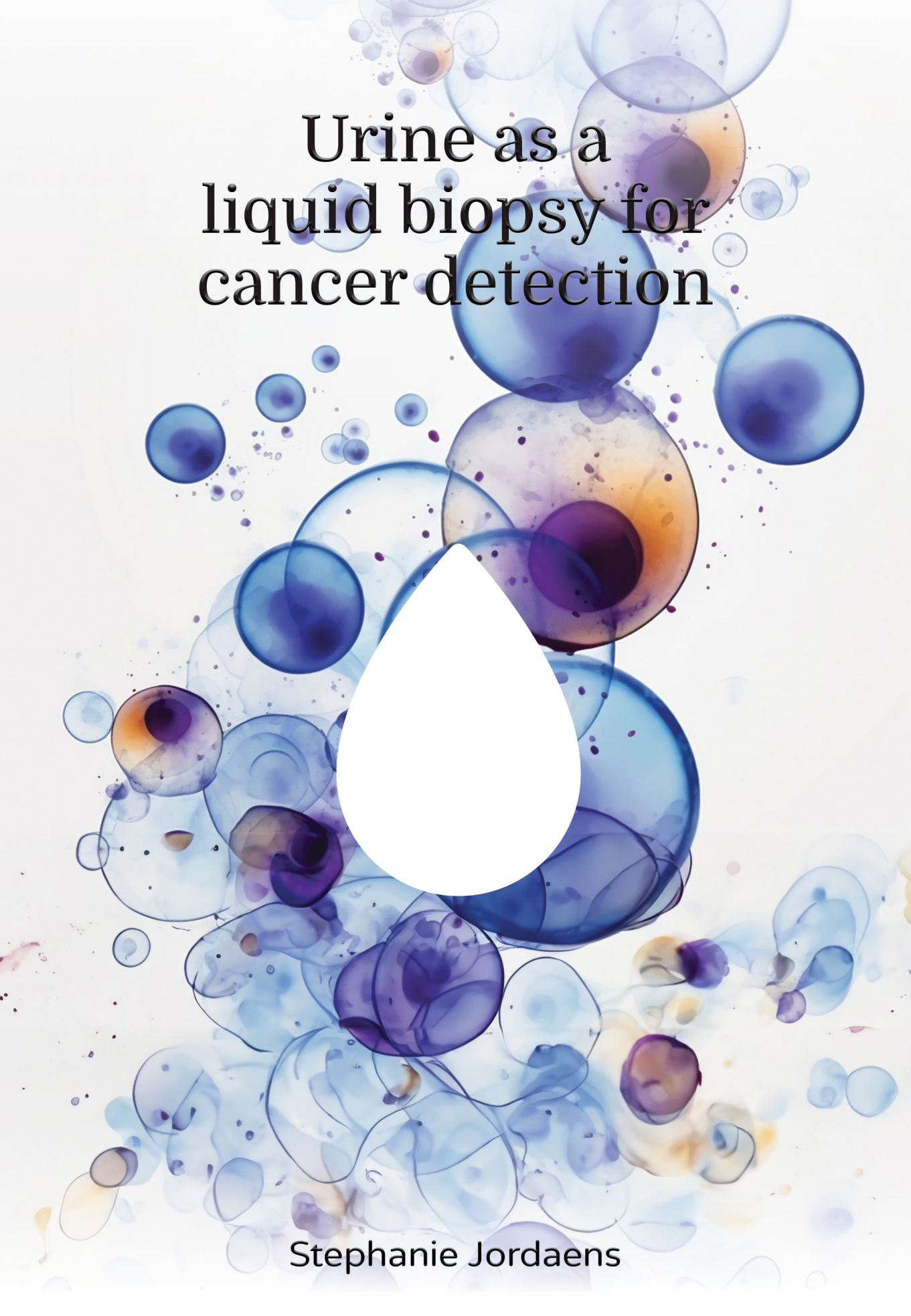
Urine as a liquid biopsy for cancer detection



