Biochemistry and Biophysics Reports 39 (2024) 101778

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

A non-invasive screening method using *Caenorhabditis elegans* for early detection of multiple cancer types: A prospective clinical study

Hideyuki Hatakeyama, Masayo Morishita, Aya Hasan Alshammari, Umbhorn Ungkulpasvich, Junichi Yamaguchi, Takaaki Hirotsu, Eric di Luccio *

Hirotsu Bio Science Inc., Tokyo, 102-0094, Japan

ARTICLE INFO

Keywords:
Caenorhabditis elegans (*C. elegans*)
Urine
Nematode-nose (N-NOSE)
Olfactory response
Multi-cancer early detection

ABSTRACT

Cancer is the second leading cause of death worldwide, according to the World Health Organization, surpassed only by cardiovascular diseases. Early identification and intervention can significantly improve outcomes. However, finding a universal, non-invasive, economical, and precise method for early cancer detection remains a significant challenge. This study explores the efficacy of an innovative cancer detection test, N-NOSE, leveraging a *Caenorhabditis elegans* olfactory assay on urine samples across a diverse patient group exceeding 1600 individuals diagnosed with various cancers, with samples from the Shikoku Cancer Center (Ehime, Japan) under approved ethical standards. Current cancer screening techniques often require invasive procedures, can be painful or complex, with poor performance, and might be prohibitively costly, limiting accessibility for many. N-NOSE addresses these challenges head-on by offering a test based on urine analysis, eliminating the need for invasive methods, and being more affordable with higher performance at early stages than extensive blood tests or comprehensive body scans for cancer detection. In this study, N-NOSE demonstrated a capability to accurately identify upwards of 20 cancer types, achieving detection sensitivities between 60 and 90 %, including initial-stage cancers. The findings robustly advocate for N-NOSE's potential as a revolutionary, cost-effective, and minimally invasive strategy for broad-spectrum early cancer detection. It is also particularly significant in low- and middle-income countries with limited access to advanced cancer diagnostic methods, which may contribute to the improved outcome of affected individuals.

1. Introduction

Cancer is the second leading cause of death worldwide, causing nearly 10 million deaths in 2020, and this number is expected to double by 2050 (World Health Organization, WHO). The most prevalent new cases of cancer in 2020 included breast (2.26 million cases), lung (2.21 million cases), colon and rectum (1.93 million cases), prostate (1.41 million cases), skin (non-melanoma) (1.20 million cases), and stomach (1.09 million cases). The most common causes of cancer death in 2020 were lung (1.80 million deaths), colon and rectum (916,000 deaths), liver (830,000 deaths), stomach (769,000 deaths), and breast (685,000 deaths), respectively [1]. Cancer results generally from the interplay between genetic factors and various external environmental factors, including (i) physical carcinogens (ultraviolet and ionizing radiation), (ii) chemical carcinogens like asbestos, components of tobacco smoke, alcohol, and other carcinogenic substances, and (iii) biological carcinogens such as infections caused by certain viruses, bacteria, or parasites. Cancer cases and deaths are also expected to increase significantly due to lifestyle behaviors that elevate cancer risk, such as excess body weight and physical inactivity. This trend is particularly significant in low- and middle-income countries experiencing significant economic and cultural transformations [2].

Furthermore, due to senescence, cancer incidence gradually rises with age, likely influenced by the cumulative impact of less effective DNA and cellular repair mechanisms. Early detection and treatment of cancer at earlier pathological stages are crucial to reduce cancer mortality rates. Therefore, developing an early screening method to identify various cancers, including pre-cancerous conditions, is essential, mainly before individuals exhibit any associated symptoms. It is crucial to scale up cancer screening initiatives that employ innovative technologies with

Abbreviations: C. elegans, *Caenorhabditis elegans*; N-NOSE, nematode-nose; WHO, World Health Organization; VOCs, volatile organic compounds; TNM, tumor-node-metastasis; NGM, Nematode Growth Media; *E. coli*, *Escherichia coli*.

* Corresponding author.
E-mail address: e.diluccio@hbio.jp (E. di Luccio).

https://doi.org/10.1016/j.bbrep.2024.101778
Received 26 March 2024; Received in revised form 21 June 2024; Accepted 4 July 2024

high specificity to detect cancer in asymptomatic individuals.

In recent years, significant attention has been given to biological diagnostics, particularly cancer detection. One notable approach that utilizes the olfactory system of animals for cancer detection is the use of well-trained sniffer dogs [3–5]. However, this method is impractical due to the extensive time, cost, and effort required to train dogs. *Caenorhabditis elegans* (*C. elegans*) is a multicellular organism whose entire genome sequence was first determined in hermaphrodites; it is also easy to handle and inexpensive to breed. *C. elegans*, as one of the nematodes, also has an excellent olfactory system, which includes approximately 1200 olfactory receptor-like genes (compared to around 800 genes in dogs), allowing its chemotactic behavior. *C. elegans* approach favorable odors while avoiding unpleasant smells. Hirotsu et al. first reported that the wild-type N2 strain of *C. elegans* displayed attractive chemotaxis toward human cancer cell secretions, cancer tissues, and urine from patients with colorectal, gastric, and breast cancers, whereas *C. elegans* showed aversive response to urine derived from healthy subjects [6]. This phenomenon is likely due to the response of olfactory sensory neurons, such as AWC neurons in *C. elegans*, to specific volatile organic compounds (VOCs) associated with cancer. Building upon our findings, we developed an innovative, non-invasive cancer detection method termed "Nematode-Nose (N-NOSE)" and applied it to screen various malignancies [7–11]. Recent studies by other groups have also demonstrated the efficacy of nematode-based screening for prostate cancer [12] and breast cancer [13] with high accuracy, indicating that the concept of N-NOSE for cancer detection is reproducible worldwide.

