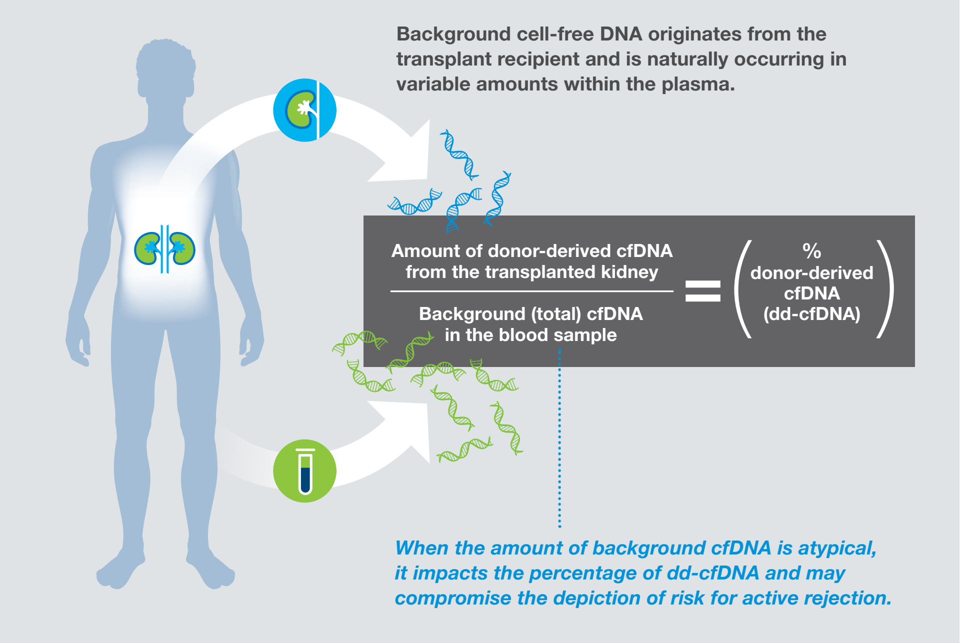
The PEDAL Study for Kidney Transplant Patients

Prospera **E**nhancement by **D**etecting Background Cell-free DNA **L**evels

PEDAL Study

Together with our study collaborators, we hope to gain a better understanding of how quantifying the absolute concentration of background cell-free DNA (cfDNA) may allow for a more precise and confident assessment of allograft rejection—especially in identifying patients at-risk of false-negative interpretations.

Defining background cell-free DNA and its influence on your result



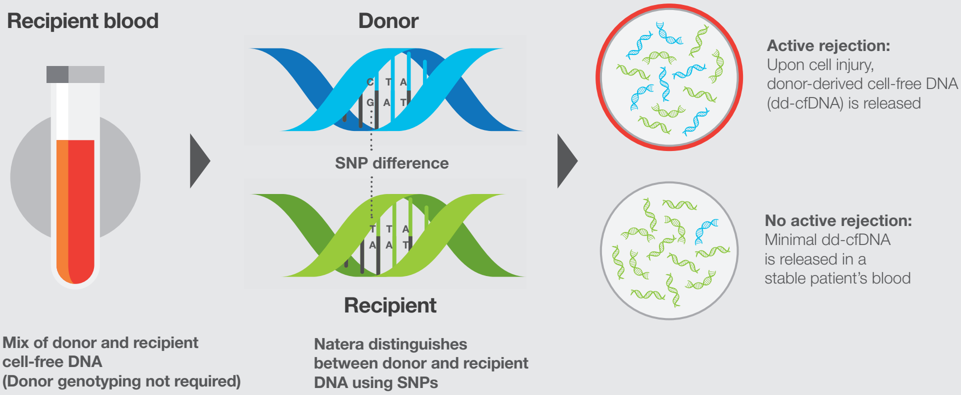
Let's understand how to more precisely assess for active rejection

Prospera™ and the PEDAL Study will delve deeper into how to better manage your transplant patients using cell-free DNA as a non-invasive biomarker for active rejection. The study will include 500 kidney transplant patients from 20 major U.S. centers to measure diagnostic capability of the update across three critical measures:

1. **Performance in both clinically indicated and surveillance biopsies**
2. **Ability to identify both antibody mediated rejection and T cell-mediated rejection**
3. **Correlation against clinical and/or histological resolution of rejection**

Prospera's core technology

Leveraging our findings from two million cfDNA tests, Prospera is designed to assess kidney transplant injury by evaluating the percentage of donor-derived cell-free DNA (dd-cfDNA) in a transplant recipient's blood. Too much dd-cfDNA in the recipient's blood is an early indication of potential organ rejection.



Based on our leadership in cfDNA innovation, Natera has now introduced two novel techniques for even greater precision in Prospera results:

Two novel techniques for greater precision

1.

Proprietary Library Preparation

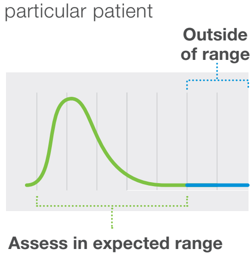
Proprietary library preparation step that adjusts for background cfDNA introduced into the sample during transport

+

2.

Quantifed Background CfDNA

Unique quantification method that can identify atypical background cfDNA that may influence the reported result for a particular patient



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Potentially fewer false negative interpretations

PEDAL STUDY ELIGIBILITY

Inclusion Criteria	Exclusion Criteria
Must be willing to provide informed consent	Cannot have other non-kidney transplanted organ(s)
Must be a kidney transplant recipient	Cannot be pregnant
	Cannot have genetically identical donor organs

We need your help to enable patients to thrive and prosper

With Prospera, we delivered a non-invasive way to identify rejection, giving you greater confidence in making treatment decisions for your organ transplantation patients. But we can do more—we're committed to continue refining this test to support you in bringing hope to these patients. Starting now.

