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Article

A Novel Cell-Free DNA Fragmentomics Assay and Its Application for Monitoring Disease Progression in Real-Time for Stage IV Cancer Patients

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Simple Summary

For patients with advanced (stage IV) cancer, imaging can take 6 to 8 weeks to show whether a new treatment is helping – a stressful delay that can keep them on ineffective therapies. A new blood test has been developed to detect treatment failure in just 2 - 3 weeks. The test measures tiny tumor-derived DNA fragments in the blood and reports a "Progression Score" from 0 to 100. High scores flag likely rapid cancer growth; low scores suggest the therapy is effective. Because the test is quick, non-invasive, and does not rely on specific genetic mutations, it can be used across many cancer types and treatments, helping doctors decide sooner whether to continue, change, or stop a patient's therapy.

Abstract

Background/Objectives: Conventional imaging assesses therapy response in stage IV solid-tumor patients in 8–12-week intervals, delaying detection of non-responders. We evaluated a real-time quantitative PCR (RT-qPCR) assay that interrogates size-distributed cell-free DNA (cfDNA) fragments to provide earlier insights into treatment efficacy. **Methods**: In this prospective study, 128 patients with metastatic lung, breast, or colorectal cancer provided plasma 12–21 days after the first dose of a new systemic regimen. RTqPCR targets multicopy retrotransposon elements in cfDNA fragments >80 bp, >105 bp, >265 bp, and internal control. A model integrates these quantities into a Progression Score (PS) ranging from 0–100; higher values indicate probable disease progression. **Results**: The PS model yielded an area under (AUC) the receiver-operating-characteristic (ROC) curve of 0.93 for predicting radiographic progression at first imaging. Scores were strongly bimodal: 92 % of patients with PS > 90 progressed, whereas 95 % with PS < 10 did not. Intermediate scores (10–90) comprised a mixed cohort. Assay performance was unaffected by tumor genomic profile. **Conclusions**: This cfDNA-based Progression Score (PS) assay enables tumor- and therapy-agnostic, non-invasive monitoring of treatment response as early as two weeks after initiation. By flagging ineffective regimens well before standard imaging, the test can accelerate clinical decision-making, reduce exposure to fu-tile therapy, and potentially improve outcomes in stage IV cancer. Early treatment plan changes may also avoid the high drug and administration costs of ineffective treatments, prevent downstream toxicity-related hospitalizations, and free up limited imaging and infusion-suite capacity—yielding savings for patients, payers, and healthcare systems.

Keywords: tumor response; cfDNA; therapy monitoring; fragmentomics; retrotransposons; metastatic cancer; liquid biopsy

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1. Introduction

Cell-free DNA (cfDNA) has been widely studied as a cancer biomarker and has been proposed for oncologic applications, including early detection, recurrence monitoring, prognosis, and therapy monitoring [1–4]. Unfortunately, until recently, unlike circulating-tumor DNA (ctDNA), cfDNA has lacked the clinical specificity required for practice [5–9]. With the advent of fragmentomics as a cancer biomarker, there has been renewed interest in cfDNA combined with fragmentomics as a cancer biomarker [10–15]. Here, a cfDNA fragmentomics assay is proposed, hereafter referred to as the Progression Score (PS) assay, with the analytic sensitivity and clinical specificity necessary to provide clinicians, in real time, the ability to identify stage IV cancer patients who are experiencing disease progression.