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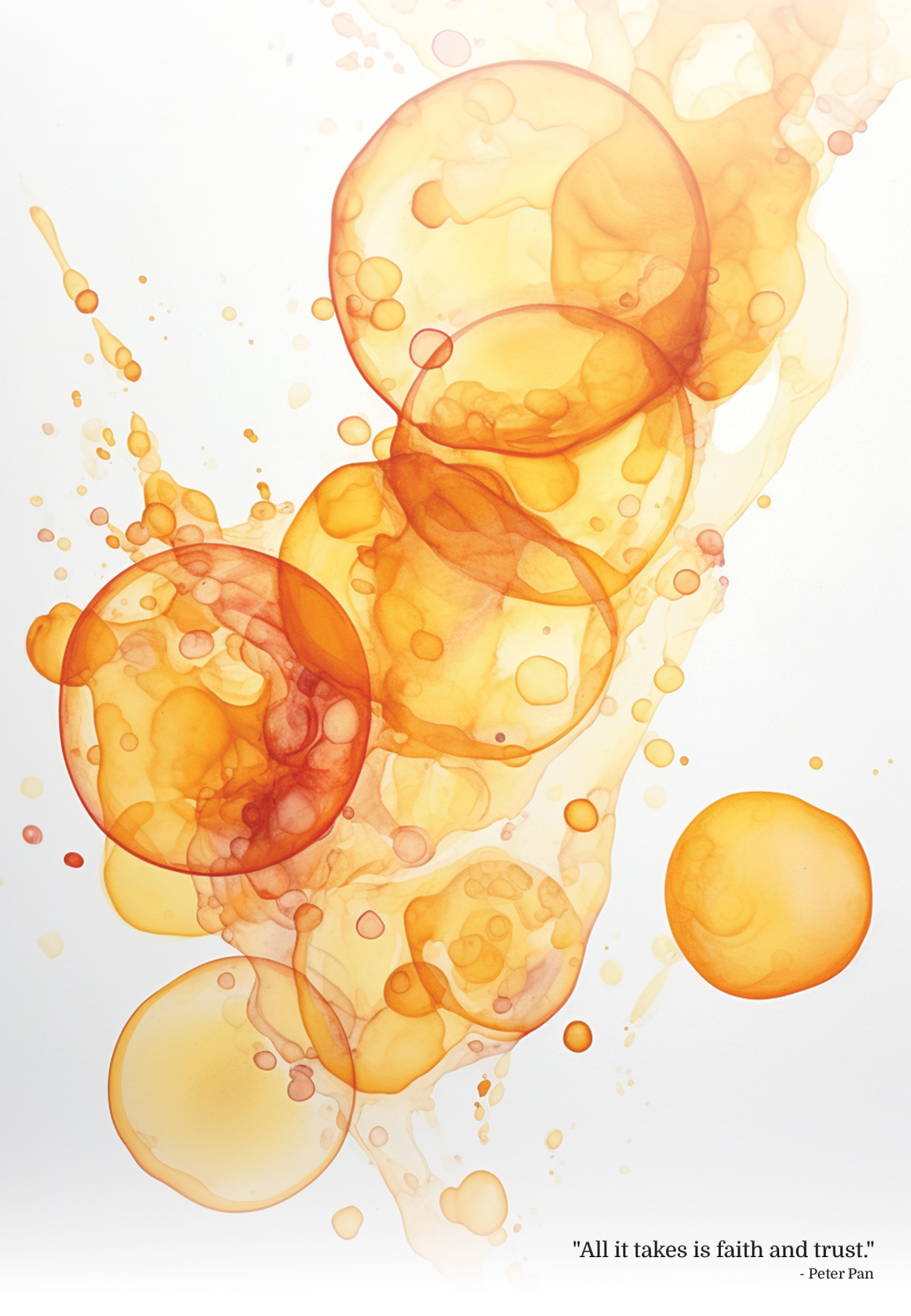
"All it takes is faith and trust."
- Peter Pan

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University of Antwerp

Urine as liquid biopsy for cancer detection

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AICR American Institute for Cancer Research
AUC Area under curve
Avg. Average

BCA Breast cancer
BCP Breast cancer patient
BRISQ Biospecimen Reporting for Improved Study Quality

CC Cervical cancer
CE European Conformity
cfDNA Cell-free DNA
cfNA Cell-free nucleic acids
cfRNA Cell-free RNA
cfTNA Total cell-free nucleic acids
CLIA Clinical Laboratory Improvement Amendments
CRC Colorectal cancer
CTC Circulating tumor cell
ctNA Circulating tumor nucleic acids

ddPCR Digital droplet polymerase chain reaction
DRE Digital rectal examination

EBP Evidence-based practices
EC Endometrial cancer
EDRN Early Detection Research Network
EDTA Ethylenediaminetetraacetic acid
EIT European Institute for Innovation and Technology
ELISA Enzyme-Linked Immunosorbent Assay
EMA European Medicine Agency
ER Estrogen receptor
EU European Union
EV Extracellular vesicle

FDA U.S. Food and Drug Administration

GBCI Global Breast Cancer Initiative
gDNA Genomic DNA

HBV Hepatitis B virus
HCC Hepatocellular carcinoma
HCI Human Computer Interaction
HCV Hepatitis C virus
HE Human Engineering
HER2 Human epidermal growth factor receptor
HFE Human Factors Engineering
HMW High-molecular-weight
HNC Head and neck carcinoma
H-NMR Proton nuclear magnetic resonance
HPLC High-performance liquid chromatography
HPV Human Papilloma virus
hrHPV High-risk HPV
HVF Healthy female volunteer
HVM Healthy male volunteer

IARC International Agency for Research on Cancer
IFU Instructions for use
IHC Immunohistochemistry
ISEV International Society for Extracellular Vesicles
ISO International Organization for Standardization
IVDR In-vitro diagnostic device regulations

KIC Knowledge and innovation community

L

LDT Laboratory-developed test
LMW Low-molecular-weight
lncRNA Long non-coding RNA
LOC Lab on chip

M

miRNA microRNA
MISEV2018 Minimal Information for Studies of Extracellular Vesicles 2018
MRI Magnetic resonance imaging
MS Mass spectrometry