Through PEDAL and other studies, we look to you as our partner in delivering innovations that offer a second chance to patients. Because together, we can make meaningful changes—in individual lives and the field of organ transplantation.

1 Organ Donation Statistics. U.S. Department of Health and Human Services. U.S. Government Information on Organ Donation and Transplantation. https://www.organdonor.gov/statistics-stories/statistics.html. Published March 31, 2016.
2 Kidney Disease Statistics for the United States. National Institute of Diabetes and Digestive and Kidney Diseases. https://www.niddk.nih.gov/health-information/health-statistics/kidney-disease. Published Dec. 1, 2016.
3 Stegall et al,Through a Glass Darkly: Seeking Clarity in Preventing Late Kidney Transplant Failure, *J Am Soc Nephrol*. 2015; 26 (1):20-9
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5 Sigdel TK, et al. *J. Clin. Med*. 2019, 8, 19.

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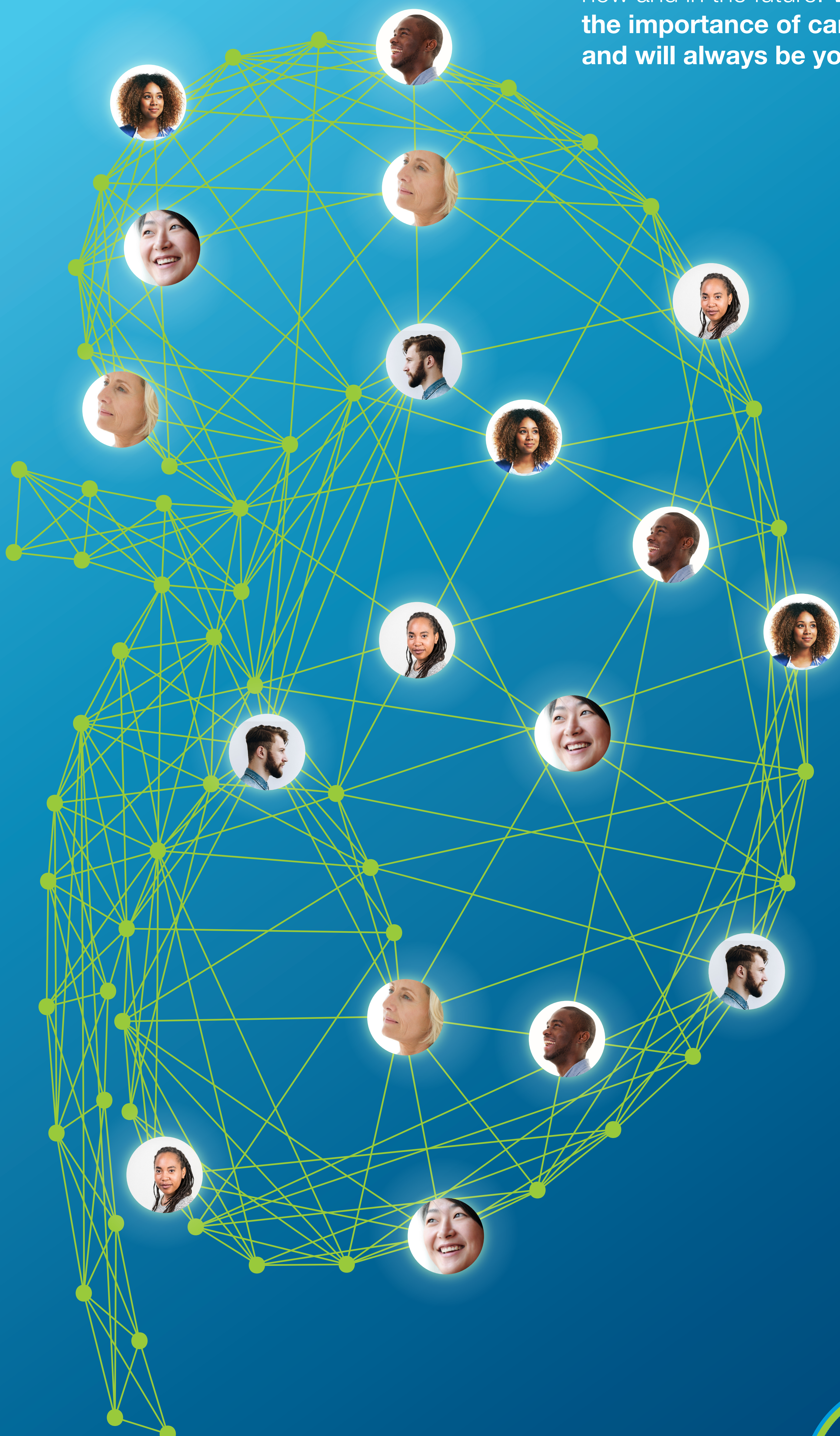
The PEDAL Study for Kidney Transplant Patients

Prospera **E**nhancement by **D**etecting Background cfDNA **L**evels

A kidney transplant is the greatest gift that can be given or received. Such a precious treasure must be cared for with the utmost diligence and attention.