In this study, we assessed the broad applicability of the N-NOSE method for over 20 types of malignancies. A cohort of more than 1600 patients with different cancer types was included. N-NOSE method successfully detected the most common cancer types globally, such as lung, stomach, colorectal, breast, cervical, and prostate cancer, even at earlier stages, demonstrating higher sensitivities. These results suggest that N-NOSE is a sensitive, non-invasive primary screening method for the early detection of multiple cancers.

2. Materials and methods

2.1. Study population

The study design was approved by the Ethics Committee of the National Hospital Organization Shikoku Cancer Center (Ehime, Japan). Urine samples were collected from 1664 patients diagnosed with malignancies at the center between May 2017 and March 2021. The cohort included 951 males (mean age 67.5 ± 11.0 years, range 23–95) and 713 females (mean age 60.8 ± 13.8 years, range 18–93). Clinical examinations strictly adhered to the WMA Declaration of Helsinki principles. All patients provided written informed consent before registration to this study. The authors also guarantee the accuracy and completeness of the data and analysis, along with the fidelity of this study regarding technical and bioinformatic protocols. One limitation of this prospective clinical study is the lack of a non-cancer control group, which is essential for determining specificity. Our previous studies (references [6,10]) have demonstrated the specificity of the N-NOSE test in distinguishing healthy individuals from cancer patients, with reported specificities of 90 % and 95 %, respectively. It provides important context for the current findings. Notably, this study employed the same cutoff as our previous clinical studies [6,10] to separate the healthy control group from the cancer-patient group, reinforcing the significance of our findings and facilitating comparisons with our previous research.

This study defined a cancer patient as someone histopathologically identified as having cancer. The tumor-node-metastasis (TNM) staging was performed according to the TNM Classification of Malignant Tumors of the UICC.

2.2. Culture and maintenance of C. elegans

C. elegans (wild-type N2) was cultured with standard methods at 20 °C on Nematode Growth Media (NGM) seeded with *Escherichia coli* (*E. coli*) strain NA22 as a food source [6,14]. *C. elegans* is a type of nematode, about 1 mm long, becomes an adult in 3–4 days, and lays 100 to 300 eggs.

2.3. Measurement by N-NOSE

Chemotaxis analysis of nematodes was performed according to standard protocols used in previous nematode studies [6,15,16]. *C. elegans* (wild-type N2) was cultured at 20 °C under well-fed and uncrowded conditions with the *E. coli* strain NA22 as a food source. Chemotaxis assays were performed on 9 cm plates containing 10 mL of 2 % agar, 5 mM KPO_4, 1 mM $CaCl_2$, and 1 mM $MgSO_4$, as previously described. Briefly, 0.5 μL of 1 M sodium azide was spotted on two points at both ends of the plates, and then 1 μL of urine sample diluted at 10-fold and 100-fold with ultra-pure water was added to two points. Sodium azide is the anesthetic used to minimize the effects of adaptation and is added before adding the nematodes to the plate. Only sodium azide is added to the non-urine side of the plate. In summary, sodium azide is added to 4 points of the plate (urine side and non-urine side). Urine is added on two points (on the urine side and the sodium azide location). We confirmed that nematodes showed no chemotaxis behavior to 1 μL of water (data not shown). Approximately 100 adult nematodes were collected, washed three times with chemotaxis buffer (0.05 % gelatin, 5 mM KPO_4, 1 mM $CaCl_2$, and 1 mM $MgSO_4$), and placed in the center of the plate. After removing the excess buffer, the nematodes were allowed to roam for 30 min (Fig. 1). The chemotaxis index was calculated using the following equation: Chemotaxis index = (A - B)/(A + B), where (A) is the number of nematodes near the urine samples, and (B) is the number of nematodes in the region without the samples. For each sample, the chemotaxis assays were performed at both 10-fold and 100-fold urine dilution (n = 10 for each dilution). A negative index (−1 to 0) indicates repulsion to the sample, and a positive index (0–1) indicates an attraction to the sample; it was considered "positive" if the chemotaxis indexes were positive in at least one of two dilutions [10]. When estimating sensitivity, "negative" was defined as only when both indexes in 10-fold and 100-fold dilution were less than 0. Previous studies have demonstrated that the worm consistently shows repulsive behavior to urine samples from the non-cancer group [6,10]. This threshold was kept unchanged throughout all experiments, which were done under the same conditions as before.

2.4. Statistical analyses

All statistical analyses, including One-way analysis of variance (ANOVA) and Wilcoxon signed-rank tests, were performed using JMP® 14 (SAS Institute, Cary, NC, USA). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Cancer patients

The N-NOSE chemotaxis assays were performed using two different conditions of 10-fold and 100-fold urine dilution. Urine samples were obtained from a total of 1664 patients, consisting of 951 males with a mean age of 67.5 ± 11.0 years (age range: 23–95) and 713 females with a mean age of 60.8 ± 13.8 years (age range: 18–93). Statistical analysis indicated no significant correlations between N-NOSE results and age or sex. This suggests that N-NOSE may be a generalized screening tool applicable to a broad patient population. Overall, 1199 out of 1664 patients (72.1 %) tested positive for N-NOSE (Fig. 2).