N

NCI National Cancer Institute
NGS Next-generation sequencing
NSCLC Non-small-cell lung cancer
NTC No template control

O

OC Ovarian cancer

P

PCa Prostate cancer
PCP Prostate cancer patient
PR Progesterone receptor
PRW Pregnant women
PSA Prostate-specific antigen

Q

qPCR Real-time quantitative polymerase chain reaction

R

RCC Renal cell carcinoma
RT Room temperature
RT-qPCR Reverse transcription qPCR

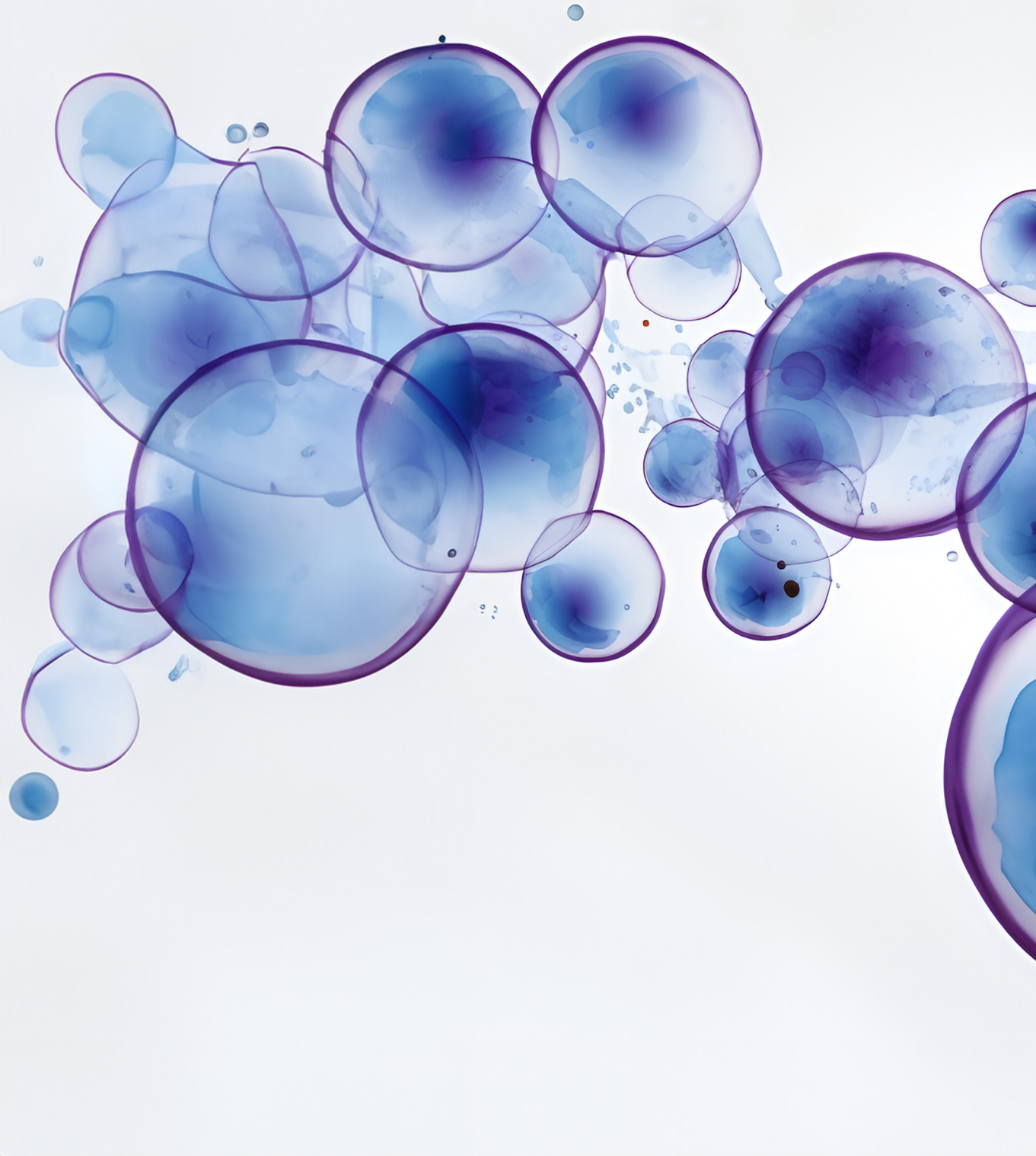
STI Sexually transmitted infections

THCA Thyroid cancer
TOI Target of Interest
trtDNA Trans-renal tumor DNA
TRUS Transrectal ultrasound

UCD User-centered design
UE Usability Engineering
UZA Antwerp University Hospital

VLAIO Flanders Innovation and Entrepreneurship
VOM Volatile organic metabolite

WGA Whole Genome Amplification
WHO World Health Organization



CHAPTER

01

Introduction

The way to get started is to quit talking and begin doing.
(Walt Disney, himself)

Cancer

01

1.1. Cancer as a major public health concern

Cancer is considered a worldwide major challenge for public health. Cancer is uncontrolled cell growth leading to the destruction of healthy organs. It is a disease which will affect one in three men and women. Indeed, more than 19.3 million new cases of cancer occurred in 2020, and it is estimated that by 2040 the incidence will increase to 30.2 million new cases. The three most common cancers in women are breast, colorectal and lung cancer, while in men lung, prostate and colorectal cancer are most frequent. Annually, about 9.96 million people die from cancer worldwide (Table 1)[1]. Prevention is therefore of the utmost importance.