A precise, non-invasive biomarker for rejection gives you the confidence you need to know that all is well. Natera will continue to refine and improve Prospera now and in the future. **We understand the importance of caring for patients and will always be your best partner.**



The PEDAL Study
A Study For
KIDNEY TRANSPLANT PATIENTS

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natera™ | Signatera™ Residual disease test (MRD)

Seeing beyond the limit: Detect residual disease and assess treatment response

Summary of analytical and clinical data for Signatera™, the first ctDNA assay custom-built for MRD detection and treatment response

Introduction

Non-invasive monitoring of circulating tumor cells and molecular alterations has been an established method of detecting minimal residual disease in hematologic malignancies.[1,2] However, progress in blood-based monitoring for solid tumors has been limited by the rarity and heterogeneity of circulating tumor cells and the extremely low concentrations of circulating tumor DNA (ctDNA) in the blood.[3] With recent advances in next generation sequencing (NGS) technologies, there has been renewed interest in using ctDNA profiling as a tool for detecting residual disease, detecting relapse early, and assessing treatment response in solid tumors.[3,4]

Signatera™ is the first ctDNA assay that has been custom-built for detecting molecular residual disease (MRD) and assessing treatment response, with the ability to detect ctDNA at a variant allele frequency (VAF) of <0.1% of cell free DNA (cfDNA) from plasma.[5-9] This white-paper reviews the differences between Signatera and other ctDNA detection assays, summarizes data from the analytical and clinical validation of Signatera, and presents potential research applications of Signatera in clinical studies.

The Signatera approach

Currently available assays for detecting ctDNA from patient plasma tests for a fixed panel of hotspot or actionable mutations. Given the heterogeneity of cancer, even large generic panels targeting up to more than a hundred of genomic loci might detect only a few mutations from a given individual's primary tumor.[10-12] Mutations identified in these panels may not be tumor derived, making such approaches less specific.[10-12] Additionally, the plasma-level VAF limit of detection (LOD) starts at approximately 0.1–1% for these conventional technolgies, which is 10–fold lower than the 0.01% VAF LOD achieved with Signatera.[13-17]

For the Signatera approach, somatic variants are identified by whole–exome sequencing of the primary tumor and the matched normal (whole blood) sample. Following this, a bespoke assay of 16 clonal, somatic variants are generated for each patient. The resulting "tumor signature," individualized to each patient's tumor, is monitored throughout the patient's disease course to detect the presence of tumor DNA in the plasma.[6-9]

There are several advantages with the Signatera approach. Compared to other ctDNA approaches, analytical sensitivity and specificity of Signatera is enhanced due to improved library preparation and molecular recovery, significantly reduced PCR error, and advance knowledge of specific variants present in a patient's tumor. Furthermore, focusing on patient-specific variants enables ultra-deep sequencing (100,000X average depth of coverage) of each target to obtain a high level of confidence for a positive-ctDNA call, effectively lowering the limit of detection into the single–molecule range. The limit of detection for Signatera, measured in VAF, is 0.01%. This is equivalent to one mutant haploid genome in a background of 10,000 normal haploid genomes. Signatera is optimized to achieve high analytical specificity of >99.5%. Combining a low limit of detection and advanced knowledge of clonal, tumor-specific variants is how Signatera achieves high sensitivity and specificity in ctDNA detection.[6]

Steps in the Signatera process

To monitor for cancer recurrence or to detect residual disease with ctDNA, the Signatera process starts with whole exome sequencing of the tumor tissue and the buffy coat from matched normal whole blood for each patient. Based on sequencing results, a list of somatic single-nucleotide and indel variants specific to each patient are bioinformatically identified and prioritized. Next, 16 somatic single-nucleotide and indel variants for multiplex PCR primer design are selected based on several factors, including the clonality, detectability, and frequency of the variants identified in the tumor tissue DNA. For longitudinal surveillance, cell free DNA (cfDNA) libraries are prepared from each blood sample, followed by patient-specific, 16-plex PCR. The amplicon products are tagged with sequencing barcodes and pooled for ultra-deep next generation sequencing, followed by data analysis to detect the presence or absence of ctDNA.[6]

Steps in the Signatera work flow are outlined below (figure 1):

Step 1: Primary tumor tissue and matched normal blood are collected from each patient. Genomic DNA from tumor tissue and buffy coat are extracted, whole-exome sequenced, analyzed, and filtered for patient-specific somatic mutations.

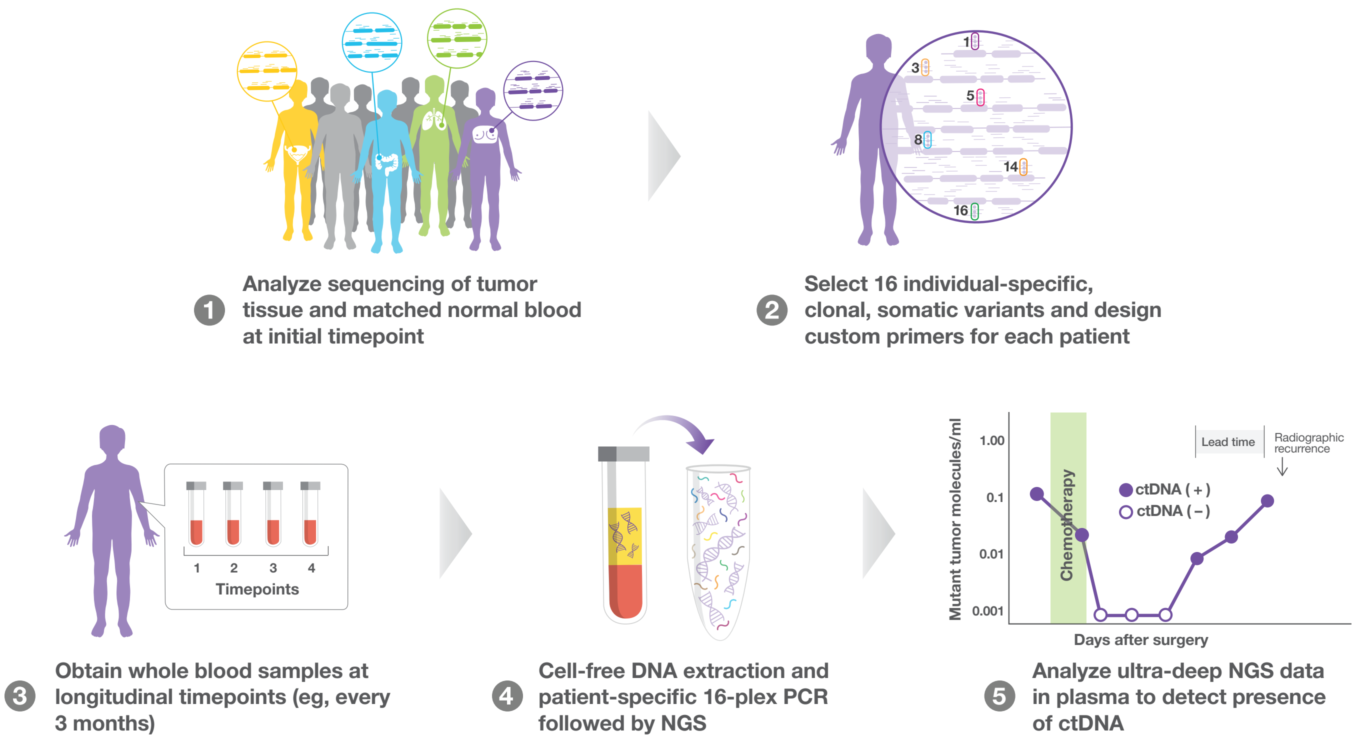
Step 2: The top 16 somatic variants are selected based on clonality, detectability, and frequency of mutations. Multiple-PCR compatible primers are designed for each of the 16 somatic variants.

