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C. elegans odour detection in the diagnosis of prostate cancer

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Author:
Laura Rinta-Kanto

Supervisor:
Docent Päivi Koskinen, PhD
Department of Biology

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There are about 200 distinct forms of cancer, each of which is diagnosed and treated differently. Early identification and treatment of cancer is especially important as the population ages and cases become more frequent. In Finland prostate cancer is the most prevalent cancer in men with approximately 5000 new cases and 900 deaths yearly. There are nearly 1,3 million cases globally every year. Despite advances in recent years, prostate cancer continues to be a significant medical issue for afflicted men, with overtreatment of benign illness and lack of effective medicines for metastatic prostate cancer.

Prostate cancer rarely displays symptoms until it is incurable. Present technologies are unable to reliably identify between those tumours that will advance so slowly that they will not generate symptoms and those that are likely to cause death. As there is no sure way to prevent prostate cancer, the only way to reduce suffering and mortality is via early identification and competent patient care. Early detection followed by appropriate treatment plays a big role in the recovery while economical and accessible diagnosis methods are of vital importance.

Presently prostate-specific antigen (PSA) screening is frequently employed for detecting prostate cancer in its early state. However, it is considered a controversial method, since around 67% of men with an elevated PSA level obtain a false positive result and are not diagnosed with prostate cancer, whereas 15% of men with a negative result will develop cancer.

The *Caenorhabditis elegans* nematodes are widely used in research as a model organism since they are easy to maintain and possess many well-defined features. These include an efficient chemosensory system, which allows *C. elegans* to distinguish between substances in its surroundings and move towards or away from them. The chemosensory neurons in the head of *C. elegans* express around 700 distinct G protein–coupled receptors (GPCRs) that are able to recognize soluble or volatile compounds (VOCs) from the urine and exhaled air of cancer patients. It is thought that cancer metabolism generates particular odorants that could be useful in the early detection of the disease.

The goal of this study was to determine how well the *C. elegans* nematodes can detect prostate cancer from human urine samples. Chemotaxis assays were performed with urine samples of men with either benign hyperplasia of the prostate or malignant prostate cancer. Results obtained during this study were fairly variable and not reliable enough to recommend the method for clinical use. However, several factors may have influenced the results that were not in line with previously published data. Thus, further studies on this topic are definitely needed.

Key words: Cancer, prostate cancer, *C. elegans*, nematode, olfaction, chemotaxis, diagnostics

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1 Introduction

1.1 Cancer

The term cancer refers to a versatile group of diseases, usually characterized by abnormal cells that grow rapidly and can circulate to other organs in the body causing metastatic growths. In these malignancies cells cease to function properly, growing and dividing in uncontrolled ways throughout the body producing tumours that may progress over time. Cancer is an illness caused by mutations in the genes that control the activity of the affected cells (Miller, 2018). These cells have gained the ability to divide uncontrollably due to disrupted mechanism of cell cycle regulation. As cancer is regarded as an organ illness, malignancies are often named after the organ from which they originated. When cancerous cells spread to other tissues throughout the body, they become more destructive (Miller E., 2016). The resulting illness will vary based on the cell undergoing division and the type of tissue invaded. Untreated cancer cells that have migrated to other tissues will finally disrupt organ function and lead to death.

Worldwide cancer is the second leading cause of death and a substantial obstacle to prolonging life expectancy. As stated by the World Health Organization (WHO), cancer is one the most significant causes of mortality before the age of 70 in 60% of countries (Sung et al., 2021). Stroke and coronary heart disease mortality rates have declined substantially in comparison to cancer mortality rates in many regions, contributing to the increasing prominence of cancer as the leading cause of death (Bray et al., 2021). In 112 countries, prostate cancer is the most frequently diagnosed cancer in men and the second leading cause of mortality related to cancer. However, in part due to its high mortality rate, lung cancer is the leading cause of cancer death in males in 93 countries (Arnold et al., 2019).

Changes in signalling pathways influence a broad range of cellular functions, spanning from proliferation and growth to apoptosis and invasiveness in malignant cells (Vaghari-Tabari et al., 2021). Metabolism in a malignant cell undergoes extensive alterations, affecting a variety of processes, including, energy production and biosynthesis of macromolecules. Numerous therapeutic approaches in cancer treatment have concentrated on disrupting the metabolism of malignant cells. Oncogenes and tumour suppressor genes also have a substantial impact on

cellular metabolism. The metabolic needs of cancer cells for uncontrolled growth, resistance to apoptosis, and migration seem to be supported by a strong relationship between its metabolism and signalling pathways.

1.1.1 Hallmarks of cancer

In 2000 Hanahan and Weinberg (Hanahan & Weinberg, 2011) argued that as normal cells transform into a neoplasm, they generate distinct ways to enhance tumour development and metastatic dissemination. They discovered six hallmarks found in practically every human cancer and in 2011, they increased the number to eight (Figure 1). Understanding the rationalisation behind a multitude of anti-cancer medications and their mechanisms of response and resistance has been facilitated by the identification of these hallmarks. (O'Neill et al., 2018).

Currently, the 8 hallmarks include the acquired capacity to maintain proliferative signalling, evading growth suppressors, resistance to apoptosis, promoting replicative immortality, inducing angiogenesis, initiating invasion and metastasis, remodelling cellular metabolism, and evading immune destruction. Also, inflammation and genome instability are characteristics that enable cancer develop these hallmarks (Hanahan, 2022).

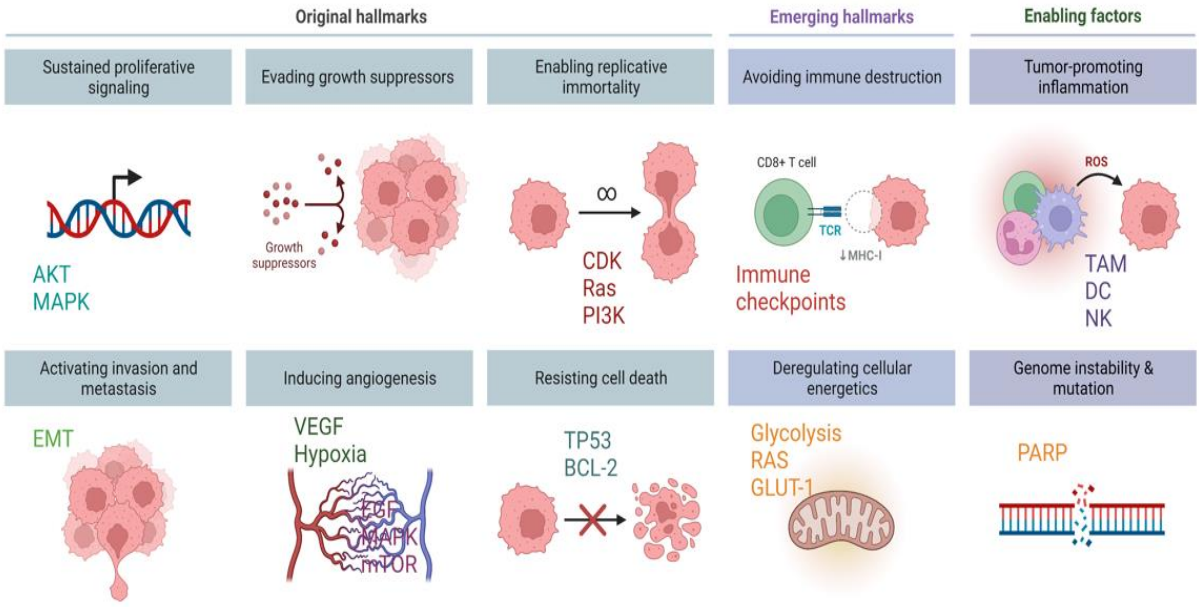

Figure 1. Hallmarks of cancer. The defining characteristics of cancer are comprised of eight biological capacities accumulated throughout the progression of the malignancy. These hallmarks serve as an organizational framework for understanding the complexity of malignant tumours. In addition, there are two factors that enable cancer to develop the hallmarks. Adapted from “Hallmarks of Cancer”, in BioRender.com by Laura Rinta-Kanto

Normal cells control cell growth and division to preserve homeostasis, but tumour cells can proliferate continuously (Vivanco et al., 2014). Proliferation is achieved via the synthesis of growth factor ligands, which increase the amount of receptor proteins on the surface of cancer cells, increasing their sensitivity to growth factor ligands. When epidermal growth factor (EGF) binds to a receptor on the cell surface, the mammalian target of rapamycin (mTOR) pathway and the mitogen-activated protein kinase (MAPK) pathway are activated. These mechanisms promote cell proliferation, survival, and motility. Additionally, continuous proliferation stimulates downstream signalling pathways or suppresses the negative feedback loops of the different signalling pathways (Zhang et al., 2016). Approximately 25% of non–small cell lung cancer (NSCLC) tumours include EGFR mutations.

