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A Novel Multiplex Immunoassay for the Early Detection of Bladder Cancer: Study Protocol for a Prospective, Observational Cohort Study

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Abstract: Introduction: Early-stage bladder cancer (BCa) has an excellent 5-year survival rates; however, outcomes decline sharply with disease progression. Established risk factors for BCa include tobacco exposure and certain occupational hazards. The objective of this study is to evaluate the feasibility and practicality of conducting a large-scale BCa screening study targeting individuals at elevated risk. Methods and Analysis: This is a prospective, multicenter, observational feasibility study conducted at Cedars-Sinai Medical Center—one of the largest hospital systems in Southern California—and the VA Long Beach Healthcare System. Between April 2022 and January 2025, 200 individuals at risk for developing BCa were enrolled. The primary endpoint is to assess the feasibility of recruiting and retaining 200 participants for longitudinal follow-up over four years. Participants undergo annual evaluations, including collection of demographics, clinical, and exposure data, as well as urine-based molecular analyses. These data will be incorporated into multivariate model to identify predictors of BCa development. Ethics and Dissemination: Given the rising incidence and mortality associated with BCa, early detection—when the disease remains highly treatable—is critical. The use of robust, non-invasive diagnostic tools in high-risk populations provides a promising strategy toward this goal. Although modest in scale, this study represents the first known effort to leverage lung cancer screening clinics as a platform for identifying individuals at risk for BCa, and the first to apply a multiplex immunoassay within this cohort. Findings from this feasibility study will inform optimal strategies for recruitment, retention, and molecular screening in future large-scale, definitive trials. Strengthens and Limitations: (1) This study is the first prospective feasibility trial to evaluate a multiplex urinary immunoassay for BCa screening within a high-risk population recruited from lung cancer screening clinics, leveraging an existing infrastructure to improve recruitment efficiency; (2) The multicenter, observational design with standardized data collection, centralized laboratory testing, and longitudinal urine sampling enhances methodological rigor and reproducibility; (3) Broad inclusion criteria and consecutive recruitment were used to minimize selection bias and reflect real-world high-risk populations; (4) As a feasibility study, the sample size and event rate are not powered to assess definitive diagnostic accuracy or clinical outcomes; (5) Reliance on self-reported

exposure data (e.g., tobacco and occupational history) introduces potential information bias despite standardized questionnaires and trained coordinators.

Keywords: bladder cancer; screening; risk factors; tobacco exposure; cohort study; urine biomarkers; multiplex immunoassay

1. Introduction

Globally, bladder cancer (BCa) ranks as the fourth most prevalent cancer in men and eighth in women [1]. Urothelial carcinoma (UC) accounts for up to 90% of primary BCa [1]. It is the second most common cancer affecting the urinary system. BCa is three to four times more prevalent in men than in women [2]. Over 400,000 new cases of BCa are diagnosed globally each year**,** including approximately 80,000 new cases annually in the US [2]. Age-standardized mortality rates worldwide range from 2.1–21.8 per 100,000 males and 1.3–5.1 per 100,000 females [1]. In 2021, an estimated 83,730 new cases of BCa were diagnosed, with 17,200 projected deaths in the US [3]. Since 2000, these figures have increased by 54% and 45% respectively [4].

Environmental exposures, particularly tobacco smoke, are well-established risk factors for multiple malignancies, including BCa. Over the past four decades, epidemiologic studies have consistently demonstrated that cigarette smoking significantly increases the risk of BCa [5]. A meta-analysis of case-control and cohort studies estimated that current smokers have approximately a threefold higher risk of developing BCa compared to non-smokers. Furthermore, a clear dose–response relationship has been observed, with risk increasing in proportion to both the number of cigarettes smoked per day and the duration of smoking. In contrast, later age at first exposure and smoking cessation are inversely associated with BCa risk [6]. In addition to tobacco exposure, occupational exposures have also been implicated, particularly in professions such as firefighting, cosmetology, and work in the dye/textile and petroleum industries.