Step 3: Whole blood is collected at predefined time points for longitudinal surveillance.

Step 4: Plasma are isolated and cfDNA extracted, followed by assaying with the patient-specific 16-plex PCR pool.

Step 5: Following multiplex PCR amplification, ultra-deep sequencing is performed. Next-generation sequencing data are analyzed to detect the presence of ctDNA.

Figure 1: Signatera workflow



Analytical validation[6,14,29]

Signatera analytical validation was performed using two different sets of titration samples built from a) mononucleosomal DNA from cancer cell lines, and b) a commercially available mutation mixture from SeraCare, “SeraseqTM ctDNA Mutation Mix v2.”

Mononucleosomal DNA from three cancer cell lines, including two breast cancer cell lines (HCC2218, HCC1395) and one lung cancer cell line (NCI–H1395), were titrated into their matched normal B lymphoblast-derived counterparts (HCC2218–BL, HCC1395–BL, and NCI–H1395–BL, respectively). Titrations of tumor into normal mononucleosomal DNA were made at average VAFs (based on DNA input) of 1%, 0.5%, 0.3%, 0.1%, 0.05%, 0.03%, 0.01%, 0.005%, 0%. Six primer pools were tested with replicate numbers from two to nine (for each pool)—increasing with the dilution factor. In addition, a commercially available control SNV mixture (SeraseqTM ctDNA Mutation Mix v2) was titrated from 0.5% to 0.005%. Starting allele fractions were confirmed by SeraCare by droplet digital PCR. Two primer pools were tested in triplicate on these mixtures.

The starting total input into library prep for each reaction was 15,000-20,000 haploid genome equivalents. SNV targets from the corresponding tumor DNA spike-in samples were amplified using the 16-plex-PCR assay primer pools. The mPCR products were tagged with sequencing barcodes, then pooled with other mPCR barcoded products, and subsequently sequenced on an Illumina HiSeq 2500 Rapid Run with 50 cycles of paired-end reads using the Illumina Paired End v2 kit with an average read depth of ~100,000/target.

The sample-level performance was derived by calculating a binomial probability for detecting at least two clonal mutations at a given ctDNA level, assuming that the majority of the custom panels have between 10 to 16 clonal variants. As shown in Table 1, ctDNA would be detected in samples with ctDNA between tumor DNA concentration of 0.01% and 0.02% for >98% of samples. Reproducibility was calculated as the percent coefficient of variation (%CV) of the median VAF of positive targets. Sample-level performance calculated from orthogonal control samples from SeraCare is shown in Table 2.

Table 1: Sample-level performance calculated when at least 2 variants are detected from a set of 16 target SNVs

Tumor DNA Concentration (%)	Tumor DNA Concentration Range (%)	Sensitivity Per Sample (%)	CV of Median VAF (%)	Data Points (n)
0.00375	0.0025-0.005	44.7-70.8	69.1	501
0.0075	0.005-0.01	58.9-83.3	44.2	562
0.015	0.01-0.02	98.5-100.0	23.8	474
0.025	0.02-0.03	99.9-100.0	25.1	278
0.04	0.03-0.05	100	17.6	289
0.0625	0.05-0.075	100	7.8	153
0.0875	0.075-0.1	100	16.1	72
0.2	0.1-0.3	100	10.1	268
0.4	0.3-0.5	100	6.4	120
0.75	0.5-1.0	100	6.6	117

Table 2: Sample-level performance calculated from orthogonal control samples from SeraCare

Tumor DNA Concentration (%)	Sensitivity Per Sample(%)	CV of Median VAF (%)	Data Points (n)
0.005	35.0-59.9	33.8	90
0.01	81.3-96.1	51.0	90
0.03	100	25.5	90
0.05	100	16.2	90
0.1	100	14.3	90
0.2	100	14.6	90
0.5	100	9.4	90

Designed without the need for molecular barcodes

Assays for low quantities of ctDNA detection often use molecular barcoding followed by hybrid capture as an approach to decrease error rates caused by process and sequencing-related artifacts. Molecular barcoding, also known as unique identifiers (UIDs), enable tagging and tracking of individual DNA molecules to distinguish somatic mutations from artifact mutations generated during the PCR and sequencing process. During research and development of Signatera, molecular barcoding approaches were also explored. However, use of molecular barcodes was found to sacrifice sensitivity without improving specificity, ultimately considered unnecessary, and not incorporated into the Signatera methodology.