Similar to normal cells, malignant cells need oxygen and nutrition, as well as the capacity to remove carbon dioxide and metabolic waste (Hanahan et al., 2010). Whilst angiogenesis is temporarily induced in healthy tissue during tissue regeneration, it is permanently active in tumour cells, as a result of a so-called angiogenic switch. Angiogenesis is stimulated early in the multiphase development of neoplasia and is a common factor among numerous tumour types.

This angiogenic switch is regulated by signalling proteins like vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) that bind to their receptors situated on endothelial cells (Tirumani et al., 2015). Hypoxia is induced by fast tumour development, causing the tumour to exceed its blood supply, which is the primary trigger for the activation of hypoxia-inducible factors. VEGF-A, which encodes ligands that facilitate blood vessel formation and signalling through receptor tyrosine kinases (RTKs), is the most extensively investigated proangiogenic factor. Frequently, tumour neo vasculature is abnormal, as shown by dilated arteries with atypical branching, microhaemorrhage and leaky capillaries (Hanahan & Weinberg, 2000). Once angiogenesis is triggered, different neovascularization patterns emerge in malignancies. While pancreatic ductal adenocarcinoma is often hypovascular, renal cell carcinomas (RCC) are highly vascular.

Often, cancerous cells exhibit genetic and epi-genetic alterations, distinguishing them from normal cells; as a result, they present antigens and promote T cell–mediated immunity (Hanahan & Weinberg, 2011). Tumour cells are identified as foreign by the immune cells of the host and are destroyed by natural killer cells and T lymphocytes. Through the activation of immunosuppressive pathways known as immune checkpoints, cancer cells may escape detection and avoid immunologic destruction. This characteristic has been reported in several cancers, including non-small cell lung cancer (NSCLC) and advanced melanoma.

The invasion and metastasis cascade is a complicated process where malignant cells circulate to other tissues in the body (Barkan et al., 2010). The first phase of metastasis is the dissemination of cancerous cells from the initial tumour. Intra- and extravasation allow cancer cells to spread via blood and lymph vessels resulting in efficient colonization, in which the cells adjust to their new environment by generating micrometastases and eventually larger tumours. The epithelial-mesenchymal transition (EMT) is a mechanism through which altered epithelial cells acquire the capacity to invade, resist apoptosis, and proliferate. These micrometastases might remain inactive for years. For instance, melanoma and breast cancer might generate macroscopic metastases decades after original tumour removal.

To acquire all of the previously described characteristics, like angiogenesis and resistance to immune destruction, tumour cell genomes must complete a series of modifications (Negrini et al., 2010). Multistep tumour growth needs many consecutive clonal divisions from a mutated

genotype. Due to the capability of the genome maintenance system to identify and repair DNA errors, the incidence of spontaneous mutations in each cell generation is limited. Cancer cells accelerate mutation rates by impairing the monitoring mechanisms that normally evaluate genomic integrity and drive damaged cells into programmed cell death or senescence.

The various abnormalities that impact distinct components of the DNA repair machinery are known as genome caretakers (Artandi & DePinho, 2010). Telomerase is one of them since the loss of telomeric DNA may result in karyotype disruption by deletion or amplification of chromosomal regions. Specific genome modifications vary across tumour types, but instability of the genome is crucial to malignant cells and fundamental for the advancement of the disease (Walsh, 2015). Poly–adenosine diphosphate ribose polymerase (PARP) enzyme family repairs single- and double-stranded DNA breaks and the inhibition of PARP-1 converts single-stranded DNA breaks into double-stranded DNA breaks. In BRCA-mutated cancers, suppression of PARP may result in genomic abnormalities and double-strand breaks, which eventually induce cell death.

Cancer cells must evade negative control of cell proliferation in addition to maintaining proliferative signals (de Caestecker et al., 2000). Tumour suppressors restrict cell growth and proliferation in diverse ways. Due to mutations in tumour suppressors, cancer cells lack gatekeepers to control cell cycle, resulting in continuous cell proliferation. For example, the transforming growth factor β-pathway hinders the proliferation of cells. Cancers damage this pathway to activate another cell program and divert signals, which may result in characteristics that are linked to high-grade malignancies (Hanahan & Weinberg, 2011).

Cyclins and cyclin-dependent kinases (CDKs) play a crucial role in sustaining cell cycle regulation (Finn et al., 2016). In order to progress across the G1 and S phases, the retinoblastoma (Rb) protein must be phosphorylated by CDK4 or CDK6. Alteration of the CDK4-cyclin D1-Rb association might result in a lack of cell cycle control. Hormone receptor-positive breast cancer shows a possible dependency on this interaction.

Apoptosis, also known as programmed cell death, is a form of defence against the development of cancer (Adams & Cory, 2007). TP53 and BCL-2 are upstream regulators and downstream effectors involved in apoptosis (Aubrey et al., 2017). When chromosomal

aberrations and DNA breaks occur, the TP53 tumour suppressor triggers apoptosis. The absence of the TP53 tumour suppressor may lead to cells escaping apoptosis. BCL-2 suppresses apoptosis by inhibiting BAX, BAK and BH3 proapoptotic proteins and reducing their activity. In chronic lymphocytic leukemia (CLL), resistance to apoptosis is related with enhanced BCL-2 protein expression (Anderson et al., 2016).

To produce macroscopic tumours, cancer cells require a limitless capacity for replication (Hanahan & Weinberg, 2011). Crisis and senescence are two main obstacles of proliferation. Senescence results in a permanent nonproliferative stage, while crisis results in apoptosis (Blasco, 2005). Telomeres are thought to be fundamental in unrestricted replication. They guard the ends of chromosomes, and the loss of this protective mechanism may result in crisis and induction of apoptosis. Telomerase is an RNA-dependent DNA polymerase that inserts telomere repeat sequences to telomeric DNA ends (Hanahan & Weinberg, 2011). It is expressed in 90 percent of malignant cells but is almost non-existent in normal cells, suggesting that telomerase inhibitors can be used as a targeted treatment.

Immune cells are present in almost all neoplastic cells, in varying numbers (Hanahan & Weinberg, 2011). Inflammation paradoxically promotes carcinogenesis and progression by delivering growth and survival factors, and extracellular enzymes that promote vasculature, metastasis, and invasion. Inflammation is widely believed to reflect the effort of the immune system to eliminate malignancies.

Neoplasia is characterized by chronic uncontrolled proliferation of cells which is dependent on an enhanced and altered energy metabolism to support cell development (Eriksson et al., 2017). Normal cells obtain the majority of their energy from mitochondrial oxidative phosphorylation and, under anaerobic circumstances, via transition to the less efficient glycolytic process. Under hypoxic tumour microenvironment, cancer cells may change their metabolic activity to favour glycolysis (aerobic glycolysis). Oncogenes, like RAS, MYC, and mutant tumour suppressor TP53, are linked to glycolytic fuelling. This metabolic transition is accomplished by tumour cells upregulating glucose transporters (particularly GLUT-1), enhancing glucose uptake, and upregulating proteins of the glycolytic pathway.

1.1.2 Cancer metabolites

Previous metabolomic studies have identified a pattern of down-regulation of specific metabolites in cancer patients in comparison to healthy individuals (Silva et al., 2011) . A plausible explanation for this tendency might be found in malignant cells that are converting some of these metabolites into other substances in order to fulfil the increasing energy needs.

Many cancers are characterized by a change in cellular metabolism. In addition to the well-described alterations in nutrient intake and waste elimination, abnormal cancer cell metabolism also leads to alterations in cytoplasmic metabolite concentration (Muñoz-Pinedo et al., 2012). Upregulation of metabolites and cancer-associated alterations in protein expression may increase the progression and onset of cancer. Even under aerobic conditions, cancer cells exhibit glycolytic hyperactivity, oxidative phosphorylation, and downregulation of the tricarboxylic acid (TCA) cycle. Several studies indicate that cancer cells have specific metabolic phenotypes, primarily related to amino acid, carbohydrate, purine, and nucleotide metabolism.

Modifications in the levels of urinary metabolites involved in metabolic pathways such as glucose, and purine metabolism, AA and TCA and urea cycle, have been suggested as possible urine indicators of prostate cancer stage and, as a result, might be beneficial in non-invasive diagnosis and prognosis (Struck-Lewicka et al., 2015). Besides PSA, additional potential biomarkers for prostate cancer are: Intracellular PSA, PSA derivatives, prostate-specific membrane antigen (PSMA), prostate cancer antigen 3 [PCA3], early prostate cancer antigen 2, annexin A3, the fusion gene TMPRSS2: ERG, human kallikrein 2, and the Engrailed-2 protein (EN2). None of these, though, are employed for screening (Khalid et al., 2015). Also, sarcosine has been distinguished as a biomarker for metastatic prostate cancer by one study, but its significance remains unclear (Sreekumar et al., 2009).