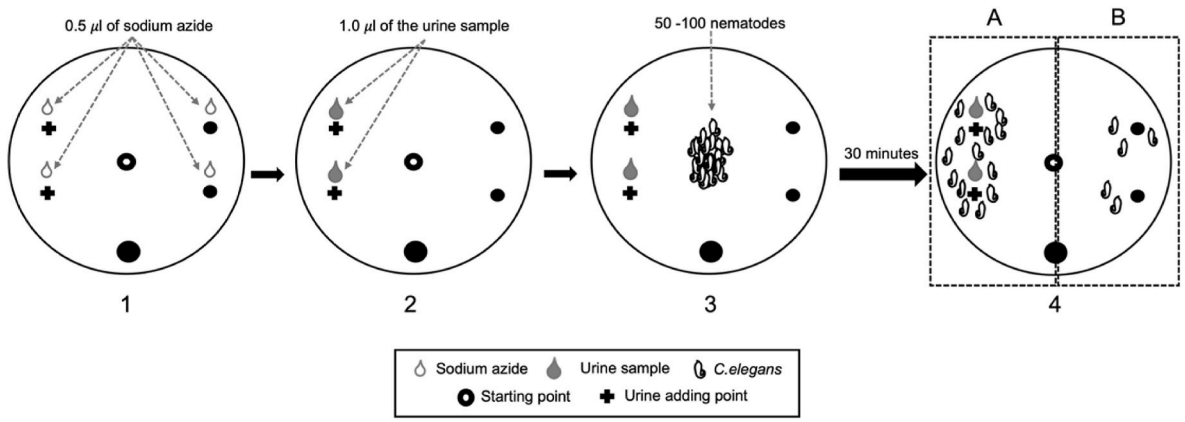

Fig. 1. Illustration of Chemotaxis Assay Procedure, including four steps: (1) Add 0.5 μL of sodium azide, (2) Add 1.0 μL of urine sample, (3) Introduce 50–100 nematodes into the center of the plate and wait for 30 min; and (4) Capture the image, where (A) represents the number of nematodes near the urine samples, and (B) represents the number of nematodes in the region without the samples.

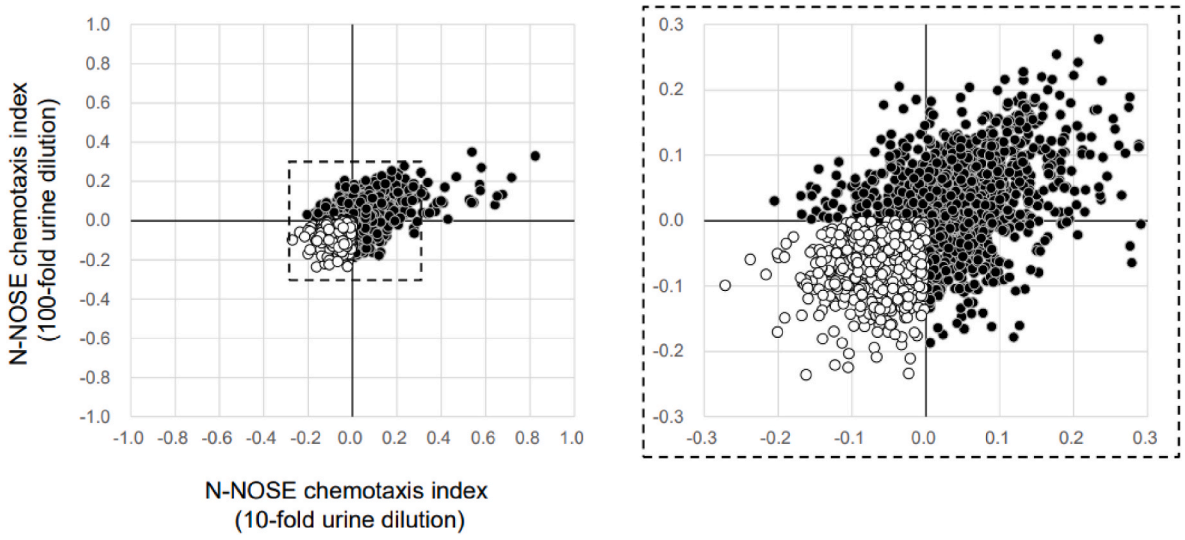

Fig. 2. The N-NOSE chemotaxis assays for all 1664 patients with over 20 different types of malignancies. The Chemotaxis Index (A–B)/(A + B) measures nematode attraction (positive values) or repulsion (negative values) to a urine sample. Assays were performed using two urine dilutions (10-fold and 100-fold, n = 10 each), and results were averaged for each dilution. A sample was considered "positive" if the chemotaxis index was positive (0–1) in at least one dilution. Closed circles indicate "positive" for N-NOSE, and open circles indicate "negative" for N-NOSE.

3.2. Sensitivity of N-NOSE in detecting several cancer types

The cancer-type-specific sensitivities and stage-specific sensitivities are summarized in Tables 1 and 2, respectively. Table 1 shows that out of eleven cancer types with more than 50 patients each, ten exhibited high N-NOSE sensitivities (69.0 %–81.0 %). Uterine cancer showed a relatively lower sensitivity of 59.0 %. Focus was then placed on the N-NOSE results for the most common types of cancer worldwide, such as lung, stomach, colorectal, breast, cervical, and prostate cancer. Stage-specific sensitivities are listed in Table 3. All six cancer types listed showed a high sensitivity for N-NOSE, even at an earlier phase (stage 0 + I): lung (67.7 %), stomach (73.1 %), colon, and rectum (80.0 %), breast (67.2 %), cervix (83.7 %), and prostate (73.6 %). We also found that N-NOSE can detect pancreatic cancer (Table 4) and ovarian cancer (Table 5) with high sensitivity at all stages, respectively. These findings strongly suggest that N-NOSE successfully detects over 20 types of cancer with high sensitivity, even at earlier stages. Additionally, only a few patients enrolled and showed positive for N-NOSE, including thymic carcinoma, pleural mesothelioma, parotid gland cancer, peritoneal cancer, mediastinal cancer, ureteral cancer, and various types of oral cancer such as gum cancer, listed as 'Others' in Table 1.