There are several reasons why sensitivity and specificity may be compromised by approaches that utilize molecular barcoding, including hybrid capture and one-sided PCR:

- The depth and uniformity of sequencing is poor with hybrid capture, which decreases the quality of data across target sets. Specificity can also be variable across targets with non-uniform depth of read. At a minimum, a 5X to 10X sequencing depth per target input molecule is required to distinguish errors from mutations in the original target
- Hybrid capture has been reported to cause DNA oxidative damage, such as 8-oxoguanine and cytosine deamination, which could lead to false positive results[19-21]
- We have observed the formation of chimeric molecules from the hybrid capture process, which can appear as an original target molecule and contribute to false positive calls. In principle, chimeric molecules should also occur with 1-sided PCR approaches and lead to false positive calls.
- The use of molecular barcodes is not robust enough for error correction with respect to input mass in hybrid capture and one-sided PCR approaches. In cases where there are low concentrations of ctDNA and more input mass is required, specificity will suffer

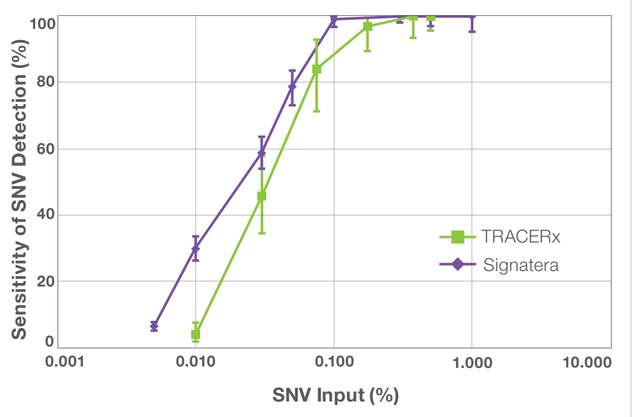
Due to the reasons above, Natera has developed other methods in the workflow to optimize the sensitivity and specificity of Signatera without use of molecular barcoding.

Signatera prototype in the TRACERx study

A prototype of the Signatera assay was used in the non-small cell lung cancer (NSCLC) TRACERx study to phylogenetically profile and detect cancer tumor DNA recurrence up to 346 days earlier than standard radiological confirmed relapse.[5] A sensitivity of 93%, with no false positives, was demonstrated for early relapse detection in patients with stages I–III lung cancer. When results produced in the TRACERx study for the same lung cancer cohort were compared with a generic lung panel, TRACERx identified 10 out of 10 ctDNA positive early-stage lung cancer cases compared to 7 out of 10 cases detected using a generic hotspot lung panel.[5]

Following the TRACERx study publication, multiple improvements have been made to produce a more automated and scalable work flow with higher molecular recovery and lower error. The analytical validation for the assay in TRACERx was performed using synthetic SNV spikes and mixing libraries directly into the multiplex PCR test, which does not account for molecular loss during library preparation or differing DNA fragmentation. In contrast, the analytical validation for Signatera started with cfDNA mixtures from the tumor and matched normal samples during the library prep process. A comparison of the analytical sensitivity between the prototype and Signatera, as shown in Figure 2, demonstrates the sensitivity improvements that have been made since the TRACERx study at tumor DNA concentration levels below 0.5%.[5,6,18]

Figure 2: Comparison of Analytical Sensitivity for Single SNV Detection between TRACERx and Signatera



Pan-cancer clinical summary of Signatera

Since the TRACERx study in lung cancer, the potential clinical usefulness of Signatera has also been demonstrated in colorectal, breast, and bladder cancers (tables 3-5, figures 3-5).[7-9]

Table 3: Study overview of Signatera in colorectal cancer[7]

Colorectal Cancer Study Overview	
N	125
CRC stage	I to III
Prior treatment received	Received curative surgery and adjuvant chemotherapy
# of blood time points	795
# of relapses	16
Average lead time	8.7 Months
Median lead time	10.1 Months
Maximum lead time	16.5 Months
Specificity	98%

Table 4: Study overview of Signatera in breast cancer[9]

Breast Cancer Study Overview	
N	49
Breast cancer stage	I to III
Subtype (n)	HR+/HER2-=34 HER2+= 8 TNBC= 7
Prior treatment received	Adjuvant chemotherapy within 3 years of study entry
# of blood time points	208
Average lead time	9.5 months
Median lead time	8.9 months
Maximum lead time	2 years
Specificity	100%

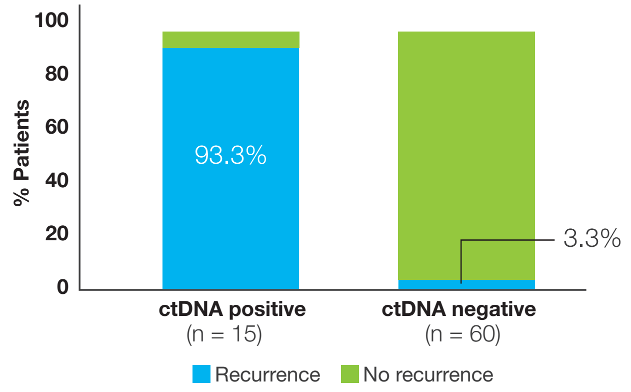
Table 5: Study overview of Signatera in muscle-invasive bladder cancer (MIBC)[8]

MIBC Study Overview	
N	68
Prior treatment received	Received neoadjuvant or first-line chemotherapy before cystectomy
# of blood time points	656
# of relapses	16
Average lead time	2.8 Months
Median lead time	3.2 Months
Maximum lead time	8.2 Months
Specificity	100%

Figure 3. Relapse-free survival of local or regionally advanced CRC patients, stratified by ctDNA status
Detection of positive ctDNA after surgery and after adjuvant chemotherapy is associated with higher risk of relapse.