To date, there are no diagnostic tools capable of detecting BCa at an early, preclinical stage, prior to clinical presentation. As a result of this limitation, nearly 30% of patients initially present with muscle-invasive disease (stage 2 or higher). Prognosis at these stages remains poor, with a 5-year survival of approximately 50% for stage 2 disease and less than 20% for stage 3–4 disease. Although prevention would be ideal, this has not yet been realized. Therefore, it is reasonable to speculate that if patients currently diagnosed with stage 2–4 disease could instead be detected at stage 1—where the 5-year survival exceeds 94%—meaningful improvements in overall BCa survival could be achieved [7–9]. This unmet need underscores the importance of developing biomarkers for early detection. Such markers could then be evaluated in large-scale studies to enable earlier identification of patients at a stage when more effective therapeutic interventions are available.

Previously, we have identified a BCa-associated signature comprise of 10 urinary protein biomarkers [10–13] which has been confirmed in several large cohort studies [14–19]. This BCa associated diagnostic signature has been incorporated into a validated multiplex immunoassay [20,21] which has reported the ability to denote biomarker changes as early as five years prior to the clinical presentation of BCa [22].

A comprehensive review of the literature regarding markers of early detection of BCa identified limited reporting. Messing et al. deployed a chemical reagent strip (urine dipstick) for hemoglobin detection to screen a high risk population (i.e.*,* men ages 50 years and older with a significant tobacco history; >40 pack years) for BCa [23]. (This dipstick testing has a sensitivity of 50% and specificity of 54% for BCa detection [7]. The subjects were randomized to no screening versus screening utilizing the urinary dipstick test. Participants who had positive urinary dipstick test underwent standard urologic evaluation, including cystoscopy and imaging. If an abnormality was noted, then the patient underwent transurethral resection of a bladder tumor (TURBT). The proportion of high-grade invasive (late stage) BCa was substantially lower among screened men (10%) compared to unscreened men (60%; $p = 0.002$). At 14 years of follow-up, no deaths attributable to BCa were observed in the screen-detected group, whereas 20.4% of men diagnosed outside of screening had died of disease ($p = 0.02$). These findings suggest that screening facilitates detection at earlier stages and may be associated with a reduction in BCa-specific mortality with cancers identified through standard clinical presentation.

In addition, the Bladder Cancer Urine Marker Project (BLU-P) study has evaluated the feasibility of population-based screening for BCa while simultaneously assessing a multi-marker screening algorithm designed to reduce reliance on cystoscopy. This algorithm incorporates NMP22, FGFR3, microsatellite analysis (MA), and MLPA (a custom methylation-specific assay). To date, 1611 men have been enrolled, of whom 23.5% tested positive for hematuria. The incorporation of these molecular screening tools prior to referral for cystoscopy reduced the number of cystoscopic procedures from 378 to 66, representing an 82.5% decrease [24]. However,

comprehensive performance metrics, including overall sensitivity and specificity, as well as clinical outcomes such as cancer detection rates, stage at diagnosis, and BCa–specific mortality, have not yet been reported.

Building upon Dr. Messing's groundbreaking work in screening at risk individuals [23], we propose to leverage a nested case-control study in which we will gather well annotated serial urines from individuals who were enrolled in longitudinal studies and who developed BCa to test for the presence of our BCa-associated signature in voided urine samples prior to the clinical manifestation of BCa.