Disease progression is a commonly used endpoint for determining the efficacy of therapeutic agents. Response evaluation criteria in solid tumors (RECIST 1.1) are a widely used criterion for assessing the status of a patient's disease for clinical studies [16,17]. In clinical practice, while most physicians do not strictly follow RECIST criteria for determining whether to adjust a patient's treatment plan, almost all periodically image their patients to assess whether the patient's disease remains under control. It is generally recognized that imaging for assessing therapy efficacy has weaknesses.[18]. For example, some patients with so-called stable disease frequently have progressive disease, or patients start to develop progressive disease long before imaging can detect tumor growth [19,20]. With the rapid expansion of immunotherapy, clinicians are increasingly acknowledging that standard imaging often fails to distinguish between true progression and treatment-related changes in the early phases of care. Notably, pseudo-progression may mimic tumor enlargement on radiographic studies even when the underlying disease is improving [21]. This is particularly problematic for immunotherapy clinical studies that use disease progression as an endpoint. "Mixed responses" on imaging – where cancer seems to be growing in one area but perhaps stable in another – is another challenging clinical area, as is residual positivity on PET scan. In clinical practice, physicians are left with difficult treatment decisions when an image shows a patient as progressing. Clinical decision making can be made more difficult if the patient experiences hyper-progression from immunotherapy.

Cancer biomarkers such as CEA, PSA, CA19-9, and CA-125 are often used to monitor patients, but their lack of sufficient clinical specificity makes them inadequate for use in clinical decision-making [22,23]. Up to one third of patients do not express these tumor markers.

A blood-based assay that quantifies tumor-derived circulating cell-free DNA and demonstrates high clinical specificity for the early detection of disease progression would satisfy a critical unmet need in oncologic care. Earlier insight would enable timely chang-es to the treatment plan, lessen toxicity from ineffective drugs, lower the cost of futile care, and make better use of therapeutic, hospital, and administrative resources.

2. Materials and Methods

Following two previously successful proof of concept studies [24–26] demonstrating the potential for a cfDNA fragmentomics assay to identify in real-time stage IV cancer patients whose disease has progressed, we prospectively enrolled participants into an observational study to develop a practical assay that could be used by physicians in clinical practice to monitor the tumor response of the patient's under their care.

2.1. Study Design

An observational study was designed to prospectively collect blood samples from cancer patients at two time points during treatment. Patients could be enrolled at any point in a patient's treatment regardless of the patient's treatment plan. Two blood draws were collected. The first draw was taken within two days prior to the infusion of the first cycle of treatment delivered following the

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baseline scan. The second blood draw was taken 12 to 21 days following the first treatment and prior to the next infusion of therapy.

Patients were only enrolled if a CT scan was planned between 9- and 12-weeks following the first blood draw. Participants were enrolled regardless of which drugs were delivered, regardless of the line of therapy, and regardless of where they were even starting a new line of therapy. The enrollment period for each patient was from baseline scan to assessment scan, which was required to be performed between 9 to 12 weeks apart. Board-certified radiologists measured lesions from each scan. Oral medications were taken according to the regular schedule. The flow diagram of the CADEX-0001 study is pro-vided in Figure 1.