- Molecular relapse was detected at an average of 8.7 months before clinical relapse

A. Post-surgical detection of minimal residual disease



B. Relapse-free survival after surgery and adjuvant chemotherapy

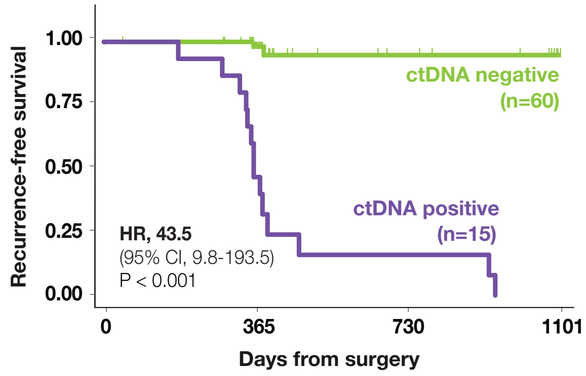
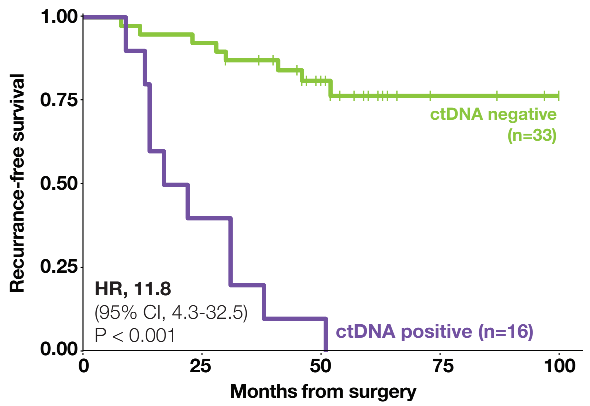


Figure 4. Relapse-free survival of local or regionally advanced breast cancer patients, stratified by ctDNA status
Detection of positive ctDNA after surgery and after adjuvant chemotherapy is associated with higher risk of relapse.

- Molecular relapse was detected at an average of 9.5 months

A. ctDNA status at first timepoint



B. ctDNA status at any timepoint

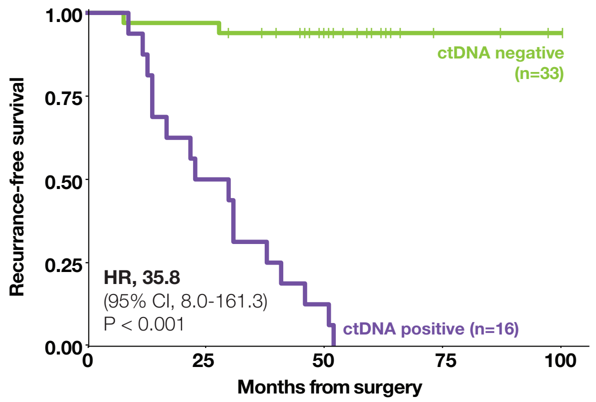
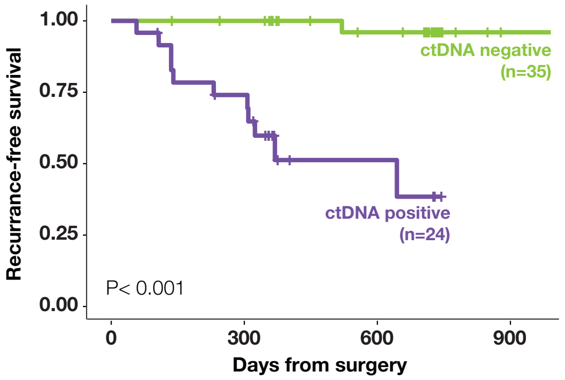


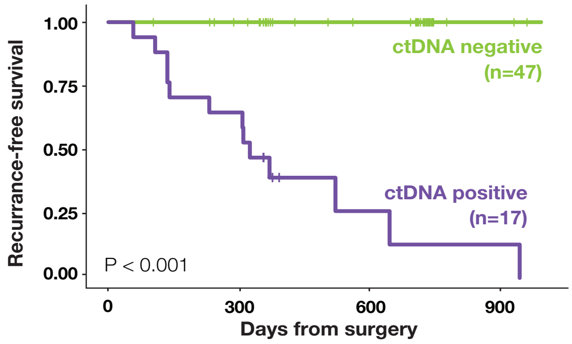
Figure 5. Relapse-free survival of MIBC patients, stratified by ctDNA status
Significantly shorter relapse-free rates were observed for patient who are ctDNA-positive at diangnosis and post cystectomy.

- Molecular relapse was detected at an average of 2.8 months before clinical relapse

A. ctDNA status before chemotherapy



B. ctDNA status after cystectomy



Potential applications of Signatera in the clinical setting and in drug development

Unlike other ctDNA assays that are being developed for early cancer detection or as a tissue biopsy replacement for identifying actionable mutations in metastatic disease, applications of Signatera ctDNA analysis in the clinical setting could include predicting the likelihood of relapse at diagnosis, monitoring response to neoadjuvant treatment, detecting minimal residual disease, monitoring for recurrence after adjuvant treatment, or monitoring for treatment resistance (table 6, figure 6).