We have identified a BCa–associated biomarker signature that has been validated across several large cohorts [14–19]. Notably, this signature demonstrates the ability to detect even small tumors (~1 cm), which exhibit elevations in one or more of the 10 biomarkers at the time of diagnosis, although typically to a lesser magnitude than that observed in larger, primary *de novo* tumors (~3 cm) [16]. Consistent with these findings, we have reported that increases in tumor size, grade, and stage are associated with corresponding increases in the absolute levels of one or more biomarkers comprising this signature [16]. At present, this BCa–associated signature is being evaluated in four large prospective studies: (i) detection of BCa in patients with gross hematuria; (ii) detection of BCa in patients with microscopic hematuria; (iii) surveillance of patients with a history of BCa; and (iv) prediction of response to intravesical BCG therapy. In a prior nested case-control study, significant differences in individual biomarker levels between cases and controls were observed, including ANG, APOE, MMP10, PAI1, SDC1 at 12 months ($p < 0.05$), as well as at 48 months ($p < 0.05$) and 60 months ($p < 0.05$), and VEGF at 12 months ($p < 0.05$). Furthermore, a preliminary predictive model incorporating IL8, CA9, PAI1, and APOE demonstrated excellent performance for forecasting subsequent bCa, with an AUC of 0.98, sensitivity of 88%, and specificity of 100% [22]. Collectively, these data support the robustness of this BCa–associated signature and highlight its potential utility for the early detection of BCa in at-risk populations.

Study Objectives

Therefore, we propose a feasibility study with the following primary objective: to evaluate the ability to recruit and retain an appropriate high-risk cohort in a 4-year longitudinal study designed to monitor for the development of BCa. Participants will be followed from initial screening through annual assessments, with systematic collection of both clinical data and urine-based molecular measurements.

Our secondary objective is to report the performance of our multiplex immunoassay in this feasibility study.

2. Methods and Analysis

2.1. Study Design

This is a single arm multicenter prospective, observational cohort study initiated in April 2022 with ongoing monitoring (Clinicaltrials.gov NCT05347342; version 1.8 12/16/2025).

2.2. Study Setting

Cedars-Sinai Medical Center (Los Angeles, CA, USA) opened the study to accrual in April 2022, while the Long Beach VA Healthcare System open the study to accrual in March 2024. The study enrolled 200 from April 2022 to January 2025. The study is closed to enrollment, but as it is a longitudinal study, the study still has over 3 years before it is complete. The manuscript adheres to the guidelines set forth by strengthening of the reporting of observational studies in epidemiology (STROBE) statement [25].

Subjects' medical records will be reviewed for the purpose of screening. The minimal variables needed to review would include age, gender, tobacco use as well as co-morbid conditions. The research coordinator trained and assigned to this study will have this responsibility.

Recruitment strategies were diverse. Subjects were first approached via mail/email (see attached patient recruitment letter) or in person at Primary Care Physician (PCP) Clinic (Cedars-Sinai) by someone on the clinical team (physician, nurse/care partner, assistant) asking the patient's permission before a member of the research team approaches the subject. To better explain BCa and how to prevent BCa, the healthcare team had access to handouts from the American Urological Association (AUA) on BCa. With only 23 subjects recruited over the ensuing 18 months, in October 2023, we transitioned from targeting PCP clinics to our lung cancer screening clinics, which encompassed activating Long Beach VA Healthcare System in March 2024. Lung cancer screening clinics are geared towards patients 50 years of age and older with this significant tobacco history which mirrors the at-risk population for developing BCa. In the subsequent 18 months, 177 subjects were enrolled.

Participants will receive a total compensation of $100. Specifically, participants will be compensated with a $20 gift card for each of the 5 study visits over 4 years, thus totaling $100. This compensation will help to offset the costs of travel for study visits, parking at Cedars-Sinai for study visits, and participants' time.

3. Eligibility Criteria

3.1. Inclusion Criteria

Participants must be:

(a) Age 50 years or older
(b) ≥20 pack year history of tobacco exposure
(c) Free of any malignancy except for the following: adequately treated basal cell or squamous cell skin cancer, adequately treated Stage I or II cancer from which the patient is currently in complete remission, or any other cancer from which the patient has been disease free for five years. Patients with localized prostate cancer who are being followed by an active surveillance program are also eligible.
(d) Willing and able to give written informed consent
(e) Willing to provide voided urine sample
(f) Be able and willing to complete annual research clinic visits for 4 years

3.2. Exclusion Criteria

Participants must not have:

(a) History of hematuria (microscopic or gross) within 2 years of signing consent.
(b) Previous history of BCa
(c) A known active urinary tract infection or urinary retention
(d) Active stone disease (renal or bladder) or renal insufficiency (creatinine ≥2.0 mg/dL)—Serum creatinine value can be up to 2 years before consent, otherwise repeat.
(e) Ureteral stents, nephrostomy tubes or bowel interposition
(f) A recent genitourinary instrumentation (within 7 days prior to collection of voided urine sample)
(g) An active pregnancy.