Table 1: Cancer burden of top 10 most common cancer types for 2020

Cancer type	Incidence	Mortality	New cases estimated for 2040
Breast cancer	2 261 419	684 996	3.19M
Lung cancer	2 206 771	1 796 144	3.63M
Colorectal cancer	1 931 590	935 173	3.08M
Prostate cancer	1 414 259	375 304	2.43M
Stomach cancer	1 089 103	768 793	1.77M
Liver cancer	905 677	830 180	1.40M
Cervical cancer	604 127	341 831	798k
Esophagus cancer	604 100	544 076	957k
Thyroid cancer	596 202	43 646	761k
Bladder cancer	573 278	212 536	991k
Cancer as disease	19 292 789	9 958 133	30.2M

Resource: 1

1.2. Etiology of cancer

Before a normal cell evolves into a cancer cell, multiple genomic changes within the cell are required. This multistep process is called carcinogenesis and involving changes in multiple genes[2]. The process of carcinogenesis is often divided into three phases, (1) initiation, (2) promotion, and (3) progression. Initiation involved the induction of an irreversibly altered cell and is often equated with a mutational event. Promotion is the process by which the initiated cell expands clonally into a visible tumor. These cells must undergo one or more additional genomic, mRNA and epigenetic changes during progression to a malignant neoplasm[2]. The necessity for a malignant cell to acquire multiple, genomic alterations at independent genetic loci explains, at least in part, the sometimes long latency period for cancer[2].

The changes that make a normal cell progress into a tumor cell are the result of the interaction of several risk factors. Environmental, exogenous and endogenous factors, as well as individual genetic factors contribute to the development of cancer[3]. There are three categories of external agents that can lead to genetic changes or chronic inflammation, including: (a) physical carcinogens, such as ultraviolet and ionizing radiation; (b) chemical carcinogens, such as asbestos, components of tobacco smoke, alcohol, aflatoxin (food contaminant), and arsenic (drinking water contaminant); and (c) biological carcinogens, such as infections from certain viruses, bacteria, or parasites. Furthermore, genetic changes are the result of endogenous factors such as errors that occur as cells divide and changes that were inherited from the parents.

Cancer can be considered a genetic disease, which implies multiple genomic changes in cells. These genetic changes can be classified as gene mutations, chromosome rearrangements, gene amplification, or aneuploidy[2]. The three main types of genes affected by these genetic changes are called the drivers of cancer and are: (1) proto-oncogenes, (2) tumor suppressor genes, and (3) DNA repair genes. Proto-oncogenes and tumor suppressor genes are involved in normal cell growth and division, but when these are altered in certain ways or expressed at higher levels, cells may divide in an uncontrolled manner. DNA repair genes are involved in repairing damaged DNA, cells with mutations in these genes tend to develop additional mutations in other genes and changes in their chromosomes, such as duplications and deletions of chromosome parts.

Researchers have found that certain mutations are common in many types of cancer. Many cancer treatments are now available that target gene mutations found in cancer[4].

Importantly, several infectious agents have been identified as carcinogens of which Helicobacter pylori, high-risk Human Papilloma Virus (HPV), Hepatitis B virus (HBV), and Hepatitis C virus (HCV) have been classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC) These four most important infectious pathogens are responsible for more than 90% of infection-related cancers worldwide[5]. Therefore, high-level action proposals and statements have been made by the scientific and public health community regarding strategies of the eradication and elimination of these pathogens, as well as scaling up effective vaccination, screening and treatment[6, 7]. Vaccination and screening programs for HBV and HPV have already proven to have an important impact in high-risk environments against liver and cervical cancers, respectively[8].

Furthermore, it has been demonstrated that approximately 40% of cancers are preventable as they are associated with modifiable risk factors[3, 8, 9]. Of the remaining 60%, a small portion is probably due to inherited genetic susceptibility and some of the cancer burden may simply be due to bad luck, most cancers are coincidential[8].

Some of these modifiable risk factors can be addressed by changing lifestyle habits. Lifestyle habits which are modifiable risk factors include tobacco use, alcohol consumption, physical inactivity and unhealthy diet. The American Institute for Cancer Research (AICR) created an educational infographic to illustrate the difference between physical activity and sedentary behavior (Figure 1). Recently published reviews and reports from health organizations reported that there is moderate evidence to suggest that high levels of sedentary time are associated with an increased risk of colon, endometrial, and lung cancer[9].