Significant intracellular and extracellular alterations are associated with tumour formation and cellular transformation and urine contains the metabolic signs of the condition (Khalid et al., 2015). During tumour formation, protein alterations in malignant cells result in peroxidation of components on the cell membrane. These components can create volatile organic compounds (VOCs) that are detectable in the headspace of the cells (Bax et al., 2018). Importantly, cancer cells create patterns of VOCs that can be detected from urine. Increasing

number of studies associate various malignancies with either an increase or a reduction in VOC levels. It is connected with the odour of cancer, which is the result of metabolic alterations causing VOC patterns that are prevalent in malignancies (Daulton et al., 2021).

Several biomarkers have been identified that are specific to prostate cancer (Mcdunn et al., 2013). Glycine, threonine, and alanine have been found in the urine of prostate cancer subjects utilizing a GC–MS-based method. In addition, these amino acids were shown to substantially correlate with Gleason Pattern Progression in a bioinformatics-based investigation on the metabolism of prostate cancer. Also, a substantial drop in glutamine levels in the urine of prostate cancer patients has been observed (Struck-Lewicka et al., 2015). Other metabolites that have been found to exhibit alterations in the urine of prostate cancer patients compared to healthy individuals are associated with the TCA cycle. The reduction of acinitate, isocitrate, and succinate in the urine of men with prostate cancer relative to the control group suggests a disturbance in energy metabolism, including the TCA cycle.

1.2 Prostate cancer

The prostate is around the size of a walnut at younger ages, but it may get enlarged as men age, which increases the risk of malignant prostate cells (Miller E., 2016). Primarily affecting males over the age of 65, prostate cancer is seldom identified before the age of 40. Approximately 14% of men will be diagnosed with prostate cancer during their life.

Globally, prostate cancer is a substantial public health issue since it is the second most common form of cancer in men (Sung et al., 2021). With over 1,4 million new cases and 375,304 deaths in 2020, prostate cancer is the fifth major cause of cancer-related mortality in men globally. It is the most prevalent cancer in males in more than half of the world's nations (105 out of 185) including Finland where 5000 men are diagnosed with prostate cancer every year. Because of its latent character, histological differentiation and non-specificity of the existing diagnostic methods prostate cancer has such a high mortality rate but, in many nations, mortality has decreased owing to screening, early diagnosis, and better treatment (Carlsson & Vickers, 2020).

1.2.1 Symptoms and classification

Prostate cancer is mainly diagnosed in men over the age of 50 who present symptoms. These include erectile dysfunction, lower urinary tract symptoms (LUTS) and visible haematuria (Merriel et al., 2018). LUTS are also frequent in benign disorders affecting the prostate, such as prostatitis and benign prostatic hyperplasia (BPH), making the diagnosis difficult. The severity of LUTS does not usually correlate with the risk of prostate cancer or its stage. LUTS are generically categorized as storage, voiding, and post-urination symptoms (Osteroi Jakupsstovu & Brodersen, 2018). According to several studies, men with LUTS have an increased risk of localized prostate cancer, but no relationship with advanced prostate cancer has been found (Weight et al., 2013) (Bhindi et al., 2017). It is believed that people seeking medical care for LUTS are at a greater chance of being diagnosed with prostate cancer due to increased rates of testing and are mostly diagnosed with early-stage cancer (Weight et al., 2013).

The grading and staging of prostate cancer relate to the development and progression, along with the specific histology and cellular alterations in tumours (Table 1) (Schatten, 2018). The TNM staging addresses the size and location of the tumour (T), the spread of the tumour to lymph nodes (N), and the migration of the tumour to different areas of the body (M). These results are merged to identify the cancer stage. There are five stages to evaluate the degree of cancer, with stage 0 indicating the absence of cancer and stages I through IV describing the progression of disease.

Table 1. TNM classification for staging prostate cancer by size and dissemination.
Adapted from “Localized Prostate Cancer” by Rosario, E., & Rosario, D. J. (2022).

Classification	Definition
Tumour (T)	
Tx	Tumour cannot be evaluated
T0	No evidence of a primary tumour
T1	Tumour not detected during DRE or imaging
T1 a/b/c	Incidental finding or detected on biopsy
T2	Tumour detected during DRE but is present in the prostate only
T2a	In half or less than one lobe of the prostate
T2b	In more than half of one prostate lobe
T2c	In both prostate lobes
T3	Tumour extends outside of the prostate
T3a	Extends to the prostate capsule
T3b	Has spread to the seminal vesicles
T4	Tumour has spread to tissues near the prostate other than the seminal vesicles
Regional lymph nodes (N)	
Nx	Nearby lymph nodes are not evaluated
N0	No cancer cells found in nearby lymph nodes
N1	Cancer cells found in nearby lymph nodes
Distant metastasis (M)	
M0	Cancer has not spread beyond the prostate
M1	Cancer has spread beyond the prostate
M1a	Cancer has spread to distant lymph nodes
M1b	Cancer has spread to bone
M1c	Cancer has spread to another organ

1.2.2 Diagnosis

In the United States, a serum PSA cut-off of 4 ng/ml is part of an FDA-approved screening program (Catalona et al., 2000). PSA testing is not routinely utilized for screening elsewhere

due to its poor sensitivity, which is predicted to be around 21% for identifying any prostate cancer and 51% for detecting high-grade tumours (Wolf et al., 2010). At this threshold, the false negative rate may reach up to 20%. Only 25-30 percent of men with elevated PSA values between 4 and 10 ng/mL during screening develop prostate cancer (Naughton et al., 2000). A substantial number of malignancies identified are of low grade and do not lead to symptoms or disease-related death. Therefore, intensive therapy might reduce the life quality of the patient without affecting their lifespan, which is why there is a need for a new diagnostic method (Roine et al., 2014).

Partly due to the conflicted state of PSA screening, there have also been several studies reporting that dogs may be taught to identify prostate, lung, bladder, skin, ovarian, and breast cancer from breath and urine samples (Pickel et al., 2004). The outcomes of canine scent detection studies have been somewhat conflicting and there is variation in the performance of the dogs within and between studies. In a study by Cornu et al. (2011) trained dogs were shown to detect prostate cancer from urine with high sensitivity. However, in another study by Elliker et al. (2014) two dogs were taught to distinguish prostate cancer from urine samples of healthy individuals using a double-blinded test, but the dogs were unable to distinguish malignancies from controls.

It has been shown that malignant prostatic cells alter their growth media (Roine et al., 2012). The distinctive odour of prostate cancer is directly induced by chemicals that malignant cells secrete into the urinary system. Using urine headspace, Roine et al. investigated the capacity of an electronic nose to distinguish prostate cancer from benign hyperplasia of the prostate. The eNose is an instrument comprised of a combination of generic sensors and it has been investigated for a variety of medicinal uses, including the early diagnosis of cancer from exhaled air (Roine et al., 2014). They achieved a sensitivity of 78% and a specificity of 67% in a LOOCV analysis with the eNose method.

Gas chromatography/mass spectrometry (GC/MS) can be applied in separating and identifying volatile organic compounds (VOCs) responsible for the odour of urine (Khalid et al., 2015). A simple, non-invasive screening is required to identify cancer in its early stages with less financial and physical burden. Less invasive than blood samples, a non-invasive cancer screening method utilising bodily fluids such as urine to identify volatile biomarkers is a promising option (Bax et al., 2019). Using molecular biomarkers in urine to produce cancer

screening and diagnostic tests has advanced rapidly in recent years, from the use of analytical methods to the use of biological organisms like nematodes.

1.3 *C. elegans*

C. elegans is a nematode that lives on bacteria and fungi from decaying fruit in the soil (Hunt, 2017). At just 1 mm in length, adults are barely visible to the naked eye. *C. elegans* research has been crucial in elucidating various fundamental features of biology, such as apoptosis, RNA interference, and miRNA function since Sydney Brenner's original characterization of the model in the 1960s. In addition to being simple to maintain, *C. elegans* has a short generation period and life span. Because of these characteristics, *C. elegans* has become a major model organism in biological study (Muschiol et al., 2009).

1.3.1 Life cycle and behaviour

C. elegans has a short life cycle of just 3 days from egg to adult at 20°C and generally lives as a self-fertilizing hermaphrodite, though less than 0,2 % of the nematodes are male (Corsi et al., 2015). At 20 degrees, *C. elegans* gestation lasts roughly 16 hours (Figure 2). After fertilization, the embryo forms independently of the mother shielded by an impenetrable eggshell. Embryos typically evolve inside the hermaphrodite until around the 24-cell stage, when the eggs are laid. The hermaphrodite embryo develops into a larva of the first stage (L1). The larvae begin to feed and undergo four stages of larval development (L1-L4). The L1 stage lasts around 16 hours, and other stages approximately 12 hours. Each stage concludes with a phase of inactivity known as lethargus, during which a new outer collagenous layer is produced (Raizen et al., 2008).