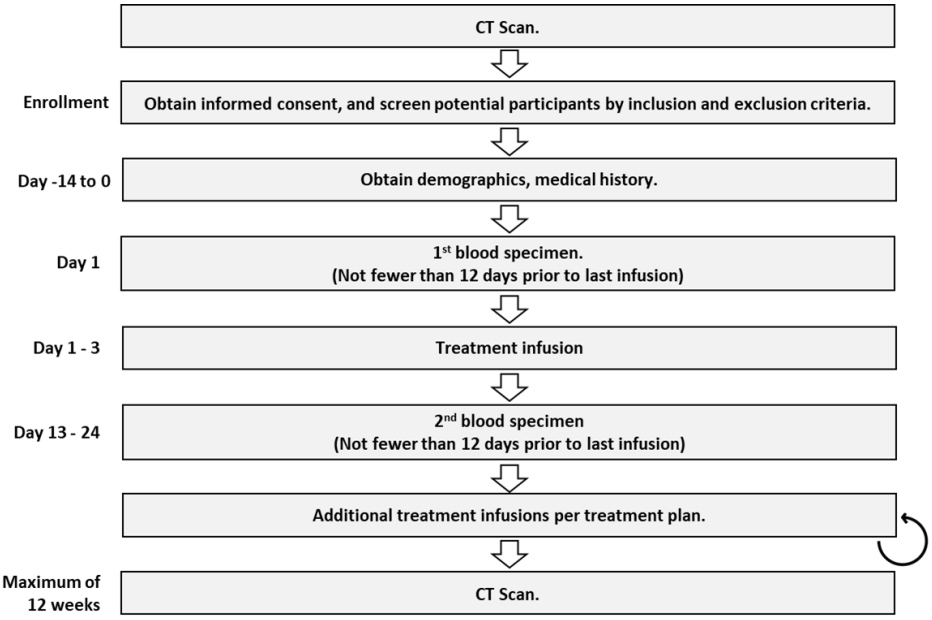

Figure 1. CADEX-0001 study flow diagram.

2.2. Participants

We enrolled 146 stage IV breast, colorectal, and lung cancer patients at 11 sites. For most sites, the study was approved by the Institutional Review Board (IRB) for human subjects at WCG Clinical. For all remaining sites, the study was approved by the respective IRBs of the institution. All study participants provided signed consent for the collection and cfDNA analysis of their blood.

Patients were excluded from the study if they had a secondary malignancy, were being actively treated for autoimmune disease, or had DVT, PE, or sepsis within the past 12 days. Participants were withdrawn from the study if these conditions developed within 12 days of the second blood draw. Of the 146 patients enrolled in the study, 128 were included in the analysis; for a variety of reasons, 18 were not included in analysis – see Table A1 in the supplementary section provides a summary of patients enrolled in the cancer cohort.

2.3. Sample Collection and Transportation

One to three tubes of 8-10 ml of peripheral blood samples in each tube were collected in a Streck™ tube. The specimens were transported at ambient temperature via overnight courier service to Cadex Genomics' lab in New Orleans. Since the delay in the processing of blood samples affects the concentrations of cell-free DNA [27]. Any participant whose first or second blood draw sample was received at the laboratory more than 120 hours after the blood sample was collected was excluded from the study.

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2.4. Plasma Separation

A two-step centrifugation protocol was used to separate plasma. First, the Cell-Free DNA BCT (Streck) tubes were centrifuged at 1,600 × g for 10 min at 15°C. Then, the plasma was centrifuged again in a 1.5 mL tube at 16,000 × g for 10 min at room temperature. The plasma aliquots were transferred to 2 mL cryogenic tubes and stored at -80^{0}C

2.5. Cell-free DNA Extraction

cfDNA was extracted from 500 µL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Germantown, MD) following the kit protocol for a plasma volume of 1 mL with the following modifications: (1) omission of carrier RNA from the ACL buffer, (2) addition of 500 µL of 1 X PBS buffer (Molecular Biologicals International, Inc. / Growcells.com, Irvine, CA) to the 500 µL of plasma to increase the sample volume to 1 mL, and (3) extend the proteinase K digestion time from 30 minutes to a 1-hour incubation. Subsequently, cfDNA was eluted in 60 µL of the kit elution buffer. Each plasma sample was extracted in duplicate. Baseline and after-treatment samples from the same patient were extracted together to avoid batch effects.

2.6. Analytical Methods

Two human-specific retrotransposons, Alu Yb8 and SVA[28–31], were selected as amplification targets to quantify different fragment sizes of cell-free DNA in patient plasma. qPCR primers and probes for each target were designed using the PrimerQuest™ Tool from Integrated DNA Technologies (Coralville, IA). Short (Alu Yb8 = 80 bp, Alu Yb8 = 105 bp) and long (SVA = 265 bp) primers were multiplexed to create two primer mixes: an 80-265 primer mix and a 105-265 primer mix. The SVA primers and probes present in the multiplex serve as both a quality control measure and an enhancement for Alu marker amplification by blocking non-specific amplification.