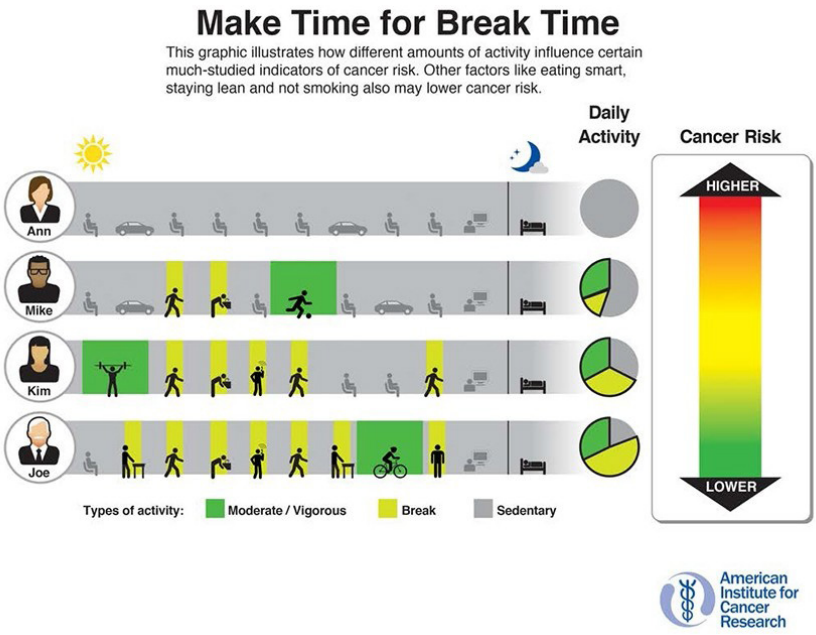


Figure 1: Comparison of physical activity and sedentary behavior as indicators of cancer risk. Reference: https://www.aicr.org/resources/media-library/make-time-break-time/

Current 'gold standard' in screening and diagnosis

02

Currently, worldwide initiatives are ongoing to reduce cancer deaths and improve patients' quality of life, including the Europe's Beating Cancer Plan from the European Commission and the World Health Organization's (WHO) Global Breast Cancer Initiative (GBCI). Two cancer types will be discussed more broadly in this thesis, namely breast and prostate cancer. Therefore, only the current standard detection and screening methodologies of these two will be discussed.

2.1. Breast cancer

Breast cancer can be divided into different subtypes based on the molecular landscape of the tumor. The first subtype of breast cancer is estrogen receptor (ER) and progesterone receptor (PR) positive. This cancer subtype responds to endocrine therapy. The second subtype is human epidermal growth factor receptor (HER2) positive and is characterized by the overexpression of HER2 oncogene. This cancer subtype can be treated with HER2 antibody therapy such as trastuzumab. The last subtype are triple-negative tumors, which lack ER, PR and HER2. This subtype does not respond to any current treatment and is associated with high mortality[10]. It has been shown that when breast cancer is diagnosed at an early stage, survival rates increase dramatically[11].

2.1.1. Diagnosis

Breast cancer is mostly diagnosed because of experiencing certain symptoms (e.g., skin changes or a palpable mass) or screening that calls for a diagnostic examination[12]. Usually, breast examination, mammography, or ultrasound are sufficient for local-regional staging of newly diagnosed breast cancer patients[12,13].

However, MRI is sometimes recommended when a patient is younger, a genetic mutation or multifocal disease is suspected, or when a mammogram or ultrasound is inconclusive[12, 13]. These imaging techniques can be used as effective tools for the detection of breast cancer[13]. Together, the diagnosis of breast cancer relies on radiology and clinical evaluation, confirmed by a tissue biopsy[14].

Ancillary tests to tissue biopsies can include immunohistochemistry (IHC) and molecular tests, which can be performed to assist in the characterization of ambiguous morphology[12]. IHC analysis of paraffin-embedded sections is routinely performed for the evaluation of ER, PR, and her-2/neu (HER2) status. Paraffin-embedded tissue samples can also be tested for their DNA and RNA presence using in situ hybridization assays, DNA micro-arrays and high-throughput reverse transcription-PCR assays. These assays can be used to categorize breast cancers into several prognostic groups, to predict the risk of recurrence and to influence decisions about therapy. Additionally, there is great interest in exploring other useful targets for precision therapy using next-generation sequencing[12].

Unfortunately, this full approach has several limitations, such as suboptimal sensitivity of radiological screening, suboptimal positive predictive power of diagnostic approaches, invasiveness and inconvenience of tissue biopsy, and the long turnaround time for the ability to retest[13, 14].

2.1.2. Screening

Screening of healthy individuals is associated with the detection of tumors that are smaller, less likely to metastasize, more prone to breast-conserving and limited axillary surgery, and less likely to require chemo- or immunotherapy. This translates into reduced treatment-related morbidity and improved survival[12]. Additionally, the imaging techniques are one of main approaches for assessing response to therapy in breast cancer patients[13]. However, the combined use of mammography, ultrasound and tissue biopsy has some limitations in the screening and monitoring setting, such as the suboptimal radiology sensitivity and the invasive nature of the tissue biopsy[14].

A 19% reduction in breast cancer mortality was shown for screening

mammography, with less benefit for women in their 40s (15%) and more benefit for women in their 60s (32%)[12]. It is important for women undergoing screening to be aware of the possibility of a false-positive results, as mammography has a 61% chance of a false-positive result over a 10-year period for women who started screening between 40 and 50 years. The risk of a false-positive test decreases with increasing age[11, 12].