Around 12 hours following the L4 molt, adult hermaphrodites begin generating offspring for a period of two to three days until they have spent all their self-made sperm; if hermaphrodite mates with a male, additional progeny can be made. Following the reproductive cycle, hermaphrodites may continue to survive for many weeks until senescence causes death (Corsi et al., 2015). *C. elegans* L2 larvae can initiate an alternative life cycle and enter into an L3 dauer stage when the animals are crowded or food bacteria are depleted from them (Hu, 2007). The shield of a dauer larva fully envelops and covers them mouth of the animal, stopping it from feeding and halting its growth (Corsi et al., 2015). The improved chemical resistance of the dauer cuticle offers stronger defence against outside stressors and caustic substances. Larvae in dauer state can persist for several months and are the most prevalent form of *C. elegans* in the wild. When dauer larvae are placed on plates containing bacteria, they undergo a transformation, and resume their development as L4 larvae.

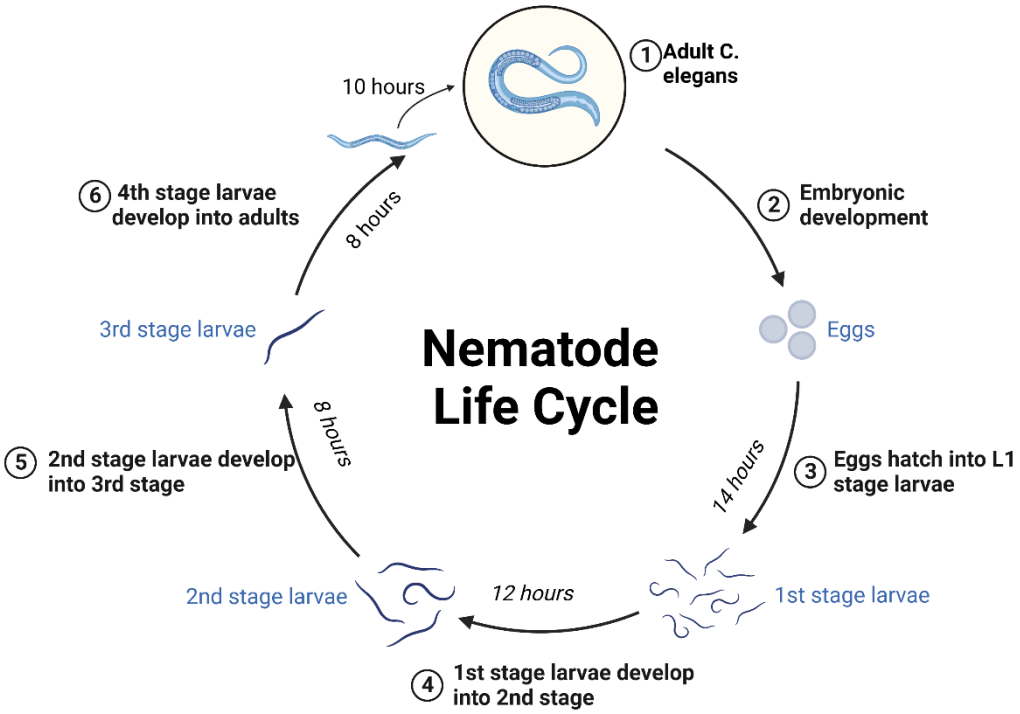

Figure 2. Nematode life cycle. The hermaphrodite life cycle of *C. elegans* is reliant on temperature and is completed in 2.5 days at 25 °C. It consists of four larval stages, and adults typically generate up to 300 offspring over the course of their lifetime. Adapted from “Nematode Life Cycle” in BioRender.com by Laura Rinta-Kanto

In the laboratory, the compact size of *C. elegans* allows for the maintenance of thousands of animals in nutrient medium in multiwell plates, allowing for the assessment of many

compounds at a broad range of concentrations in a small area (Hunt, 2017). With a reproductive potential of roughly 300 offspring by self-fertilization and a life cycle of around 3 days, millions of animals may be created quickly, and the majority of studies can be done in a week or less. *C. elegans* has a strong yet transparent cuticle, which enables observation of interior structures and structure-specific gene expression in transgenic strains. As a microscopic model organism, *C. elegans* is accessible to quick characterisation of genes using forward mutation and for example CRISPR/Cas9 and RNA interference (Waaijers & Boxem, 2014).

C. elegans is a great model for studying the genetics of several fundamental forms of behaviour, including eating, mobility, defecation, foraging, male mating behaviour, egg laying, sensory responses to taste, smell, touch and temperature, and basic forms of learning (Bargmann, 1993). Remarkably, these behaviours may be investigated at the levels of behaviour, brain circuitry, and genetics in the nematode, without the disturbance of more complicated behaviours that might impact the fundamental processes.

Due to the existence of specialised neural circuits and genes, *C. elegans* is a particularly useful model organism for studying the genetic and neurological basis of basic behaviours (Colbert et al., 1995). In contrast to other model species in which mutations in genes that control behaviour have different modes of action, a large number of *C. elegans* genes have been found to result in distinct behavioural traits, when mutated.

The naturally occurring behaviour of *C. elegans* falls into four basic categories (Goodman & Sengupta, 2019). Locomotion is affected by over 130 genes that are neuron- or muscle-specific. Through sensory input *C. elegans* can react to a variety of external stimuli, including smell, taste, touch, temperature, and osmotic cues. Survival and reproduction behaviour like feeding, defecation and mating have been studied in detail. Recently researchers have examined complex behaviours in *C. elegans*, including social behaviour, memory, and learning, as well as the interaction between experience and genes impacting the neurological system and behaviour (De Bono & Maricq, 2005). In addition, *C. elegans* has been used to identify genes implicated in the effects of medicines on the nervous system, such as anesthetics, nicotine, cocaine, fluoxetine (Prozac).

1.3.2 Chemosensation in *C. elegans*

C. elegans has a highly evolved chemosensory network that enables it to recognize a broad range of volatile and water-soluble substances linked to danger, food, or other animals (Bargmann, 2006). More than 5% of its genes and a significant part of its nervous system are devoted to environmental chemical identification. Chemotaxis, adjustments in motility, rapid avoidance, and entry into an alternate dauer stage can all be elicited by chemosensory stimuli. The 11 pairs of chemosensory neurons comprise the amphid chemosensory organs that essentially control these actions. Each sensory neuron detects a distinctive set of pheromones that serve as either attractants or repellents (Ferkey et al., 2021). The behavioural reaction of *C. elegans* to a single substance may be concentration dependent. A subset of chemical signals that are appealing at low concentrations may trigger avoidance behaviour at high doses, for instance.

Chemosensory neurons express between 500 and 1000 distinct G protein-coupled receptors (GPCRs), and these may be complemented by additional sensory pathways (Bargmann, 2006). Two major signal transduction pathways are involved in chemosensation downstream of the GPCRs: one that uses transient receptor potential (TRP) ion channels and one that utilises cGMP as a second messenger to open cGMP-gated channels. Kinases and phosphatases control and adjust these sensory pathways, as exemplified in a recent study from our group, where PIM kinases were shown to be essential for regulation of certain types of olfactory, but not gustatory sensations (Kalichamy et al., 2019). Chemosensory inclinations can be altered by associative learning, developmental history, and sensory adaptation, enabling *C. elegans* to incorporate context and experience into behaviour (Bargmann, 2006).

Chemosensory neurons are typically bilaterally symmetric pairs with anatomically identical but functionally often asymmetric left and right members in each group (Liu & Sternberg, 1995). Based on cilium and axon morphology, and synaptic targets, each pair comprises a class that can be differentiated from other classes. Other chemosensory behaviours have been discovered in other categories of neurons, and several other types of chemosensory neurons assist in mating in male *C. elegans*.

1.3.3 Chemosensory neurons and volatile organic compounds (VOCs)

There are 60 ciliated sensory neurons in *C. elegans* that detect a number of sensory modalities, such as taste, smell, temperature, touch, light, CO2, oxygen, humidity and proprioception (Bargmann, 2006). *C. elegans* uses distinct receptors to detect these environmental stimuli and many of the approximately 1000 G protein-coupled receptors (GPCRs) encoded by the *C. elegans* genome are predicted to function as receptors in sensory nerve cells. When a stimulus activates the GPCR protein receptors, the signal is mediated by two kinds of downstream ion channels which can both activate the sensory neurons by opening the voltage-gated calcium channels through calcium mobilization (Ferkey et al., 2021).

The chemosensory neurons reside in the head amphid, inner labial, and tail phasmid organs and are open to their surroundings directly or indirectly (Troemel et al., 1997). Moreover, the AWA, AWB, and AWC amphid neurons implanted inside the sheath glial cells have more complicated ciliated sensory ends, and these neurons detect mostly volatile substances. The AWB neurons are the principal mediators of aversion response to 2-nonanone and a number of other odorants. The olfactory neuron pair AWA detects volatile signals generated by bacteria to guide animals to probable food sources (Barr et al., 2018). However, there are gender variations in the attraction to some odorants, such as diacetyl.