Study trained clinical coordinators obtained informed consent.

3.3. Data Collection

Is ongoing and will focus on three main areas: Patient factors, hematuria evaluation and multiplex biomarker:

(1) Demographics: sex, gender, and race
(2) Risk factors: the age the patient began to smoke, the amount of smoking during this time, the age the patient quit smoking, exposure to secondhand smoke exposure to cigars, exposure to tobacco pipes, occupational exposure and hazardous chemical exposure
(3) Height and weight
(4) Previous hematuria (gross or microscopic)
(5) Urinary dipstick
(6) If urinary dipstick is positive, then formal complete urinalysis
(7) If hematuria confirmed them formal hematuria evaluation of cystoscopy, cytology and upper tract imaging.
(8) If a BCa is noted, then histology, tumor grade and tumor stage
(9) Annual health maintenance review
(10) Annual collection of voided urine samples for multiplex testing which consisted of the analysis of ANG, APOE, A1AT, CA9, IL8, MMP9, MMP10, PAI1, SDC1 and VEGF.

3.4. Interventions

To minimize variability in data collection, an exposure questionnaire was created and deployed. Data collectors were trained. Information bias, especially underreporting of tobacco use, is a potential issue, given the reliance on self-reporting documentation by the subjects. Furthermore, data collection focused on feasible, compliant, and easily accessible information. A schedule of enrollment, data collection, and observations activities are presented in Table 1 and study schema in presented in Figure 1.

To mitigate selection bias, patients are recruited consecutively, and eligibility criteria are kept broad.

Data quality checks and cross-verification with post-procedural assessments will be employed.

Table 1. Study calendar.

	Baseline	Month 12 (+/− 90 days)	Month 24 (+/− 90 days)	Month 36 (+/− 90 days)	Month 48 (+/− 90 days)
Informed Consent	X				
Medical History	X				
Disease/Survival Follow-up		X	X	X	X
Dipstick Urinalysis [1]	X	X	X	X	X
Complete urinalysis		As needed [2]	As needed [2]	As needed [2]	As needed [2]
Urine culture		As needed [2]	As needed [2]	As needed [2]	As needed [2]
Urinary multiplex immunoassay	X	X	X	X	X
Urinary cytology		As needed [2]	As needed [2]	As needed [2]	As needed [2]
Cystoscopy		As needed [2]	As needed [2]	As needed [2]	As needed [2]
Upper tract imaging		As needed [2]	As needed [2]	As needed [2]	As needed [2]
TURBT and/or biopsy		As needed [2]	As needed [2]	As needed [2]	As needed [2]
Urine specimen collection	X	X	X	X	X
FFPE Tumor collection		If available	If available	If available	If available

[1] Initially, urinary dipstick will be performed. If blood is detected, then a formal complete urinalysis (dipstick and microscopy). [2] As needed, i.e., if medically justified based on subject's history and if its within standard of care in the management of the patient.