3.3. Correlations between N-NOSE results and biochemical parameters in cancer patients

As shown in Table 6, biochemical blood tests revealed no significant correlations between N-NOSE results and hepatic ALT and AST, However, a positive correlation was observed between the stage of cancer and renal creatinine ($p = 0.0094$) but not renal BUN ($p = 0.9529$), possibly suggesting renal dysfunction as a side effect caused by chemotherapy. Urine general qualitative tests demonstrated significant correlations between N-NOSE results and urinary proteins (10-fold: $p < 0.0001$, 100-fold: $p = 0.0045$) and urinary ketone bodies (10-fold: $p < 0.0001$, 100-fold: $p < 0.0001$), but no correlations with urinary glucose (10-fold: $p = 0.0695$, 100-fold: $p = 0.5818$) and urinary occult blood (10-fold: $p = 0.3686$, 100-fold: $p = 0.9144$). Positive correlations were also found between the stage of cancer and urinary proteins ($p < 0.0001$) and urinary ketone bodies ($p < 0.0001$).

Table 1
Cancer-type-specific sensitivities of N-NOSE.

Cancer type	Number of patients	N-NOSE positive	Sensitivity (%)
Lung	353	250	70.8
Prostate	221	159	71.9
Colon and rectum	198	158	79.8
Stomach	155	107	69.0
Breast	128	94	73.4
Uterine	78	46	59.0
Cervix	74	53	71.6
Pancreas	63	49	77.8
Ovary	58	47	81.0
Esophagus	57	39	68.4
Bladder	54	39	72.2
Kidney	30	19	63.3
Thyroid	29	23	79.3
Lymphoma	28	24	85.7
Pharynx	15	9	60.0
Liver	11	7	63.6
Biliary tract	11	6	54.5
Tongue	9	8	88.9
GIST	7	6	85.7
Testis	6	5	83.3
Glottis	5	4	80.0
Melanoma	3	2	66.7
Others	71	45	63.4
Total	1664	1199	72.1

GIST: Gastrointestinal stromal tumor.

Table 2
Stage-specific sensitivities of N-NOSE.

Stage	Number of patients	N-NOSE positive	Sensitivity (%)
Stage 0	28	22	78.6
Stage I	626	447	71.4
Stage II	308	231	75.0
Stage III	228	159	69.7
Stage IV	384	283	73.7
Undefined	90	57	63.3
Total	1664	1199	72.1

3.4. Influence of co-existing diseases on N-NOSE sensitivity in cancer patient

Additionally, we investigated whether co-existing conditions or co-morbidities influenced N-NOSE results. Although we were able to obtain clinical information for a subset of cancer patients enrolled in this study, the findings indicated no significant impact of co-existing diseases or co-morbidities, such as diabetes mellitus, hyperlipidemia, hypertension, and infectious diseases, on N-NOSE sensitivities (Table 7). Among cancer patients with specific conditions, including diabetes mellitus only (sensitivity: 82.1 % [78/95]), hyperlipidemia only (sensitivity: 83.3 % [5/6]), hypertension only (sensitivity: 83.3 % [40/48]), infectious diseases only (sensitivity: 84.2 % [32/38]), or any combination of these conditions (sensitivity: 85.3 % [197/231]), no significant deviations were observed when compared to cancer patients without other diseases or aberrant signs (sensitivity: 87.2 % [144/165]).

4. Discussion

In this cohort study, N-NOSE demonstrated high sensitivities (60–90 %) for the detection of over 20 types of cancer, including the most common types worldwide, such as lung, stomach, colon and rectum, breast, cervix, prostate, even at earlier stages. Although the numbers of patients with liver cancer, kidney cancer, thyroid cancer, biliary tract cancer, pharyngeal cancer, and malignant lymphoma were relatively low (cases: 10–50), N-NOSE also exhibited potential for effectively detecting these malignancies. Early diagnosis improves prognosis, particularly pancreatic cancer [17–19]. However, early detection is

Table 3
Stage-specific sensitivities of N-NOSE for the most common six types of cancer worldwide.

Cancer type	Number of patients	N-NOSE positive	Sensitivity (%)
Lung	353	250	70.8
Stage 0	1	1	100.0
Stage I	154	104	67.5
Stage II	36	27	75.0
Stage III	55	37	67.3
Stage IV	103	78	75.7
Undefined	4	3	75.0
Stomach	155	107	69.0
Stage 0	1	1	100.0
Stage I	66	48	72.7
Stage II	25	18	72.0
Stage III	19	8	42.1
Stage IV	41	30	73.2
Undefined	3	2	66.7
Colon and rectum	198	158	79.8
Stage 0	1	1	100.0
Stage I	54	43	79.6
Stage II	49	41	83.7
Stage III	43	32	74.4
Stage IV	50	41	82.0
Undefined	1	0	0.0
Breast	128	94	73.4
Stage 0	7	4	57.1
Stage I	57	39	68.4
Stage II	36	28	77.8
Stage III	13	11	84.6
Stage IV	15	12	80.0
Undefined	0	0	0.0
Cervix	74	53	71.6
Stage 0	7	6	85.7
Stage I	36	30	83.3
Stage II	14	8	57.1
Stage III	7	5	71.4
Stage IV	8	4	50.0
Undefined	2	0	0.0
Prostate	221	159	71.9
Stage 0	5	5	100.0
Stage I	86	62	72.1
Stage II	59	44	74.6
Stage III	20	15	75.0
Stage IV	19	14	73.7
Undefined	32	19	59.4

Table 4
Stage-specific sensitivities of N-NOSE for pancreatic cancer.

Stage	Number of patients	N-NOSE positive	Sensitivity (%)
Stage 0	–	–	–
Stage I	4	3	75.0
Stage II	22	17	77.3
Stage III	5	5	100.0
Stage IV	31	23	74.2
Undefined	1	1	100.0
Total	63	49	77.8

Table 5
Stage-specific sensitivities of N-NOSE for ovarian cancer.

Stage	Number of patients	N-NOSE positive	Sensitivity (%)
Stage 0	–	–	–
Stage I	27	23	85.2
Stage II	4	3	75.0
Stage III	7	7	100.0
Stage IV	16	11	68.8
Undefined	4	3	75.0
Total	58	47	81.0

Table 6
Statistical analyses for the involvement of the subjects' background characteristics in N-NOSE results and cancer progression.