The calcium levels of the AWA neuron rise in response to the presence of pyrazine, diacetyl, 2-methylpyrazine, and hexyl acetate (Larsch et al., 2013). This neuron pair exhibits a calcium rise as a response to the presence of *Escherichia coli* (*E. coli*) and a calcium reduction following its removal. The capacity of AWA neurons to detect volatiles over a vast gradient of concentration is likely vital to the survival of *C. elegans*. In fact, the response qualities of AWA allow *C. elegans* to react to odorants over a 100,000-fold concentration range. AWA responses also adjust to changes of concentration as opposed to the total concentration, enabling the nematode to sense the odour concentrations that vary most quickly, so helping them to take the closest path to an odour source (Itskovits et al., 2018).

The paired AWA and AWC neurons are the primary olfactory neurons of *C. elegans*, sensing a variety of attractive scents (Jin et al., 2016). The two AWC neurons are functionally asymmetric because they express distinct G protein-coupled receptors (GPCRs) and react to

diverse odorants. In the presence of sick or starving nematodes, scents that are usually appealing may turn repellent. Under these circumstances, the AWC neurons still detect these substances, but they instead transmit repulsion. Previous studies have found AWC-ablated *C. elegans* to have a diminished capacity to detect cancer, showing that the AWC amphid neurons play a crucial role in regulating this behaviour (Lanza et al., 2021). These findings imply that the AWC neuron has a significant role in the attraction of cancer biofluids. Yet, since this neuron is associated with attraction, it cannot explain the repulsion.

VOCs are the final products of cellular metabolism and are likely generated by oxidative stress of cell membranes in cancer cells as a result of gene or protein modifications (Amann et al., 2014). Over 1700 volatile compounds have been found in skin emanations, exhaled breath, saliva, urine, human breast milk, faeces, and blood. Alcohols, aldehydes, ketones, N-heterocycles, sulphur-containing chemicals, and hydrocarbons are commonly detected in the urine of healthy individuals and may be produced from nutrients, intermediates, or environmental toxins.

VOCs may indicate any metabolic alterations in response to necrosis, inflammation, cancer, or microbiota modification, or they might be due to external influences such as pollution, medications, and food (Peng et al., 2010). These compounds are released into the circulation, and they are eliminated from the body through the alveoli or renal tubules.
Four volatile compounds: 3-octanone, 2,6-dimethyl-7-octen-2-ol, 2-octanone, and pentanal have been found to be down-regulated or less commonly present in the urine of prostate cancer patients (Khalid et al., 2015). With the excessive generation of reactive oxygen species known to trigger lipid peroxidation, aldehyde synthesis has been associated to cancer and inflammatory diseases. This may account for the increased incidence of pentanal seen in the urine of prostate cancer patients. These urinary VOC levels have been found to be elevated along with the PSA value of men exhibiting symptoms of prostate cancer.

1.3.4 *C. elegans* in research – chemotactic experiments

The experimental advantages and similarities between several biological processes in *C. elegans* and other animals, such as organelle structure and function, metabolism, protein biology and gene regulation, have made *C. elegans* an ideal model organism in research

(Culetto & Sattelle, 2000). At least 70 % of human genes have an ortholog in the genome of *C. elegans*, and 40 % of genes that are linked to human disease have orthologs in the *C. elegans* genome. Hence, several *C. elegans* findings are applicable to the study of human health and illness (Rankin, 2002). Moreover, *C. elegans* may be employed to study how substances like alcohol, antidepressants and anaesthetics affect the nervous system and behaviour.

The chemotaxis assay was first created by Samuel Ward in 1973, and it has since had several implementations (Ward, 1973). *C. elegans* has showed olfactory adaptation, a basic form of memory and cognition, utilising chemotactic experiments which has especially benefitted neurobiology (Colbert & Bargmann, 1995). The assay has also been used to demonstrate that *C. elegans* can acquire ethanol tolerance, a finding that not only reveals the behavioural plasticity of the nematodes but also suggests that they may be highly valuable in the study of alcoholism in humans (Lee et al., 2009). Using chemotactic experiments, it has been established that *C. elegans* is capable of short- and long-term memory by demonstrating that they form associations between food (OP50) and chemoattractants (Troemel et al., 1997). Furthermore, the chemotaxis behaviour of *C. elegans* has been modified multiple times by generating mutations which has been possible due to the extensive amount of knowledge presently accessible on the *C. elegans* genome.

Chemotaxis is a strategy utilized by different organisms to find food, avoid harmful substances, and mate (Margie & Palmer, 2013). *C. elegans* possesses powerful chemotaxis behaviour. Placing the nematodes in a distinct location and following the movement induced by an odorant is the concept behind assessing the *C. elegans* reaction to an odorant. Although there are many assays available, there is still much to do in terms of maximising the starting position of *C. elegans* in relation to both the control and test areas, reducing interaction, and maintaining a sufficient sample size.

Since its development in 1973, the chemotaxis assay has undergone several modifications and been used in a variety of fields (Ward, 1973). Some assays have attempted to determine the precise path *C. elegans* use to reach a destination. Ward created the prototype for this kind of assay. Pierce-Shimomura et al. (1999) designed an assay to determine the precise nature of chemotactic movement. Several experiments have examined the reaction of a vast population of *C. elegans* to various substances. A two-quadrant chemotaxis assay has been utilised to

study the functions that different receptors, neurons, and signal transduction molecules play when *C. elegans* is exposed to diverse substances (C. Bargmann et al., 1993). Existing techniques for measuring the chemotactic response of *C. elegans* are continuously modified to improve their simplicity, efficiency, and specificity.

Several studies have employed *C. elegans* and its chemosensory abilities in the detection of cancer. Hirotsu et al. (2015) found that *C. elegans* was attracted to cancer tissues, cancer cell secretions, and urine from individuals with gastric, breast and colorectal cancer. Cancer urine attraction and aversion to control urine was found to peak at 10^{-1} dilution. Included in the study were also six cases of early-stage cancer, which indicates that *C. elegans* chemotaxis may be useful for cancer screening. Based on these findings Hirotsu et al. develop a nematode scent detection test (NSDT) which has a sensitivity of 95.8 % and a specificity of 95.0 %. The method was later further tested and developed by adding a 10^{-2} dilution which increased the sensitivity of the technique.
Identifying the GPCRs and the sensory neurons involved in the recognition of cancer odorants have further shed light to the mechanisms behind the chemotactic behaviour of *C. elegans* (Lanza et al., 2021). Employing mutant strains that lack AWC neurons in chemotaxis assays, the involvement of these olfactory neurons in *C. elegans* attraction towards cancer odours has been proven. Calcium imaging has also been used to record the activity of olfactory neurons to determine their role in the detection of specific odorants and improve the accuracy of the chemotaxis assay.

1.4 Aim of the study

It is crucial to detect cancer in the early stage in order to facilitate treatment and increase the likelihood of recovery. Existing screening techniques for early cancer diagnosis have several disadvantages, including high invasiveness, high expense, and limited sensitivity to malignancies in their early stages. Currently, prostate-specific antigen (PSA) screening is commonly employed to identify prostate cancer, but it is a controversial method because more than half of men with an elevated PSA level have a false positive result and are not diagnosed with prostate cancer, whereas 15% of men with a negative PSA result will develop prostate cancer later on. Also, even a false positive result has to be confirmed with biopsy which is an invasive method that can result in further complications.

The aim of this study was to determine whether *C. elegans* nematodes could be reliably used in diagnosing early-stage prostate cancer to differentiate malignancies from benign growth and whether the method is sensitive and specific enough. The end goal would be to develop an affordable, and easy cancer diagnosis method with a high degree of accuracy that could be used in a clinical setting to complement other existing techniques.

2 Results

2.1 Chemotaxis index (CI) with 10- fold and 100- fold dilutions

In the first part of the study, 10 urine samples of prostate cancer patients and 6 samples of patients with benign prostatic hyperplasia (BPH) were analysed with 10- and 100- fold dilutions. In general, six parallel plates were used for each sample and dilution but due to several influencing factors some plates were discarded during the experiments. This was mostly due to external factors like humidity, dryness or starvation which occasionally affected the movement of the nematodes.

Once the experiments had been performed the samples were decoded and the chemotaxis index (CI) calculated and plotted as seen in Figure 3. Dispersion was substantial in both groups between and within samples and there was no significant difference between the BPH and cancer patient urine samples when the chemotaxis index was calculated. The samples with BPH showed a slightly more consistent result as 58% of all the samples had a negative chemotaxis index whereas 55% of the prostate cancer urine samples had a positive CI.