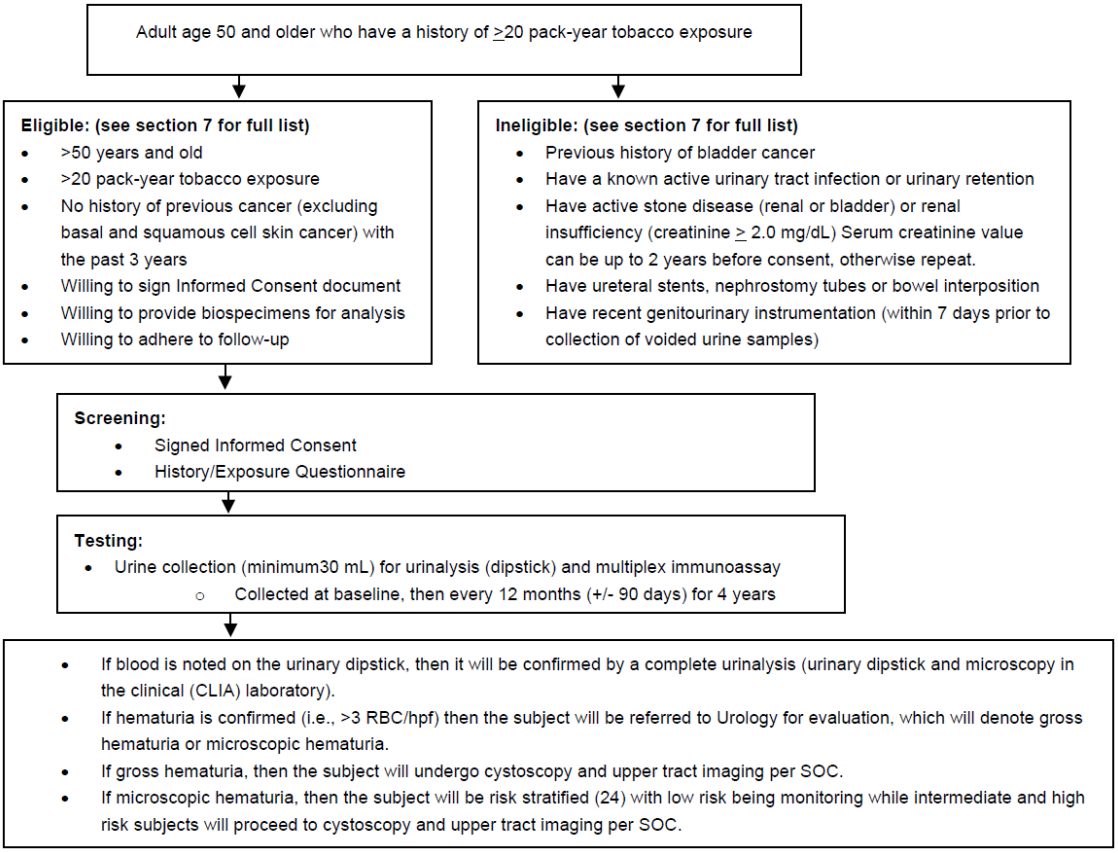

Figure 1. Study schema.

3.5. Data Management and Quality Control

Data will be recorded on case report forms (CRFs) with our electronic data capture (EDC) system, RedCap (21 CFR Part 11 compliant). The data monitoring team, independent from the clinical coordinators, will conduct annual data accuracy checks using de-identified source documents. Any discrepancies between the CRFs and the source documents will be thoroughly review and corrected. RedCap is a web-based EDC [26] where study related data will be securely stored on a dedicated server with access limited to the study personnel, ensuring confidentiality and integrity.

3.6. Outcomes and Definitions

The primary outcome is the recruitment and retention of high-risk subjects into this 4-year longitudinal study.

3.7. Sample Size Justification

This prospective study is a pilot study to a) generate high quality data and b) show feasibility, which we will define as know-how to launch workflow associated with a longitudinal screening study and to learn about the correlations between the urinary biomarkers of interest over time. Pearson's correlation coefficients with a 5% significance level (two-sided) and 99% power require sample sizes of 95 (4 cancer subjects), 120 (5 cancer subjects), 150 (6 cancer subjects), or 200 (8 cancer subjects) patients to detect a difference between the null hypothesis correlation of zero and the alternative hypothesis correlation of 0.42, 0.38, 0.34, or 0.31 respectively.