To detect inhibitors in the sample, a 172-bp synthetic nucleotide sequence was used as an internal positive control (IPC) and added to each primer mix. The hybridization probe for the short target was labeled with FAM, the long target with Cy5, and the IPC with HEX. All HPLC purified primers and probes were purchased from Integrated DNA Technologies (Coralville, IA). The primer mixes contain primers and probes for each target (short, long, and IPC) along with PCR enhancer additives. Standard curve assays were conducted on the ABI 7500 or the QuantStudio™ 5 Real-Time qPCR system (Applied Biosystems, ThermoFisher Scientific, Waltham, MA).

Standard DNA was extracted from a single donor's blood using organic extraction (Proteinase K/SDS digestion, phenol/chloroform extraction, ethanol precipitation, and dissolution in Tris-EDTA buffer - 10 mM Tris, 0.1 mM EDTA, pH 8.0) and calibrated against NIST Human DNA Quantitation Standards SRM 2372 Components B (National Institute of Standards and Technology, Gaithersburg, MD). Two microliters (2 µL) of standard DNA or unknown extracted cfDNA were amplified in triplicate in a 20 µL reaction volume, which included 7.7 µL of primer mix, 0.3 µL of the ROX reference standard (diluted to 6 µM), and 10 µL of Brilliant Multiplex QPCR Master Mix (Agilent Technologies). The PCR conditions consisted of one enzyme activation cycle for 10 minutes at 95°C, followed by 40 cycles of a 2-step qPCR (15 seconds at 96°C and 2 minutes at 64°C combined annealing/extension time). DNA samples were quantified using both the 80-265-IPC primer mix and the 105-265-IPC primer mix. qPCR data analysis was performed using the automatic baseline feature of the QuantStudio-5 Design and Analysis Software v1.5.1 (Applied Biosystems, ThermoFisher Scientific, Waltham, MA).

The results of the analytical evaluation of the qPCR multiplex 80-265-IPC are presented in the supplementary section (Table A2).

2.6. Statistical Analyses

The following were measured in each blood sample.

- SM1 - Concentration level for >80bp cfDNA levels, first blood draw

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- MM1 - Concentration level for >105bp cfDNA levels, first blood draw
- SM2 - Concentration level for >80bp cfDNA levels, second blood draw
- MM2 - Concentration level for >105bp cfDNA levels, second blood draw

These four derived variables were used in logistic regression models to find the best predictor of progression.

- Frag1 = SM1 - MM1 (Frag1 floor = 0.0)
- Frag2 = SM2 - MM2 (Frag2 floor = 0.0)
- FragDiff = Frag2 - Frag1
- MMDiff = MM2 - MM1

Models were fit using R version 4.4. The area under the curve [AUC] was calculated for each model, and the model with the highest AUC was selected as the preferred model. The selected model was then analyzed using the bootstrap method[32] to determine the cut-point for making a progression call.

3.1. Model Selection

Using data analyzed from blood samples collected from the cancer patient cohort, a statistical model was developed to predict which patients would exhibit disease progression, as confirmed by CT scans, at 8 to 12 weeks. The radiology reports from each patient were analyzed to identify whether a patient's disease had progressed during the 8- to 10-week period between scans. A patient's disease was determined to have progressed if and only if the sum of the diameters of the tumor lesions had increased by 20% or more. Several potential logit regression models were pre-identified based on biological hypotheses. These models were statistically tested with the best result from the model with FragDiff + MMDiff as the predictors with the ROC AUC = 0.93, p-value < 0.001, being selected (Figure 2). The Bonferroni multiple testing adjustment was used to control the error rate.

Following the example of the 21-gene recurrence score assay[33]a progression score was generated with a range of 0 to 100.