Biochemical parameters	N-NOSE results		Stage of cancer
	10-fold	100-fold	
Hepatic ALT	$p = 0.9347$	$p = 0.9550$	N/A
Hepatic AST	$p = 0.1796$	$p = 0.7181$	N/A
Renal BUN	$p = 0.0032$	$p = 0.0607$	$p = 0.9529$
Renal creatinine	$p = 0.0002$	$p = 0.0052$	$p = 0.0094$
Urinary glucose	$p = 0.0695$	$p = 0.5818$	$p = 0.2650$
Urinary proteins	$p < 0.0001$	$p = 0.0045$	$p < 0.0001$
Urinary ketone bodies	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Urinary occult blood	$p = 0.3686$	$p = 0.9144$	$p = 0.7656$

N/A: Not Applicable.

Table 7
Influence of co-existing diseases or co-morbidities of cancer patients on N-NOSE sensitivities.

Co-existing diseases or co-morbidities	Number of patients	N-NOSE positive	Sensitivity (%)
Diabetes mellitus	95	78	82.1
Hyperlipidemia	6	5	83.3
Hypertension	48	40	83.3
Infectious diseases	38	32	84.2
Any combination of these	231	197	85.3
Without other diseases	165	144	87.2

challenging, and most pancreatic cancer cases (80 %) are diagnosed at an advanced stage [20–22]. In this study, in combination with our previous reports [9,11], we achieved high sensitivity for early detection of pancreatic cancer using N-NOSE across three different institutions. Successful detection and treatment of most cancers are more likely at earlier stages, underscoring the importance of "primary cancer screening" methods that can detect various types of cancer before the manifestation of cancer-related symptoms. Therefore, this study suggests that N-NOSE, a *C. elegans* scent test using urine, holds promise as a sensitive tool for multi-cancer early detection.

Additionally, we observed correlations of the N-NOSE results with urinary protein and urinary ketone bodies. Both urinary components also exhibited simultaneous correlations with cancer stages. Matsui et al. recently demonstrated that elevated levels of urinary proteins, ranging from mild to heavy (more than 2+) and trace (±), were significantly associated with increased mortality risk from cancer in a general population [23]. Although the exact relationship between specific urinary proteins and chemotactic behavior in nematodes remains unclear, this association suggests a possible link between elevated urinary protein levels as cancer biomarkers and cancer progression. Regarding urinary ketone bodies, it is hypothesized that such ketogenic status in cancer patients is caused by insufficient uptake or starvation of nutrition (glucose) at later stages. As cancer progresses, the ketogenic rate in individuals seems to be triggered by sequential alteration of energy metabolic pathway: (i) glucose depletion, (ii) fatty-acid production, and upregulated β-oxidation in the liver, followed by (iii) ketone body overproduction. A similar phenomenon is confirmed by a low-carbohydrate diet for reducing body weight. Another possibility is the influence of a ketogenic diet as a cancer therapeutic option [24–29]. Dmitrieva-Posocco et al. reported a potent tumor-inhibitory effect of ketogenic diets, recapitulated by administering the ketone body β-hydroxybutyrate [30]. Elevated levels of urinary ketone bodies (acetone, acetoacetic acid, and β-hydroxybutyrate), some of which are preferable odors for nematodes [31], may also affect the N-NOSE index, further attracting the nematode to urine derived from cancer patients. Although the N-NOSE indexes were drastically low at 100-fold urine dilution due to the decreased contents of urinary ketone bodies, further investigation is required to determine how urinary ketone bodies may impact nematode chemotaxis.

While N-NOSE demonstrated high sensitivity for detecting various malignancies, this study has some limitations, particularly in estimating specificity due to the lack of a control group comprising healthy subjects and non-cancerous individuals. Previous studies have shown that N-NOSE effectively distinguishes healthy individuals from cancer patients, with reported specificities of 95.0 % (207/218) [6] and 90.2 % (129/143, using a combined method with two different urine dilutions) [10]. However, a previous study conducted at the Saitama Medical University International Medical Center in Japan [11] used a control disease group rather than healthy subjects. This study focused on pancreatic cancer and demonstrated N-NOSE's ability to differentiate cancer from other diseases. It is important to note that using a control disease group instead of healthy subjects affects the evaluation of specificity. Particularly, N-NOSE showed a relatively lower specificity of 60 % (57/95) against the non-cancerous disease control group, which included conditions such as autoimmune pancreatitis, pancreatic pseudocyst, and common bile duct stone [11]. Therefore, while the specificity data from different studies provide important context, the variability, considering the reference groups, should be carefully considered when evaluating performance and correlation with this present study.

High specificity is crucial for practical application in cancer screening tests. It is important to note the variability in results across different studies, even when the same cancer types are investigated independently. Table 1 shows that twelve cancer types exhibited relatively lower sensitivities (less than 72.1 % overall in this study), including lung, stomach, cervix, liver, esophagus, and prostate cancers. However, compared with our previous reports, there were no marked differences in sensitivity for specific cancer types [8,11]. This consistency in sensitivity across studies reinforces the reliability of N-NOSE, although specificity data may vary due to differences in control groups.

N-NOSE is a behavioral test based on the chemotaxis of *C. elegans*, where the worms are attracted to cancer urine samples (positive chemotaxis index) and repelled by healthy urine samples (negative chemotaxis index). The consistent cutoff value (0) used in this study is the same as our previous N-NOSE clinical studies, reinforcing the relevance of this study. N-NOSE correlates with the presence or absence of cancer, as it is a behavioral test leveraging on *C. elegans*'s olfaction [8,11]. Even without a control group, tracking the N-NOSE index is sufficient for cancer screening [8,11].