3.8. Data Analysis

Data analysis has not started. Assay precision for individual markers will be estimated by coefficient of variation (CV) using data from the embedded and co-randomized quality controls (QCs). In addition, signal to noise, defined as the ratio of standard deviation (SD) of biological samples over SD of QCs will be estimated for individual markers as a surrogate measure of information content and relative dynamic range of data.

The mean of the duplicate results of each biomarker will be used as the assay value for the biomarker. Biomarker values with difference between duplicates greater than five SD over mean duplicate difference across all samples will be marked as missing. All biomarker data will be normalized into z scores after optional log-transformation to correct for possible left skewness. In addition, sensitivity and specificity of several potential predictors will be estimated.

4. Discussion

This study will build on previous research to provide new insight into the ability to identify recruit and retain at risk patients for developing BCa. We noted the herculean task of screening and recruiting from PCP offices which were previously reported by Messing et al. [23]. When we transition to screening lung cancer screening clinics which already had a captive audience of exposed similarly exposed patients, we were able to greatly accelerate our screening and recruitment efforts. Furthermore, Messing et al. previously screened these high-risk patients twice a year but we opted to reduce this to once a year in hopes of eventually seeing higher recruitment and retention rates. We also provided a rather nominal financial incentive of $20 per visit which was geared to defray costs associated with travel and parking. A critical improvement we have deployed over the past year was the ability for the patient to collect their voided urine sample at home and ship it directly on ice packs to our laboratory for testing. This way the patient does not have to take time off work or leave the comfort of their home to participate in the study. Lastly incorporating the VA healthcare system into the study was critical as they recruited a significant number of patients in a short period, which was helped by their established lung cancer screening program. Furthermore, tobacco use rates as well as occupational exposure rates tend to be higher in the military. Their integrated health system and excellent electronic medical records made them an excellent partner in this feasibility study.

This present feasibility study provides the necessary framework to conduct a larger definitive study in which these high-risk patients would be randomized to standard of care versus multiplex immunoassay for the early detection of BCa in a longitudinal cohort. Assuming an incidence rate of 0.1% of developing BCa in an at-risk population and accounting for expected dropout and missing data rate of 15%, the study requires a minimum of 100 BCa events. This threshold is needed to support the robust development of the risk prediction model. Consequently, the final target sample size has been set at 20,000 patients enrolled (10,000 randomized to arm 1 SOC and 10,000 randomized to arm 2 multiplex immunoassay testing). These calculations may change as we conclude our current feasibility study and determine the overall retention rate.

5. Trial Status

- Protocol version number v1.7 and date 24 July 2024
- Date recruitment began 4 April 2022
- Recruitment completed
- Annual clinical and molecular follow-up is ongoing for a planned duration of four years
- Last patient/last visit 5 January 2029

Author Contributions

C.J.R.: Study concept and design; G.G. and H.F.: Acquisition of samples and data; M.L.: Data analysis and interpretation. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Cedars Sinai and VA Long Beach Local Ethics Review Board (CSMC IRB No: STUDY00001895; Approved: 04/18/2022).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data available upon reasonable request.

Conflicts of Interest

C.J.R. is Chief Executive Officer of Nonagen Bioscience. All other authors declare that they have no competing interests.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

Abbreviations

UC	urothelial carcinoma
PCP	Primary Care Physician
AUA	American Urological Association
ANG	angiogenin
APOE	Apolipoprotein E
A1AT	Alpha-1 antitrypsin
CA9	Carbonic Anhydrase 9
IL8	Interleukin 8
MMP9	Matrix metalloproteinase-9
MMP10	Matrix metalloproteinase-10
PAI1	plasminogen activator inhibitor-1
SDC1	syndecan-1
VEGF	vascular endothelial growth factor
CRF	case report forms
ECC	electronic data capture
NMIBC	non-muscle invasive bladder cancer
MIBC	muscle invasive bladder cancer
CLIA	Clinical Laboratory Improvement Amendments
RBC	red blood cells
Hpf	high power field
SOC	standard of care
TURBT	transurethral resection of a bladder tumor
CV	coefficient of variation
QC	quality control
SD	standard deviation

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