The result was more persistent with the 10- fold dilution in both the BPH and prostate cancer group. In the BPH group, among all the plates (n=53) 62% had a negative CI with the 10-fold dilution. In the same group 54% of the chemotaxis indexes were negative with the 100-fold dilution. Within the prostate cancer group (n=76), 58% of the 10-fold diluted plates were on the positive side and 53% of plates diluted to a 100-fold. The ability of these experiments to identify individuals with prostate cancer (specificity) or BPH (sensitivity), can not be considered sufficient enough (Table 2). As the 10-fold dilutions seemed to give slightly more consistent results, the next phase of the experiments was carried out with only this dilution.

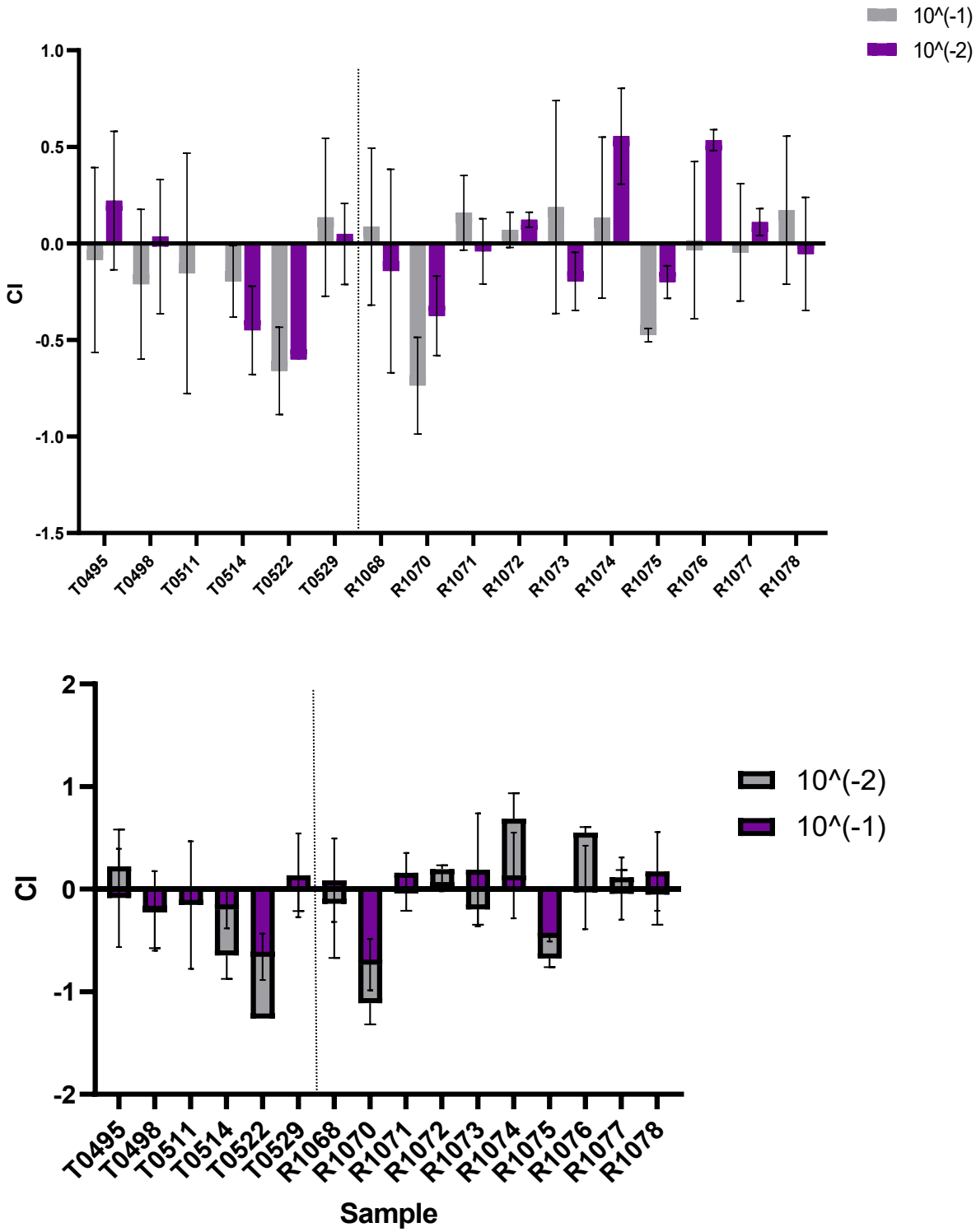

Figure 3. Chemotaxis indexes for urine samples with 10-fold and 100-fold dilutions. Replicates vary between 4-8 plates per sample and each bar represents CI of *C. elegans* response to urine samples of prostate cancer patients (R) and individuals with BPH (T). Error bars represent mean with SD.

Table 2. The number of plates with positive and negative chemotaxis indexes and the comparison of sensitivity and specificity according to dilution.

		Positive	**Negative**	**Total**	**Sensitivity**	**Specificity**
10^(-1)	Cancer	23	17	40	58%	
	BPH	11	18	29		62%
	Total	34	35	69		
10^(-2)	Cancer	19	17	36	53%	
	BPH	11	13	24		54%
	Total	30	30	60		

2.2 Chemotaxis index with 10- fold dilution

In the second part of the study the chemotaxis assays were performed using the same samples as in the previous phase and 10 additional samples of which 5 were prostate cancer samples and 5 BPH samples. Five replicates were used consistently for each sample with only the 10-fold dilution.

The chemotaxis indexes were calculated as previously and can be seen in Figure 4. The results were similar to the previous experiments. There were no significant differences in the chemotaxis indexes between the two groups and the dispersion within the groups was again substantial. Of the BPH plates (n=65) only 42% had a negative CI whereas 59% of the prostate cancer plates (n=75) had a positive CI.

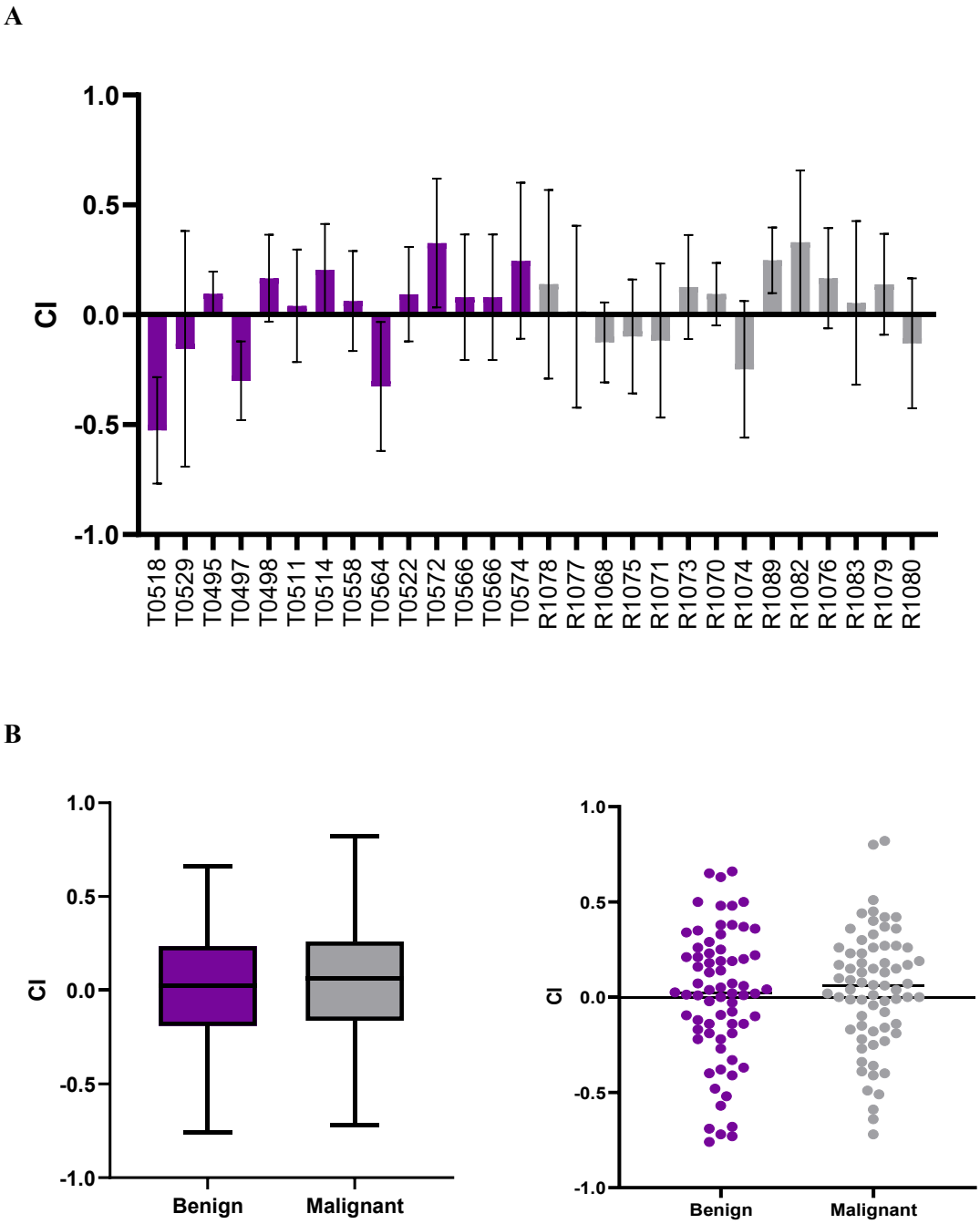

Figure 4. Chemotaxis indexes for urine samples (n=28) with 10-fold dilution (**A**) 5 plates per sample and bars represent CI of *C. elegans* response to urine samples of prostate cancer patients (R) and individuals with BPH (T). (**B**) Comparison of the two groups with all samples combined, horizontal line indicating median value. Error bars represent mean with SD.