In drug development, ctDNA status determined by Signatera may potentially be used as an entry criterion for clinical trial enrollment. Enriching for patients based on ctDNA status, which correlates with the likelihood of relapse or treatment response, may have implications in decreasing sample sizes, shortening time to completion, minimizing total cost, and increasing the likelihood of trial success. Additionally, ctDNA detection at different time points during the trial may potentially complement Response Evaluation Criteria in Solid Tumors (RECIST) as a measurement of treatment response.

Conclusions

Advances in next-generation sequencing technology have now made it possible to detect low quantities of ctDNA.[4,13] Signatera is the first individualized, pan-cancer ctDNA platform that has potential applications in oncology for detecting MRD and monitoring treatment response with a low limit of ctDNA detection. Signatera has been analytically validated as a highly sensitive and specific platform for detecting and quantifying patient-specific, somatic SNVs and indels at a VAF of 0.01%.[6] Analytical and clinical results across different tumor types suggest that Signatera may have wide-ranging clinical and drug development applications.[5-9]

Table 6: Proposed applications of Signatera

Applications	Signatera results	Implications	Trial designs	Example
Molecular residual disease after surgery	ctDNA-positive	Increased rate of relapse	Trials combining novel therapy and adjuvant therapy	Addition to SOC of anti-PD-1/PD-L1 vs placebo for NSCLC patients who are ctDNA-positive
	ctDNA-negative	Decreased rate of relapse	Trials to avoid or de-escalate treatment	
Recurrence monitoring	ctDNA-positive	Increased rate of relapse	Trials to increase surveillance during recurrence monitoring, showing correlation to outcomes	Imaging every 3 months vs 6 months in ctDNA-positive patients
Molecular relapse after adjuvant treatment	ctDNA-positive without evidence of clinical relapse	>98% certainty for clinical relapse without additional therapy	Trials to treat with additional therapy prior to clinical relapse	Extending duration of adjuvant therapy in patients who are persistently ctDNA-positive after finishing standard course of treatment
Treatment monitoring	ctDNA levels increasing or unchanged	Increased rate of therapy failure	Trials to correlate ctDNA-negative status after treatment with decrease in RFS or improvement in PFS	ctDNA to predict response to I-O therapy, distinguish pseudo-progression from true progression, and determine early trial readout
	ctDNA levels decreasing or undetectable	Decreased rate of therapy failure		

Figure 6: Applications of Signatera in detecting ctDNA

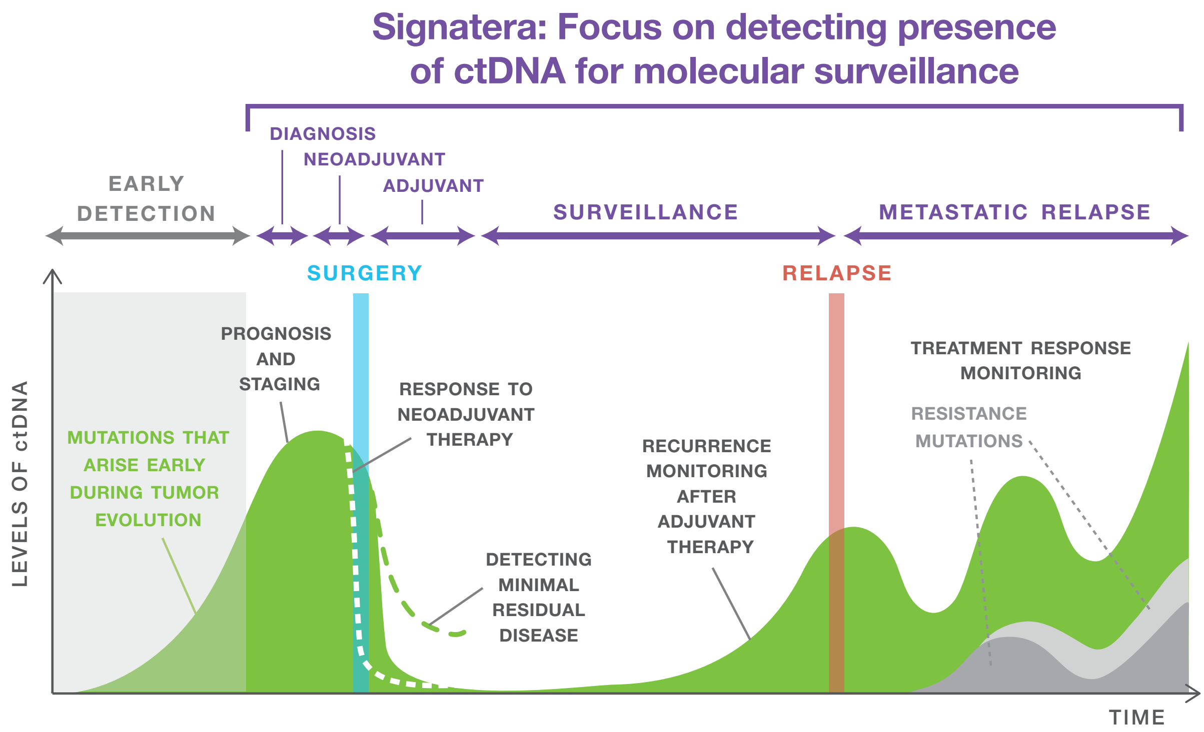
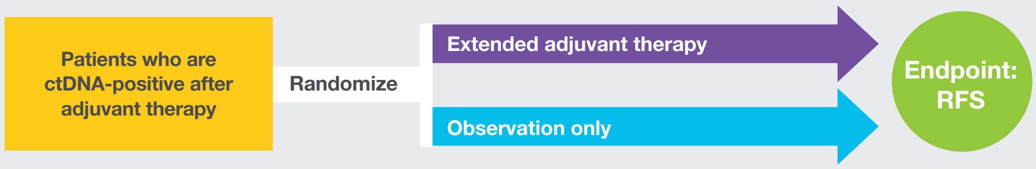