Like other novel cancer screening technologies, N-NOSE is designed for global implementation to benefit a wide range of people. Considering scaling-up operations, several procedural and environmental factors need to be considered when implementing N-NOSE cancer screening worldwide. First, careful attention must be given to the large-scale culture and maintenance of nematodes, as this critically affects their olfactory performance. Second, well-controlled ambient conditions, such as room temperature and humidity, can improve the results of N-NOSE chemotaxis assays. Third, an appropriate method for urine collection and storage is crucial. Therefore, sophisticated manipulation of nematodes and careful preparation of diluted urine samples to minimize the loss of VOCs are essential to successfully deploying N-NOSE worldwide.

The signal-to-noise ratio is typically lower in asymptomatic individuals compared to symptomatic ones. In a screening context, detecting the disease in individuals without symptoms is inherently more challenging due to less pronounced indicators of the disease, leading to a potential overestimation of a cancer test's sensitivity in a prospective analysis where cases have already been identified. The assumption of universal and perfect diagnostic testing does not hold in real-world screening programs. The variability in the receipt and accuracy of diagnostic tests can further degrade the performance of a screening test in a prospective setting. This degradation occurs because, in a prospective analysis, the selection of cases and controls may inadvertently favor scenarios where the diagnostic testing performed

exceptionally well, not reflecting the variability and imperfections encountered in routine screening. Therefore, while our study provides solid insight into the efficacy of N-NOSE cancer screening tests, the data presented may not fully capture the complexities and challenges of implementing these tests in a real-world, prospective screening program. The differences in population characteristics, such as the presence or absence of symptoms and the inherent limitations of diagnostic testing methodologies, may affect the performance metrics of screening tests when applied prospectively.

Moreover, upon receiving a positive N-NOSE result, initiating follow-up procedures to determine the specific cancer type is crucial. This typically involves further clinical investigations such as positron emission tomography (PET), computed tomography (CT), or magnetic resonance imaging (MRI) to obtain a more definitive diagnosis. While early detection offers the potential for improved treatment outcomes, it's important to acknowledge that any screening tool carries a risk of overdiagnosis. Careful consideration of the potential harms of overdiagnosis, like unnecessary treatment or psychological distress, is necessary when implementing any screening program [32]. N-NOSE offers significant value as a primary cancer screening tool despite the need for subsequent diagnostic steps. Its high sensitivity, non-invasive nature, low cost, and ease of use ideally position it for detecting a wide spectrum of cancers (over 20 types as reported in this study) in their early stages. Such early detection facilitates timely intervention and improves treatment outcomes.

Clinical guidelines and benchmarks for acceptable specificity levels are crucial in cancer screening to minimize false positives and overdiagnosis, which can lead to unnecessary stress and treatment for patients. High specificity ensures that non-cancer cases are correctly identified, reducing the likelihood of false positives. For instance, specificity levels for breast cancer screening using mammography typically range from 85 % to 95 %, as these tests aim to balance sensitivity and specificity to ensure effective screening while minimizing harm from false positives. However, in a real-world situation, sensitivities for routine breast (33.5 %), cervical (2.5 %), colorectal (24 %), stomach (37.5 % by endoscopy), lung (5.7 %) are low, as reported by the Japanese National Cancer Center Cancer Information Service "Cancer Registration/Statistics". This contrasts with the significantly higher sensitivity of N-NOSE observed across multiple cancers (Table 3). Similarly, stool-based tests for colorectal cancer, such as the fecal immunochemical test (FIT), exhibit specificity levels of around 90 %–98 %, ensuring that most non-cancer cases are correctly identified. In the context of different cancer screening methods, the specificity of N-NOSE, PET-CT, and serum CEA levels varies significantly, with N-NOSE showing higher sensitivities at the early cancer stage (Table 3).

The sensitivities reported in our previous clinical studies slightly differ from those reported in this study. Clinical studies evaluating cancer tests often exhibit variability in sensitivity and specificity across different research settings. Generally speaking, for any type of clinical study, this inconsistency can be attributed to several factors, including variations in patient populations and procedural nuances. Patient demographics like age, ethnicity, and lifestyle factors may influence the presence of cancer biomarkers and impact test results. Procedural variations can further contribute to inconsistencies generally seen in clinical study variability. Geographical or demographic factors may also play a role, as the prevalence of certain cancer types and associated biomarkers can vary across populations. However, in this N-NOSE study, the cutoff value (0) is identical to that used in all previous N-NOSE clinical studies, reinforcing the significance and consistency of our findings and facilitating comparisons with our previous research. As mentioned above, the variability of performance across N-NOSE studies depends largely on the type of control group rather than other factors.

The required specificity for cancer screening in a clinical setting is not a fixed value and depends on factors like cancer type, screening population, and consequences of false positives. While no universal threshold exists, organizations like the USPSTF and NCI emphasize a balance between sensitivity and specificity. Studies evaluating new screening methods often report specificity values, serving as benchmarks for comparison. It's important to note that sensitivity and specificity are trade-offs and that positive predictive value (PPV), the probability of a positive test truly indicating cancer, is crucial, depending on sensitivity, specificity, and disease prevalence. When selecting and interpreting cancer screening tests, healthcare providers must carefully consider these factors.

Utilizing a cancer screening test with less than optimal specificity and sensitivity for mass screening in asymptomatic populations can significantly contribute to reducing cancer mortality despite its limitations. Such tests enable early detection, leading to earlier and potentially more effective treatment options, which can improve survival rates. In contrast, acknowledging that aggressive cancers and factors beyond diagnosis impact mortality, early detection generally enhances treatment options and lowers mortality for many cancers, albeit not universally. Reducing late-stage diagnoses offers considerable benefits for patients and healthcare systems alike. Instead of dismissing screening due to imperfections, efforts should focus on personalizing screening intervals, developing better tests, and integrating screening with comprehensive prevention and treatment strategies. Even moderate performance tests can improve public health by making screening more accessible and cost-effective and encouraging broader participation. Their use drives technological and methodological advancements and boosts health awareness and preventive behaviors. Therefore, while imperfect, deploying the best available screening options provides a valuable opportunity to detect and treat some cancers earlier than otherwise, thereby offering improved treatment outcomes and reducing cancer deaths over time, underscoring such initiatives' broad public health benefits.