The chemotaxis indexes of the 10-fold diluted samples of each experiment were also compared to see the possible consistency of the result (Figure 5). The variation in the CI between these two timepoints was substantial among the samples suggesting that the chemotactic behaviour of *C. elegans* in these experiments was random or influenced by other factors than the samples.

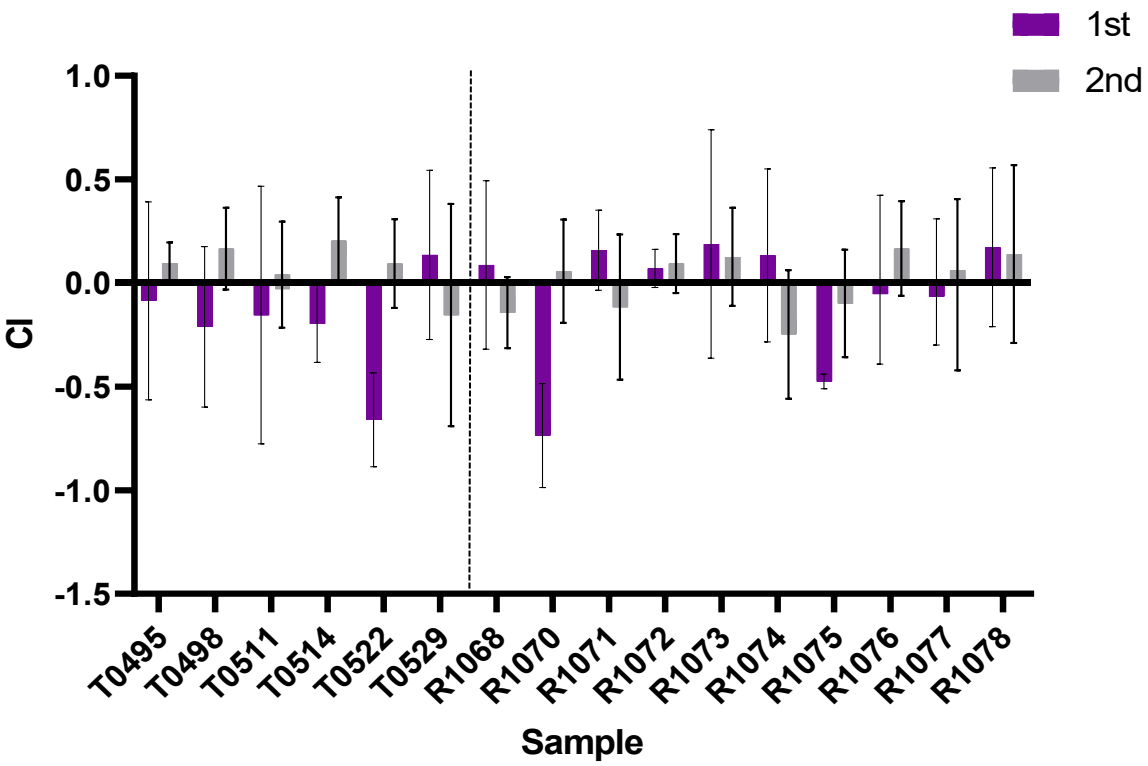

Figure 5. The comparison of the chemotaxis indexes of the 10-fold diluted samples retrieved at two different timepoints. Error bars represent mean with SD.

3 Discussion

Chemosensory receptors have an important role in the differentiation of volatile chemicals by binding them with great precision (Apfeld & Alper, 2018). Chemosensation is the primary sense in species without sensory modalities such as vision and hearing. Despite its small number of sensory neurons *C. elegans* exhibits many chemosensory receptors, enabling it to detect almost the same number of odorants as humans. Moreover, considering the abundance of knowledge already accessible about *C. elegans* genome, the behaviour of chemotaxis in *C. elegans* nematodes has been modified on multiple occasions by causing mutations.

This study aimed to demonstrate that *C. elegans* prefers urine samples from men with prostate cancer compared to subjects with benign prostatic hyperplasia. Recent evidence suggests that *C. elegans* exhibits chemotaxis toward cancer urine samples in a precise manner (Hirotsu et al., 2015). This characteristic behaviour has also been implemented in the preliminary diagnosis of cancer. Previous research on G protein mutants and the removal of olfactory neurons indicate that, these reactions are triggered by volatile chemicals (Lanza et al., 2021). However, urine is a heterogeneous biofluid comprising several volatile chemicals whose physical and chemical characteristics such as molecular weight and hydrophobicity vary between individuals.

Although being regulated by a complicated combination of neural and cellular pathways, chemotaxis may be readily and accurately measured utilising chemotaxis assays (Margie et al., 2013). Several crucial measures must be followed in order to acquire the best outcome from the tests. Initially, the staging of the *C. elegans* is crucial for producing consistent results. Nematodes at different stages exhibit distinct behaviours; hence, combining stages may influence experimental findings. Second, it is vital to ensure that all *E. coli* is removed, since leftover bacteria may impact the movement of *C. elegans*, as it is their food source. In addition, it is vital to remember that the anaesthetic, 0.5-1 M-sodium azide, can detain the nematodes within a 1 cm radius. Considering this, it is crucial that the sodium azide is kept at least 2 cm away from the source to allow *C. elegans* to advance far enough from their original spot.

Plates should also be maintained in a level to guarantee comparability between trials and to prevent gravitational biases from confounding findings (Margie et al., 2013). In addition, the

experiment should not employ more than 200 nematodes because several factors may limit *C. elegans* mobility in a crowded environment. Clustered nematodes prefer to stay together rather than go to a quadrant. Biomarker research often emphasizes the early identification of the disease; however, it has been stated that the largest unmet medical need for prostate cancer is to differentiate slow-growing or low-risk malignancies from aggressive tumours (Prensner et al., 2012). The development and validation of predictive and prognostic biomarkers will assist in the reduction of unneeded therapies that might cause more damage than good, the monitoring of progression and the targeting of therapy to those patients most likely to benefit from it. There is a need for more research leading to a better strategy for diagnosing aggressive tumours.

To detect cancer odours more accurately and quantitatively, it is essential to distinguish odours that are cancer-specific and their corresponding receptors (Matsumura et al., 2010). Many volatile organic compounds have been found as potential molecules for the detection of cancer utilising gas chromatography/mass spectrometry (GC/MS) in cancer cell lines, exhaled breath, and urine. Identification of cancer odorants and their receptors could result in the clarification of the metabolic processes of tumour cells, thereby contributing to the research and development of cancer medications, but also to the development of diagnostic methods utilizing exhaled air or urine to detect cancer.

One limitation of this study was that the concentration of the urine samples was unknown and possibly varied between the samples which can be due to the different times of the day that the urine is obtained. First pass urine would have been ideal for the set-up of this type of experiment, since it would minimize the differences in the urine concentration. The urine dilution can be determined by measuring the urine osmolality or urine creatinine to reliably define the expected VOC range (Arndt, 2009). However, in a similar study by Hirotsu et al. (2015), *C. elegans* attraction to urine was not found to be associated with the concentration of the urine, which was determined by measuring the creatinine concentration.

The fact that the disease phase cannot be precisely predicted at the time the samples are obtained is another crucial consideration. As a result, it is possible that some of the individuals who were placed in the benign group would eventually develop prostate cancer, which may affect the capacity of *C. elegans* to distinguish between the samples. Also, other

influencing factors like the stage of tumour development, BMI and/or possible hormonal changes of the sample donors were not taken into account.

It is known that hormonal changes along with food, drugs, bacterial by-products, and the overall health of the individual can influence the urine volatilome as well the time period during which the urine sample is received (Amann et al., 2014). Besides physiological or metabolic body processes VOCs can also be formed by the host in reaction to microbial infections, such as during the inflammatory response (Sethi et al., 2013). Since the ability of *C. elegans* to differentiate cancer from other samples is primarily based on the VOC profile of the urine these factors might play a crucial role in the research.