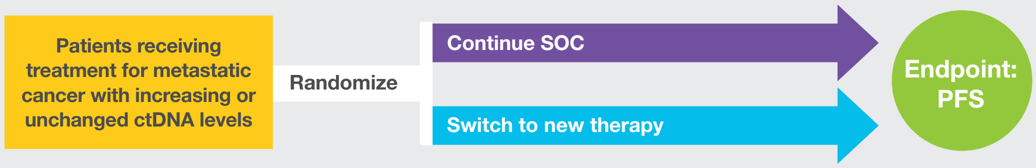
Figure 7: Potential clinical trial designs with Signatera

Trials to combine novel therapy with standard of care (SOC) adjuvant therapy for patients with high risk of clinical relapse

Trials to identify early-stage breast cancer patients with high risk of clinical relapse and treat with extended adjuvant therapy



Trials in metastatic cancer to correlate ctDNA-negative status after treatment with outcome



References

1. Paiva B, van Dongen JJ, Orfao A. New criteria for response assessment: role of minimal residual disease in multiple myeloma. *Blood.* 2015;125(20):3059-3068.

2. Brüggemann M, Raff T, Kneba M. Has MRD monitoring superseded other prognostic factors in adult ALL? *Blood.* 2012;120(23):4470-4481.

3. Abbosh C, Birkbak NJ, Swanton C. Early stage NSCLC — challenges to implementing ctDNA-based screening and MRD detection. *Nat Rev Clin Oncol.* 2018;15(9):577-586.

4. Han X, Wang J, Sun Y. Circulating tumor DNA as biomarkers for cancer detection. *Genomics Proteomics Bioinformatics.* 2017;15(2):59-72.

5. Abbosh C, Birkbak NJ, Wilson GA, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature.* 2017;545(7655):446-451.

6. Sethi H, Salari R, Navarro S, et al. Analytical validation of the Signatera™ RUO assay, a highly sensitive patient-specific multiplex PCR NGS-based noninvasive cancer recurrence detection and therapy monitoring assay. In: Proceedings from the American Association for Cancer Research Annual Meeting; April 17, 2018; Chicago, IL. Abstract 4542.

7. 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 [published online ahead of print May 9, 2019]. *JAMA Oncol.* 2019. doi:10.1001/jamaoncol.2019.0528.

8. 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 [published online ahead of print May 6, 2019]. *J Clin Oncol.* 2019. doi: 10.1200/JCO.18.02052.

9. Coombes RC, Page K, Salari R, et al. Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence [published online ahead of print April 16, 2019]. *Clin Cancer Res.* 2019. doi: 10.1158/1078-0432.CCR-18-3663.

10. Reiman A, Kikuchi H, Scocchia D, et al. Validation of an NGS mutation detection panel for melanoma. *BMC Cancer.* 2017;17:150.

11. Simen BB, Yin L, Goswami CP, et al. Validation of a next-generation-sequencing cancer panel for use in the clinical laboratory. *Arch Pathol Lab Med.* 2015;139(4):508-517.

12. Singh RR, Patel KP, Routbort MJ, et al. Clinical massively parallel next-generation sequencing analysis of 409 cancer-related genes for mutations and copy number variations in solid tumours. *Br J Cancer.* 2014;111(10):2014-2023.

13. Domínguez-Vigil IG, Moreno-Martínez AK, Wang JY, Roehrl MHA, Barrera-Saldaña HA. The dawn of the liquid biopsy in the fight against cancer. *Oncotarget.* 2018;9:2912-2922. doi: 10.18632/oncotarget.23131.

14. Lanman RB, Mortimer SA, Zill OA, et al. Analytical and clinical validation of a digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. *PLoS One.* 2015;10(10):e0140712. doi: 10.1371/journal.pone.0140712.

15. Plagnol V, Woodhouse S, Howarth K, et al. Analytical validation of a next generation sequencing liquid biopsy assay for high sensitivity broad molecular profiling. *PLoS One.* 2018;13(3):e0193802. doi: 10.1371/journal.pone.0193802.

16. Foundation Medicine, Inc. Foundation Medicine Web site. https://www.foundationmedicine.com/genomic-testing/foundation-one-liquid. Accessed March 18, 2019.

17. Oncomine™ lung cfDNA assay. Thermo Fisher Scientific Web site. https://www.thermofisher.com/order/catalog/product/A31149. Accessed March 18, 2019.

18. Zimmermann B, Salari R, Swenerton R. Personalized Liquid Biopsy: Patient-Specific Non-Invasive Cancer Recurrence Detection and Therapy Monitoring. Paper presented at: 10th Circulating Nucleic Acids in Plasma and Serum (CNAPS) International Symposium; September 20-22, 2017; Montpellier, France.

19. Costello M, Pugh TJ, Fennell TJ, et al. Discovery and characterization of artifactual mutations in deep coverage targeted capture sequencing data due to oxidative DNA damage during sample preparation. *Nucleic Acids* Res. 2013;41:e67.

20. Chen G, Mosier S, Gocke CD, Lin MT, Eshleman JR. Cytosine deamination is a major cause of baseline noise in next-generation sequencing. *Mol Diagn* Ther. 2014;18:587-593.

21. Newman AM, Lovejoy AF, Klass DJ, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. *Nat Biotechnol.* 2016;34:547-555.

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