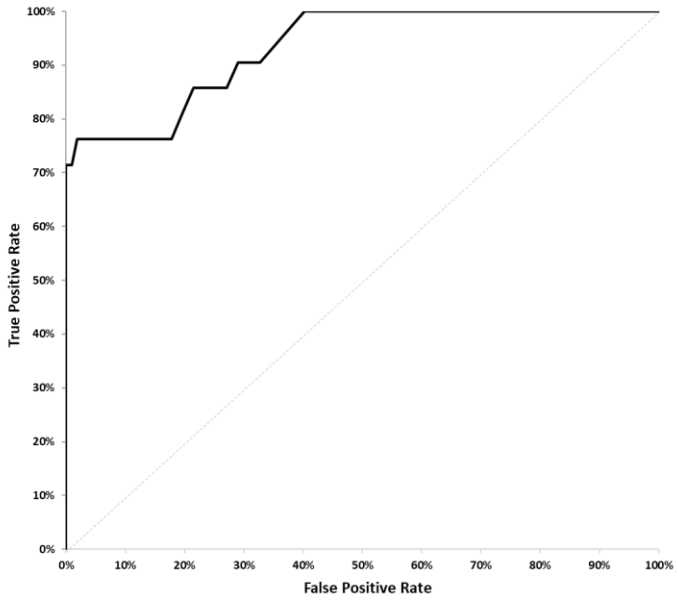

Figure 2. ROC Curve for the best-performing model.

3.2. Progression Score Cut-Point Selection

The model with the highest ROC value was analyzed to determine the appropriate cut-point for making a disease progression call. A cut-off was selected to optimize the assay for specificity and positive predictive value (PPV). Using 1,000 iterations of the bootstrap method, the expected PPV for

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each PS was calculated, and a cut-point with an expected PPV ≥99% was selected. The cut-point selected was 90.

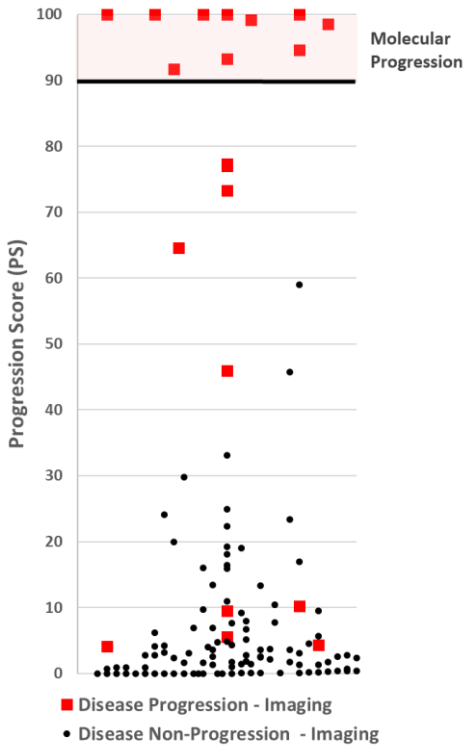

Figure 3. A scatter plot of Progression Scores (PS). No participate with non-progressive disease by imaging (black dots), had a PS over 60, well below the PS cut-off of 90 for making a molecular progression call. Five participants with progressive disease by imaging had a PS at or below 10.

3.3. Assay Performance

By design, a cut-off threshold was set to avoid false-positives, making the PPV 100%. Nine (9) patients with disease progression by imaging had a PS below 90, making the negative predictive value, 92%. The results are summarized in Table 1.

Table 1. Progression Score Assay Result Interpretation.

Progression Score (PS)	#	Interpretation	Performance
≥90	11	Progression	PPV = 100%
<90	117	Likely non-progression	NPV = 92%

4. Discussion

4.1. Role and Limitations of Fragmentomics

Our previous work has shown that cfDNA can identify disease progression and therapy futility reliably [24]. However, that work was conducted under sample handling conditions that are impractical for a commercial assay. In that study, plasma was separated at a CLIA lab within an academic medical center within hours of specimen collection. For most oncology clinics, this is not practical and in many cases impossible. Consequently, to be clinically viable, a cfDNA-based assay targeting disease progression must be able to accommodate the shipment of blood over several days without the risk of producing false-positive results. Furthermore, the clinical utility for non-response to therapy requires that common conditions unrelated to a patient's cancer do not result in producing invalid results, especially false positive results. Fragmentomics plays a significant role in the PS

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assay's ability to avoid generating false-positive results. The following data is intended to illustrate this.