A systematic review of breast cancer screening guidelines published between 2010 and 2021 was recently performed. They demonstrated that (a) most of the guidelines were created in developed countries or regions, (b) the United States accounted for the largest portion, (c) one was developed by WHO, (d) four in Europe, and (e) almost half of the included guidelines have been updated over the years[15]. They showed that most guidelines recommended mammographic screening for those with an average risk of 40-74 years, and recommended women aged 50-69 years as the optimal age group for screening. In Belgium (Flanders), women aged 50-69 years can participate free of charge in the Breast Cancer Population Screening by means of mammography every 2 years[16]. Furthermore, it was reported that agreement was found in most guidelines that annual mammographic screening or annual MRI screening should be given and that the age of onset should be earlier than the average-risk group for women at higher risk[15].

2.2. Prostate cancer

Prostate cancer (PCa) is cancer that occurs in the prostate. The prostate is a small walnut-shaped gland in men that produces the seminal fluid that nourishes and transports sperm. PCa can grow slowly and be confined to the prostate gland, where it may not cause serious harm. They are indolent and might never require treatment. However, other types are aggressive and can metastasize quickly. This aggressive form of PCa is a difficult disease to treat with a 5-year survival rate of only 30%[17]. PCa has a widespread prevalence, therefore it is important to distinguish between patients with clinically significant cancers requiring treatment and those who may be eligible for less aggressive active surveillance and avoid unnecessary treatment. The heterogenous nature of PCa makes it essential to correctly identify patients through precision medicine strategies who may be at risk for aggressive disease as well as those with indolent disease, to guide the best treatment or monitoring[17, 18].

2.2.1. Diagnosis

There is no single, specific test for prostate cancer, however, diagnosis is conventionally made by a digital rectal examination (DRE) and prostate-specific antigen (PSA) testing. However, the risk of clinically significant prostate cancer is related to age, ethnicity, family history , PSA level, free/total PSA ratio and findings on DRE. Therefore, physicians are encouraged nowadays to use risk calculators that incorporate these factors and to perform multi-parametric magnetic resonance imaging (mpMRI) before decision is made to perform a prostate biopsy. The mpMRI results are determined using the Prostate Imaging – Reporting and Data System (PI-RADS). When mpMRI is positive, targeted systematic biopsy should be done, when mpMRI is negative the biopsy can be omitted[19].

A DRE is an examination of the rectum. The healthcare practitioner inserts a lubricated, gloved finger into the lower part of the rectum and feels the surface of the prostate for any abnormalities[20].

PSA is a glycoprotein produced by the epithelial cells of the prostate gland. A PSA test measures the level of PSA in the blood of which the results are reported as nanograms of PSA per milliliter (ng/mL) of blood. The blood samples are analyzed at a PSA cut-off of 4 ng/mL. Patients have about one in four chances of developing PCa, if their PSA levels are between 4 ng/mL and 10 ng/mL. And this chance increases to 50% if the PSA is above 10 ng/mL. The PSA level is often elevated in patients with PCa, however a person's PSA level may increase due to other benign conditions such as prostatitis and benign prostatic hyperplasia[18, 20].

Confirmation of PCa diagnosis is based on the microscopic evaluation of prostate tissue obtained via needle biopsy. This medical procedure uses a thin hollow needle to collect small tissue samples. For PCa diagnosis, the biopsy can be performed through the rectal wall (transrectal biopsy) or through the skin between the anus and the scrotum. Usually, MRI or TRUS are used to locate the prostate gland during the biopsy[20, 21]. Positive MRI results can be used for targeting abnormal areas during a tissue biopsy[20]. The mpMRI, which uses specialized phases in addition to T2-weighted imaging, lead to the further increase in sensitivity. MRI demonstrated a pooled sensitivity of 89% and specificity of 73% for identifying PCa, when the standardized scoring and reporting criteria (i.e.,

PI-RADS) are applied[21]. TRUS uses a small probe that is inserted into a patient's rectum. This probe emits sound waves that pass through an organ and produce echoes. These echoes are analyzed and reported as a black and white image of the organ[20]. A systematic prostate biopsy is performed using TRUS to obtain 10 to 12 tissue samples in a grid-like pattern[21]. The biopsy procedure was further developed to MRI-directed targeting plus systematic transperineal prostate biopsy, obtaining 18 to 30 nuclear transperineal biopsy samples[22]. For each biopsy, a pathologist examines the samples and issues a primary Gleason grade for the predominant histological pattern and a secondary grade for the highest pattern, both on a scale of 1 to 5[21]. While prostate biopsy remains the standard of care, this approach misses 21% to 28% of prostate cancers and underestimates 14% to 17%[21].

2.2.2. Screening

Currently, there is no standard or routine screening test for PCa since it is not clear whether early detection and treatment decreases the risk of dying from PCa. A population-based screening study using PSA testing with follow-up of 16 years demonstrated a 25% relative reduction in PCa mortality. However, 570 men needed to be invited for screening and 18 patients needed to be treated to prevent 1 death from PCa[19].