The aim of this study was to investigate whether *C. elegans* could reliably distinguish between urine samples from prostate cancer patients and patients with benign prostatic hyperplasia and assist in the diagnostic differentiation of these conditions. The results of the study showed no significant difference between the groups and the variation between individual assays and samples was substantial which might be due to several contributing factors. The assay plates used in the experiment may have influenced the movement of *C. elegans* in different ways e.g., dryness, moisture and contamination which are all elements that are difficult to distinguish and eliminate. Besides their surroundings the nematodes themselves are affected by their handling before and during the experiment i.e., they can enter a dauer stage if they are washed from the feeding plates too early before starting the experiment. Also, the urine samples used in this study could have affected the outcome since there are several intrinsic factors that can influence the composition of urine. These factors could not be eliminated during this study but are characteristics that should be considered in the future.

4 Materials and methods

C. elegans is attracted or repelled by several volatile odorants. To investigate whether *C. elegans* detects odours secreted from cancer tissue to urine, the response of *C. elegans* nematodes to urine samples from prostate cancer patients was analysed. Chemotaxis assays were used to determine whether *C. elegans* is attracted to or repelled by the sample. Samples were provided by Turku Prostate Cancer Consortium (TPCC) and handled in a blinded manner (Table 3).

4.1 *C. elegans* culture and strain

The wild-type strain Bristol N2 was used for all the experiments in this study. The N2 strain was isolated in 1951 in Bristol, Great Britain and is commonly used in the laboratory (N2 (Strain) - WormBase : Nematode Information Resource). *C. elegans* were cultured on nematode growth medium (NGM) plates at 20°C and *E. coli* strain OP-50-1 used as food source. This strain is streptomycin-resistant unlike the parental OP-50 strain and because of this streptomycin was added to the plates to avoid contamination.

Throughout the research and before proceeding with the experiments, *C. elegans* were grown on NGM plates for several days and transferred onto new plates when necessary to ensure an adequate number of nematodes for the chemotaxis assays.

4.2 Urine sample collection and ethics

Urine as a biological material is easy and non-invasive to collect and stable during storage even for longer time periods (Remer et al., 2014). It has been shown that urine samples can be stored below -20° C for over ten years without being significantly altered. During the study the urine samples were stored in the freezer below -18° C and diluted in Milli-Q water right before the experiments. Two dilutions of 1:10 and 1:100 were used for the chemotaxis assay.

The urine samples used in this study were received from the Turku Prostate Cancer Consortium and subjects had submitted written informed consent before the samples were collected. The samples were coded and handled in a blinded manner during this research.

Table 3. Characteristics of the subjects in this study.

Number of subjects	30
Age (Years)	
Mean	70
Range	63-85
PSA (ng/ml)	
Mean	8.5
Range	0.025 – 25
Classification	
T1	9
T2	13
T3	7
NX	13
N0	12
N1	4
M0	11
M1	4

4.3 Chemotaxis assay

4.3.1 Synchronization

Only synchronized *C. elegans* were used for the chemotactic experiments to ensure that they were all in young adult stage (Stiernagle, 1999). The nematodes were washed from plates with M9 buffer (9mM NaCl, 22mM KH_2PO_4, 41mM Na_2HPO_4, 19mM NH_4Cl, ddH_2O) and sodium hypochlorite solution (1M NaOH, bleach, ddH_2O) was added to the tube to retrieve the eggs and eliminate any bacteria. The following day larvae were plated and let grow for three days.

4.3.2 Behavioural assay

To determine the behaviour of *C. elegans* and to practise the chemotaxis assay, butanone and 1-octanol were used for the chemotaxis assay before conducting experiments with urine samples. 1-octanol is known to repel C. elegans hermaphrodites while butanone acts as a

chemoattractant. (Williams et al., 2018) Three dilutions of 1:100, 1:500 and 1:2500 in ethanol were used for both chemicals.

After synchronization, the nematodes were grown on NGM (2% Agar, 54mM NaCl, 11mM Tryptone, ddH_2O, 1M KPO_4 [pH6], 1M $CaCl_2$, 1M $MgSO_4$, cholesterol [5mg/ml in EtOH]) plates seeded with *E. coli* for three days before the chemotaxis assay. Experiments were carried out for a maximum of three days before repeating the synchronization. 10 cm Petri dishes with NGM were prepared for the assay by drawing the plates accordingly (Figure 6). The plates were divided into two sections i.e., odorant and control area, divided by a separate 1cm section in the middle.

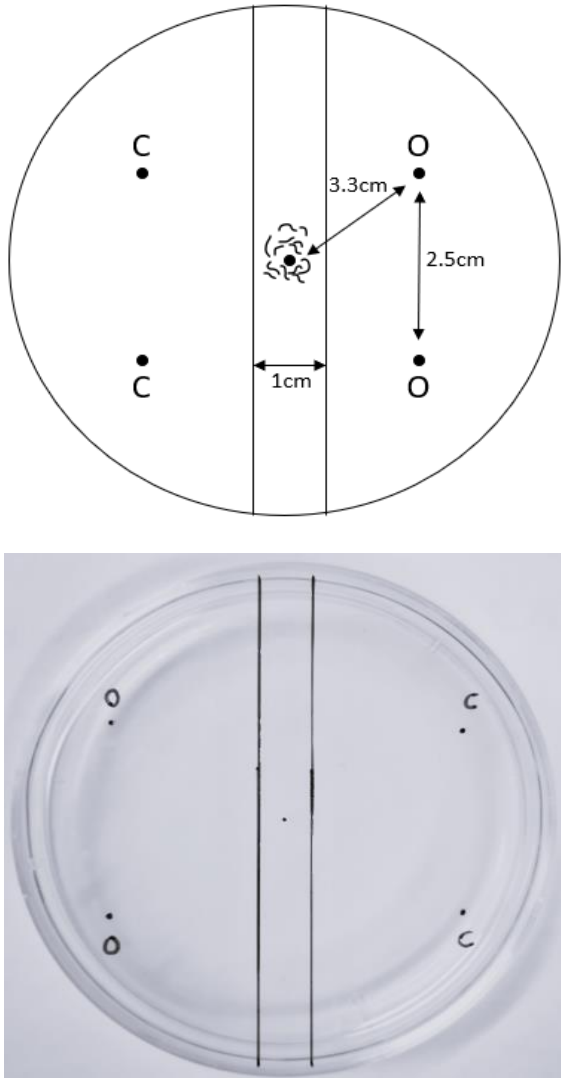

Figure 6. The chemotaxis plate set up on a petri dish. *C. elegans* are placed in the middle circle and NaN_3 and MilliQ-H_2O or urine sample on each side.

C. elegans were washed from the feeding plates with S-basal buffer (0.1M NaCl, 44mM KH_2PO_4, 6mM K_2HPO_4, 3mM Cholesterol, ddH_2O) twice to maximize the number of nematodes for the experiment. The collected nematodes were then washed 3 times to ensure there were no bacteria left to impact the movement of the animals.

A 5 µl sample (approximately 70-100 nematodes) was placed in the centre of the prepared plate and 1 µl of 1M NaN_3 was dropped to each spot to ensure that the nematodes are immobilized once they reach their target (Hirotsu et al., 2015). 1 µl of Milli-Q water was used as a control on the control side of the plates and 1 µl urine sample was placed on the odorant side of the plate. Urine samples were diluted in Milli-Q water at 1:100 and 1:10.

Animals were let move for about 60 minutes and then calculated under a microscope. Chemotaxis index was calculated as the number of *C. elegans* near the region of the urine samples subtracted from the number of *C. elegans* near the region of the control divided by the total number of the nematodes. A positive index value indicates attraction to the sample, whereas a negative index indicates repulsion by the sample.

$$(CI) = O\text{-}C \,/\, O\text{+}C$$

(CI = Chemotaxis index, O = Odorant, C = Control)

Approximately six plates / sample / dilution were used for the experiments and the waiting time varied between 45–60 minutes. The experiments were conducted on two different occasions using the same samples. GraphPad Prism 8.1.2 was used for graphing the results.

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6 Abbreviations

TP53	Tumour protein p53
BCL-2	B-cell lymphoma 2
BPH	Benign prostatic hyperplasia
C. elegans	*Caenorhabditis elegans*
CDKs	Cyclins and cyclin-dependent kinases
CI	Chemotaxis index
E. coli	*Escherichia coli*
EGF	Epidermal growth factor
GPCRs	G protein–coupled receptors
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinase
NGM	Nematode growth medium
OP-50	*Escherichia coli* strain
PARP	Poly–adenosine diphosphate ribose polymerase
PSA	Prostate-specific antigen
TCA cycle	Tricarboxylic acid cycle
VEGF	Vascular endothelial growth factor
VOCs	Volatile organic compounds

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