Table 2 presents four study participants whose blood arrived at our lab with evidence of common-shipment white blood cell lysis. The first three participants (2022, 4019 and 8003), when measuring the concentration levels of all cfDNA above 80 bp, exhibited strong evidence of disease progression. However, by imaging, none of those patients had disease progression. Conversely, participant 2021 had a small decrease in the cfDNA concentration levels above 80bp, evidence of non-progression. By imaging, that patient was experiencing disease progression. By employing the fragmentomics components of the assay, the PS Assay made the correct call on all four of these patients.

Table 2. A sample of participants with high levels of short and long cfDNA changes due to high levels of white blood cells lysis, and the impact of fragmentomics on assay performance. Considering only the change in concentration levels of >80bp cfDNA fragments, versus the Progression Score.

Participant	PD by Imaging	Δ >80bp cfDNA	PS
2022	No	10.1-fold increase	8.6
4019	No	8.8-fold increase	0.0
8003	No	3.8-fold increase	0.1
2021	Yes	2.2% decrease	100.0

While fragmentomics clearly improves the performance of the PS assay, there is evidence that limitations exist in making cfDNA cancer specific. Several non-cancer clinical conditions are known to increase cfDNA in the patient's plasma [34,35]. We evaluated the plasma of non-cancer patients for cfDNA concentrations using fragment analysis. Using a third-party vendor, blood was drawn from 30 consenting adult patients who had the following events in the past 12 days: acute stroke (3 patients), asthma requiring hospitalization (2 patients), COPD exacerbation requiring hospitalization (3 patients), diabetic ketoacidosis (3 patients), severe inflammatory bowel disease (4 patients), myocardial infarction, severe rheumatoid arthritis (5patients), severe seizure (3 patients), and viral infection (5 patients). Additionally, blood was drawn from nine healthy adults with consent. Using the average values from the nine healthy patients as the baseline for the PS assay, we measured the PS score for each of the 30 patients suffering an acute event. The results are presented in Table A3 of the Appendix in the supplementary section. While comparing the cfDNA from a healthy patient to that of a patient who has just experienced a severe event is a suboptimal way to understand how these events impact the PS assay, it is a good indicator of which events are likely to have the most significant impact (keeping in mind these are events that occur between the first and second blood draw). It also highlights the fact that additional work is required to understand the types of events that will provide an inaccurate PS assay test result. Our investigation indicates that conditions such as Acute Stroke, Myocardial Infarction, COPD – hydroxyurea, and Rheumatoid Arthritis patients undergoing treatment with methotrexate may interfere with the proposed CfDNA assay. However, this was a minimal study with only 2-5 patients in each category and needs further investigation. Ideally, a validation study would be performed on treated cancer patients who develop various conditions before blood draws. There are practical limitations to conducting such a study due to the infrequency of such conditions occurring in this population.

This study demonstrates that cfDNA combined with fragmentomics can be highly cancer-specific to measure tumor burden changes in stage IV cancer patients accurately. The study, however, is preliminary and has limitations; further studies are needed. One such limitation is the broad time difference between the timing of PS testing and radiographic imaging. For some participants, it is possible that the tumor only began to grow after the second blood draw was collected. In those cases, the results would appear as a false negative. In an earlier study conducted with MD Anderson, where the blood was collected longitudinally at various time points, there was evidence of this occurring in some subjects[24].

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Finally, this study does not consider the impact of the PS assay on clinical decision-making or how it affects patient quality of life and outcomes.

The assay presents four clear opportunities to enhance clinical management. First, it enables the rapid identification of patients experiencing disease progression, allowing clinicians to promptly transition them to alternative therapeutic strategies. Its real-time monitoring capability further permits the evaluation of low-probability treatment regimens after a single cycle, thereby providing early insight into patient responsiveness.