Emerging field of liquid biopsies

03

In the era of precision oncology, the main objective is to provide the most accurate and effective treatment strategy based on the molecular profile of cancer and the individual patient himself. The main challenge for the clinical implementation of precision oncology is to detect molecular biomarkers that make it possible to estimate the prognosis of the disease, predict the sensitivity or resistance to specific therapies, and monitor the subsequent responses, ultimately leading to the selection of treatments[23, 24].

Tissue biopsy is the gold standard method for cancer detection, staging, and prognosis[25, 26]. However, tissue biopsy has many challenges such as difficulties in accessing the tumor and obtaining tissue samples in quantity and quality, it fails to reflect tumor heterogeneity or monitor dynamic tumor progression, the timing of biopsy is relative to treatment selection, and it is unrealistic to use tissue biopsy for cancer screening and early diagnosis when tumors have not formed yet[23, 25, 26]. Currently, there are other screening methods that have proven useful for cancer detection, such as mammogram for breast cancer, Pap test for cervical cancer, and screening for colorectal cancer, but all these screening methods have limited sensitivity and specificity and are only applicable to a single cancer type[25]. To perform largescale cancer screening in healthy individuals in the future, a more general cost-effective approach is needed[25].

A new promising and emerging field in this regard is the field of liquid biopsy (Figure 2). The term liquid biopsy refers to the investigation of analytes from various biological fluids, including blood, saliva, stool, and urine[23, 27]. Compared to tissue biopsies, liquid biopsies have many advantages. They are more feasible and less invasive. They have the potential to detect material shedding from multiple metastatic sites rather than analyzing a small piece of tissue, and therefore, have the potential to better evaluate inter- and intratumor heterogeneity[23, 25-27]. However, liquid biopsies also have some limitations such as the small amounts

of components released in the fluids, which can lead to difficult and expensive detection and sequencing methods. Standardization across laboratories is necessary to ensure reproducibility[27]. The rapid development of next-generation sequencing (NGS) technologies leads to higher sensitivities and the possibility to design assays for different purposes[25].

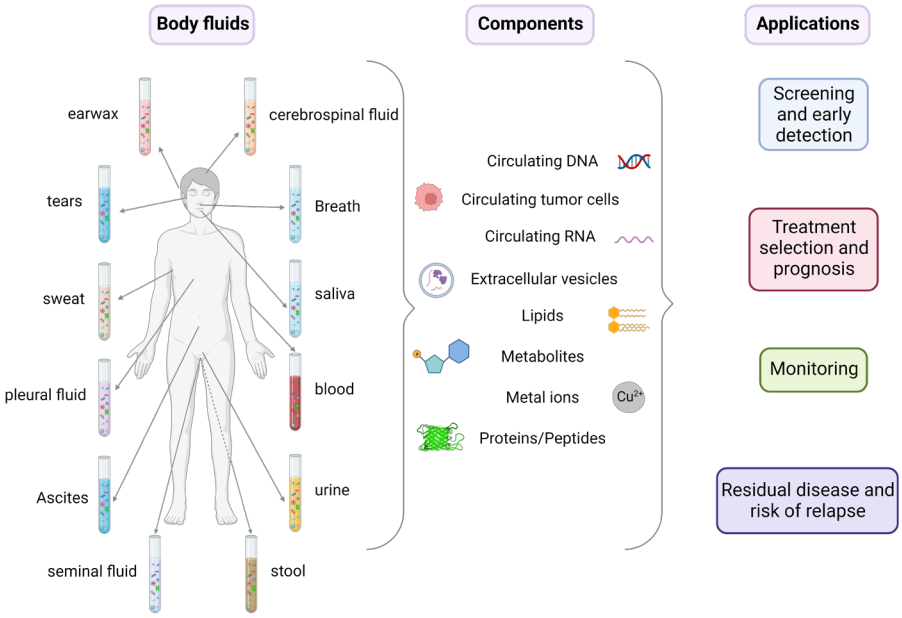


Figure 2: The emerging field of liquid biopsies. Some body fluids used as liquid biopsy are not depicted such as bone marrow, interstitial fluid and lymphatic fluid. For liquid biopsy components some general terms include multiple types such as circulating DNA includes cellular DNA, cell-free DNA, methylated DNA and circulating RNA includes mRNA, microRNAs, long non-coding RNA and other RNA types.

The field of liquid biopsies has opened up more opportunities for precision medicine in the treatment and care of cancer (Figure 2). Liquid biopsies, due to their minimally invasive nature, are associated with significantly less morbidity and can be scheduled more frequently to provide a personalized snapshot of disease at sequential time points, enabling detection, screening and real-time monitoring of disease progression and therapy response[26, 27].

3.1. Types of liquid biopsies

Liquid biopsies are minimally invasive or even non-invasive procedures for obtaining tumor-derived information from body fluids[27]. Theoretically, any fluid that circulates in or associates with the human body is applicable (Figure 2)[27-29]. Some body fluids require minimal intervention by medical personnel for collection, such as ascites, blood, bone marrow, cerebrospinal fluid, exhaled breath, earwax, interstitial fluid, lymphatic fluid, pleural fluid, and tears[27, 29, 30]. Other body fluids are easy to be collected without the need for specialized personnel such as saliva, seminal fluid, sputum, stool, sweat and urine[29-31]. Liquid biopsy consists of collecting disease-derived genetic information, protein signatures, or micro-and nanovesicles shed directly from tumor tissue[30].