5. Conclusion

N-NOSE successfully detects over 20 types of cancer with high sensitivities, making it a promising tool for early multi-cancer detection. It offers affordable, non-invasive primary screening, significantly enhancing early cancer detection opportunities. This is particularly significant in low- and middle-income countries with limited access to advanced diagnostic methods. Early detection through N-NOSE allows for timely treatment initiation, which can substantially improve patient outcomes. The widespread adoption of N-NOSE in Japan, which has already facilitated the screening of over 600,000 individuals, attests to its practicality and effectiveness as a commercially accessible tool.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Funding

The authors received no funding in relation to this article.

Ethics declarations

Institutional Review Board Statement. The study was conducted per the Declaration of Helsinki and approved by the Ethics Committee of the National Hospital Organization Shikoku Cancer Center (permission number 3045-1).

Consent to publish

Institutional Review Board Statement Informed Consent Statement. Informed written consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

CRediT authorship contribution statement

Hideyuki Hatakeyama: Writing – original draft, Data curation, Conceptualization. **Masayo Morishita:** Writing – review & editing, Investigation. **Aya Hasan Alshammari:** Writing – review & editing, Validation. **Umbhorn Ungkulpasvich:** Writing – review & editing, Formal analysis. **Junichi Yamaguchi:** Writing – review & editing, Formal analysis. **Takaaki Hirotsu:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Eric di Luccio:** Writing – review & editing, Validation, Supervision, Formal analysis, Data curation.

Declaration of competing interest

Takaaki Hirotsu is the CEO and founder of Hirotsu Bio Science Inc.; Eric di Luccio, Hideyuki Hatakeyama, Masayo Morishita, Aya Hasan Alshammari, Umbhorn Ungkulpasvich, and Junichi Yamaguchi are Hirotsu Bio Science Inc. employees.

Acknowledgments

We are grateful to many medical doctors and staff (National Hospital Organization Shikoku Cancer Center, Japan) for study design approval at their institutional review board, for patients' recruitment with written informed consent, and the collection of urine from the patients and their clinical data. We also thank Mamie Egashira, Hisayo Noguchi, Satoka Okuda, Kumiko Ishihara, Rie Nakamura, Kaori Sadasue, Nagisa Matsumura, and Nobuaki Kondo (Hirotsu Bio Science Inc., Japan) for their helpful assistance in N-NOSE data acquisition and statistics.