Second, there is a growing awareness of the overtreatment of stage IV cancer patients. In addition to the potential toxicological harm of a treatment on a patient's health, there is the time toxicity and financial toxicity of cancer treatments. This PS assay may give physicians additional flexibility to remove agents or reduce the dosing of a patient's treatment plan or give patients a break from treatment altogether. This can potentially lower the level of toxicity and morbidity delivered to patients, improving their quality of life and perhaps extending overall survival. Two-arm randomized clinical studies could potentially demonstrate that the PS assay improves patient outcomes and quality of life while reducing the cost of cancer treatment. The ability to "de-escalate" treatment – to stop ineffective treatment without changing the survival of patients can improve quality of life and minimize financial toxicity. This is particularly important in LMIC (Low and Middle Income Countries) where patients often pay for treatment out of pocket.

Third, the PS assay alleviates both patient burden and clinical trial costs by swiftly identifying non-responders. Early recognition of ineffective treatment spares participants unnecessary exposure to therapy and reduces overall study expenses. Moreover, incorporating the PS assay into dose-finding and pharmacodynamic investigations provides real-time feedback, streamlining study design and accelerating decision-making in clinical trials.

Finally, immune checkpoint inhibitors (ICIs) have revolutionized cancer therapy by harnessing the patient's own immune system to eradicate tumor cells. To date, the U.S. Food and Drug Administration has approved three classes of ICIs - targeting CTLA-4, PD-1/PD-L1, and LAG-3 - with more agents in development, across over 20 tumor types in the neoadjuvant, adjuvant and metastatic settings [31,32]. Unlike cytotoxic therapies, ICIs can precipitate immune-related adverse events (irAEs) of varying severity (grades 1–4), which may occur at any point during or even after treatment, reflecting excessive immune activation [33,34]. Moreover, unconventional response patterns such as pseudoprogression - an initial increase in tumor size followed by regression - and hyperprogression - accelerated disease growth seen in up to 30% of patients - pose significant clinical challenges, often leading to premature discontinuation of effective therapy and poorer outcomes [35,36]. By providing an early, dynamic readout of tumor-derived cfDNA changes, the PS assay could help distinguish true progression from transient immune phenomena and identify non-responders before severe irAEs arise, thereby optimizing patient selection and improving the safety and efficacy of ICI treatment.[36–38]

5. Conclusions

The PS assay has the potential to be a powerful tool to provide clinically valuable information to physicians regarding disease progression and non-progression in real time, helping physicians to optimize treatment plans and improve drug-development studies. By combining cfDNA with fragmentomics, the PS assay can identify stage IV breast, colorectal, and lung cancer patients with disease progression as quickly as 12 days after initiation of treatment. These findings demonstrate that, when combined with fragmentomic information, cfDNA is potentially an important cancer marker for measuring tumor burden.

Author Contributions: Conceptualization, SKS, WH, KK, WS, PH and HB; methodology, SKS, HB, WH, PH, KK and WS.; software, WH and WS; validation, SKS, HB, PH, WH and WS data curation, HB, WH, PH, SKS and WS; writing—original draft preparation SKS, WH; writing—review and editing, SKS, PH, WH, KK, HB and WS; supervision, SKS and WH. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The blood samples were collected in accordance with the Declaration of Helsinki and approved by the Institutional Review Board. Blood samples from normal individuals were collected with informed consent using Advera corporation IRB Approval #201601790, with an approval date of September 14, 2016. The patient samples for the prospective clinical study were collected using the protocol and the consent form approved by the Institutional Review Board (IRB) for human subjects at the WCG clinical corporation (WIRB Tracking Number: 20183188), approved on November 11, 2018. The protocol and consent forms were also reviewed and approved by some of the study's participating institutions.

Informed Consent Statement: Informed consent was obtained from all human subjects involved in the study.

Data Availability Statement: All data and supporting results reported in this article, excluding confidential patient information, are available from the authors upon request.

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Conflicts of Interest: SKS, BH, and PH are present or former employees of Cadex Genomics. SKS and HB are employees of InnoGenomics, and KK and BS are consultants to Cadex Genomics.

Abbreviations

The following abbreviations are used in this manuscript:

Bp	Base pair
ctDNA	Cell-free circulating tumor DNA
cfDNA	Cell-free DNA
DNA	Deoxyribonucleic acid
ICI	Immune checkpoint inhibitors
IPC	Internal positive control
PCR	Polymerase chain reaction
PS	Progression score
qPCR	Quantitative polymerase chain reaction

Appendix A

A Novel Cell-Free DNA Fragmentomics Assay and Its Application for Monitoring Disease Progression in Real-Time for Stage IV Cancer Patients.

Table A1. Participant Summary.

Participant Outcome	#	%
Protocol violations, incomplete follow-up	4	2.8%
Withdrawn / Death / Hospice	12	8.3%
Exceeded 72-hour sample stability threshold	2	1.4%
Completed	128	88.9%
Total	146	100.0%
Age	128	100.0%
<60	41	32.0%
≥60	87	68.0%
Average	62.75	
Sex	128	100.0%
Female	73	57.0%
Male	55	43.0%
Race and Ethnicity	128	100.0%

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Asian	2	1.6%
Black	21	16.4%
Hispanic	*0*	*0%*
Non-Hispanic	*19*	*14.8%*
Not Reported	*2*	*1.6%*
White	102	79.7%
Hispanic	*1*	*0.8%*
Non-Hispanic	*95*	*74.2%*
Not Reported	*6*	*4.7%*
Other	3	2.3%
Tumor Type	128	100.0%
Breast	20	15.6%
Colorectal	50	39.1%
Lung	58	45.3%
NSCLC	*50*	*39.1%*
SCLC	*8*	*6.3%*
Therapy Type	128	100.0%
Chemotherapy	63	49.2%
Targeted Therapy	7	5.5%
Targeted Therapy + Chemotherapy	7	5.5%
Immunotherapy	14	10.9%
Immunotherapy + Chemotherapy	37	28.9%

Table A2. Analytic Validation summary.

Metric	Value
Limit of Blank (LoB)	95th percentile: • LoB for >80bp cfDNA: 0.06614 pg/µL. • LoB for >265bp cfDNA-0.03602 pg/µL
Limit of Detection (LoD)	LoD=LoB+CβSD: • LoD for >80bp cfDNA: 0.138 pg/µL. • LoD for >265bp cfDNA: 0.139 pg/µL.
Limits of Quantitation (LoQ)	• Upper: LoQ for >80bp and >265bp cfDNA: 20ng/µL. • Lower: LoQ for >80bp and >265bp cfDNA: 0.0006 ng/µL. Upper and lower LoQs are set to the highest and the lowest DNA standard concentration, respectively.
Linearity and Reportable Range	• Upper: LoQ for >80bp and >265bp cfDNA: 20ng/µL. • Lower: LoQ for >80bp and >265bp cfDNA: 0.0006 ng/µL. Upper and lower LoQs are set to the highest and the lowest DNA standard concentration, respectively.

Table A3. Confounding conditions (Progression Score PS impact).

Events (within 12 days of blood draw)	**n**	**PS Change**
Acute Stroke	3	+/-100.0
Asthma	2	+/-4.5
COPD exacerbation	2	+/-43.4
COPD - hydroxyurea	1	+/-98.1
Diabetes out of control, diabetic ketoacidosis	3	+/-26.0
Inflammatory Bowel Disease	4	+/-47.1
Myocardial Infarction	2	+/-100.0
Rheumatoid Arthritis	3	+/-14.4
Rheumatoid Arthritis - methotrexate	2	+/-87.2

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Seizure	3	+/-45.0
Viral Infection	5	+/-15.1

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