References

[1] WHO, Cancer. https://www.who.int/news-room/fact-sheets/detail/cancer, 2022. (Accessed 26 September 2023).
[2] L.A. Torre, R.L. Siegel, E.M. Ward, A. Jemal, Global cancer incidence and mortality rates and trends—an update, Cancer Epidemiol. Biomarkers Prev. 25 (1) (2016) 16–27.
[3] M. McCulloch, T. Jezierski, M. Broffman, A. Hubbard, K. Turner, T. Janecki, Diagnostic accuracy of canine scent detection in early-and late-stage lung and breast cancers, Integr. Cancer Ther. 5 (1) (2006) 30–39.
[4] G. Horvath, G.a.K. Järverud, S. Järverud, I. Horváth, Human ovarian carcinomas detected by specific odor, Integr. Cancer Ther. 7 (2) (2008) 76–80.
[5] H. Sonoda, S. Kohnoe, T. Yamazato, Y. Satoh, G. Morizono, K. Shikata, M. Morita, A. Watanabe, M. Morita, Y. Kakeji, Colorectal cancer screening with odour material by canine scent detection, Gut 60 (6) (2011) 814–819.
[6] T. Hirotsu, H. Sonoda, T. Uozumi, Y. Shinden, K. Mimori, Y. Maehara, N. Ueda, M. Hamakawa, A highly accurate inclusive cancer screening test using *Caenorhabditis elegans* scent detection, PLoS One 10 (3) (2015) e0118699.
[7] H. Kusumoto, K. Tashiro, S. Shimaoka, K. Tsukasa, Y. Baba, S. Furukawa, J. Furukawa, T. Suenaga, M. Kitazono, S. Tanaka, Behavioural response alteration in *Caenorhabditis elegans* to urine after surgical removal of cancer: nematode-NOSE (N-NOSE) for postoperative evaluation, Biomarkers Cancer 11 (2019) 1179299X19896551.
[8] H. Kusumoto, K. Tashiro, S. Shimaoka, K. Tsukasa, Y. Baba, S. Furukawa, J. Furukawa, T. Niihara, T. Hirotsu, T. Uozumi, Efficiency of gastrointestinal cancer detection by nematode-NOSE (N-NOSE), in vivo 34 (1) (2020) 73–80.
[9] A. Asai, M. Konno, M. Ozaki, K. Kawamoto, R. Chijimatsu, N. Kondo, T. Hirotsu, H. Ishii, Scent test using *Caenorhabditis elegans* to screen for early-stage pancreatic cancer, Oncotarget 12 (17) (2021) 1687.
[10] S. Inaba, N. Shimozono, H. Yabuki, M. Enomoto, M. Morishita, T. Hirotsu, E. di Luccio, Accuracy evaluation of the *C. elegans* cancer test (N-NOSE) using a new combined method, Cancer Treatment and Research Communications 27 (2021) 100370.
[11] M. Kobayashi, A. Fujita, T. Ogawa, Y. Tanisaka, M. Mizuide, N. Kondo, Y. Imaizumi, T. Hirotsu, S. Ryozawa, *Caenorhabditis elegans* as a diagnostic aid for pancreatic cancer, Pancreas 50 (5) (2021) 673–678.
[12] M. Thompson, N. Sarabia Feria, A. Yoshioka, E. Tu, F. Civitci, S. Estes, J.T. Wagner, A *Caenorhabditis elegans* behavioral assay distinguishes early stage prostate cancer patient urine from controls, Biology Open 10 (3) (2021) bio057398.
[13] E. Lanza, M. Di Rocco, S. Schwartz, D. Caprini, E. Milanetti, G. Ferrarese, M. T. Lonardo, L. Pannone, G. Ruocco, S. Martinelli, *C. elegans*-based chemosensation strategy for the early detection of cancer metabolites in urine samples, Sci. Rep. 11 (1) (2021) 17133.
[14] S. Brenner, The genetics of *Caenorhabditis elegans*, Genetics 77 (1) (1974) 71–94.
[15] C.I. Bargmann, E. Hartwieg, H.R. Horvitz, Odorant-selective genes and neurons mediate olfaction in *C. elegans*, Cell 74 (3) (1993) 515–527.
[16] K. Yoshida, T. Hirotsu, T. Tagawa, S. Oda, T. Wakabayashi, Y. Iino, T. Ishihara, Odour concentration-dependent olfactory preference change in *C. elegans*, Nat. Commun. 3 (1) (2012) 739.
[17] H. Furukawa, S. Okada, H. Saisho, J. Ariyama, E. Karasawa, A. Nakaizumi, S. Nakazawa, K. Murakami, T. Kakizoe, Clinicopathologic features of small pancreatic adenocarconoma: a collective study, Cancer, Interdisciplinary International Journal of the American Cancer Society 78 (5) (1996) 986–990.
[18] A. Kanno, A. Masamune, K. Hanada, M. Kikuyama, M. Kitano, Advances in early detection of pancreatic cancer, Diagnostics 9 (1) (2019) 18.
[19] A.D. Singhi, E.J. Koay, S.T. Chari, A. Maitra, Early detection of pancreatic cancer: opportunities and challenges, Gastroenterology 156 (7) (2019) 2024–2040.
[20] K.Y. Bilimoria, D.J. Bentrem, C.Y. Ko, A.K. Stewart, D.P. Winchester, M. S. Talamonti, National failure to operate on early stage pancreatic cancer, Ann. Surg. 246 (2) (2007) 173.
[21] D.P. Ryan, T.S. Hong, N. Bardeesy, Pancreatic adenocarcinoma, N. Engl. J. Med. 371 (11) (2014) 1039–1049.
[22] J. Kleeff, M. Korc, M. Apte, C. La Vecchia, C.D. Johnson, A.V. Biankin, R.E. Neale, M. Tempero, D.A. Tuveson, R.H. Hruban, Pancreatic cancer, Nat. Rev. Dis. Prim. 2 (1) (2016) 1–22.
[23] M. Matsui, K. Tsuruya, H. Yoshida, K. Iseki, S. Fujimoto, T. Konta, T. Moriyama, K. Yamagata, I. Narita, M. Kasahara, Trace proteinuria as a risk factor for cancer death in a general population, Sci. Rep. 11 (1) (2021) 16890.
[24] D.D. Weber, S. Aminazdeh-Gohari, B. Kofler, Ketogenic diet in cancer therapy, Aging (Albany NY) 10 (2) (2018) 164.
[25] D.D. Weber, S. Aminzadeh-Gohari, J. Tulipan, L. Catalano, R.G. Feichtinger, B. Kofler, Ketogenic diet in the treatment of cancer–where do we stand? Mol. Metabol. 33 (2020) 102–121.
[26] F. Plotti, C. Terranova, D. Luvero, M. Bartolone, G. Messina, L. Feole, S. Cianci, G. Scaletta, C. Marchetti, V. Di Donato, Diet and chemotherapy: the effects of fasting and ketogenic diet on cancer treatment, Chemotherapy 65 (3–4) (2020) 77–84.
[27] W.H. Talib, A.I. Mahmod, A. Kamal, H.M. Rashid, A.M. Alashqar, S. Khater, D. Jamal, M. Waly, Ketogenic diet in cancer prevention and therapy: molecular targets and therapeutic opportunities, Curr. Issues Mol. Biol. 43 (2) (2021) 558–589.
[28] L. Barrea, M. Caprio, D. Tuccinardi, E. Moriconi, L. Di Renzo, G. Muscogiuri, A. Colao, S. Savastano, E. Obesity Programs of nutrition, Research, A. group, Could ketogenic diet "starve" cancer? Emerging evidence, Crit. Rev. Food Sci. Nutr. 62 (7) (2022) 1800–1821.
[29] L. Yang, T. TeSlaa, S. Ng, M. Nofal, L. Wang, T. Lan, X. Zeng, A. Cowan, M. McBride, W. Lu, Ketogenic diet and chemotherapy combine to disrupt pancreatic cancer metabolism and growth, Med 3 (2) (2022) 119–136. e8.
[30] O. Dmitrieva-Posocco, A.C. Wong, P. Lundgren, A.M. Golos, H.C. Descamps, L. Dohnalová, Z. Cramer, Y. Tian, B. Yueh, O. Eskiocak, β-Hydroxybutyrate suppresses colorectal cancer, Nature 605 (7908) (2022) 160–165.
[31] S.E. Worthy, G.L. Rojas, C.J. Taylor, E.E. Glater, Identification of odor blend used by *Caenorhabditis elegans* for pathogen recognition, Chem. Senses 43 (3) (2018) 169–180.
[32] S. Srivastava, E.J. Koay, A.D. Borowsky, A.M. De Marzo, S. Ghosh, P.D. Wagner, B. S. Kramer, Cancer overdiagnosis: a biological challenge and clinical dilemma, Nat. Rev. Cancer 19 (6) (2019) 349–358.