3.1.1. Minimally invasive liquid biopsies

The most commonly used minimally invasive liquid biopsy is blood. It is obtained by a routine blood draw and usually requires 6-10 mL[24]. It requires a short time and sampling is considered low cost[24]. However, the key consideration is that taking a blood sample is not always easy and problems may arise depending on the age and condition of the patients[30]. Another disadvantage is the lack of standardization of methodologies[24]. Blood contains many components such as cell-free DNA (cfDNA), circulating tumor cells (CTC), extracellular vesicles (EVs), mRNA, microRNA, platelets, and proteins[25].

Other minimally invasive liquid biopsies include earwax, pleural fluid, and tears.

3.1.2. Non-invasive liquid biopsies

The most commonly used non-invasive liquid biopsies are saliva, stool, and urine.

Saliva

Saliva is a complex fluid derived from major and minor salivary glands, its main functions are lubrication to protect the oral mucosa, initiating food digestion, cleaning the oral cavity, antimicrobial action, buffering oral pH and maintaining supersaturated calcium phosphate concentrations[30]. Saliva offers practical

advantages because it can be collected with simple equipment, is cost effective and is easily accessible[28,30]. Saliva contains many molecules, including EVs, metabolites, nucleic acids, and proteins[28,30]. Salivary biomarkers have potential in cancer diagnosis, detection, screening, and monitoring treatment efficacy[28, 30]. The most studied cancer type for salivary biomarkers is oral squamous cell carcinoma, as saliva said to contain molecules, cells and EVs that shed directly from the primary site of the tumor and directly from the close tumor tissue microenvironment of the primary site[30]. Additionally, salivary biomarkers have shown potential for non-small cell lung carcinoma and pancreatic cancer[28].

Stool

Currently, the analysis of stool, also known as fecal samples, is performed to detect occult blood, which is associated with chronic inflammatory diseases like Crohn's disease or ulcerative colitis and colorectal cancer. Stool contains different biomarkers such as cells, EVs, microbiota and molecules. For stool, a number of established assays have been proposed over the years to detect markers related to gastrointestinal tract pathologies[30].

Urine

Urine is gaining interest as liquid biopsy for non-invasive cancer diagnosis because it has additional benefits over blood. This sample type will be discussed in greater detail in section 4.

Other

Other non-invasive liquid biopsies are seminal fluid and sweat.
Sweat carries a range of biomolecules including small ions and molecules as well as large proteins and antibodies. Sweat has been used for the medical diagnosis of cystic fibrosis and to determine the fluid and electrolyte balance in athletes. Furthermore, studies have shown that various metabolites and neuroimmune biomarkers can be found in sweat[30].

Seminal fluid is composed of secretions from glands in the male urogenital tract. Usually, semen is collected by masturbation in sterile cups, at home or in special urological laboratories. Seminal fluid contains numerous components including ions, lipids, nucleic acids, proteins, small metabolites, and sugars.

Its composition is mainly driven by prostatic activity, which is why it is highly enriched in prostate cancer biomarkers. The most well-known use of seminal fluid storage is the cryopreservation of spermatozoa for further use in Assisted Reproductive Technologies[30].

3.2. Components of liquid biopsies

Originally, "liquid biopsy" was introduced as diagnostic concept in 2010 for the analysis of CTCs in blood and has now been extended to the analysis of all components of the different body fluids (Figure 2)[31]. A number of components can be isolated from liquid biopsy and can be divided into two categories: (1) cell-free or subcellular structure-free large and small molecules such as carbohydrates, lipids, nucleic acids, proteins, small metabolites and metal ions; (2) cellular or subcellular structures including circulating cancer-associated fibroblasts, circulating mitochondria, CTCs, EVs, immune cells, tumor-educated platelets and other potential cellular compartments[26, 29, 31, 32]. All these components can be released by or derived from cancer cells, the primary tumor, and metastatic sites[24]. The different components of liquid biopsies encompass information such as DNA mutations, copy number alterations, transcriptome and proteome profiling, epigenetic alterations, and metabolite profiling[28].

3.2.1. Circulating tumor cells

CTCs are cells that are released from the primary tumor, travel through the circulatory system and are responsible for the development of metastatic tumors at distant sites in the body[28]. CTCs usually remain in the circulation for only 1-2.5 hours before being destroyed by the immune system, but a small fraction can survive and metastasize[27]. Therefore, the percentage of CTCs in the circulation is quite low, with nearly one CTC found per million leukocytes[28]. Additionally, CTC levels have been shown to change in a dynamic manner, parallel to the tumor condition[28, 31]. Interestingly, CTCs vary in shape, depending on the stage and/or type of tumor[28]. The characterization of CTCs based on their size, cell-surface marker expression and their content (e.g., DNA, proteins, RNA) has shown promising results in early diagnosis and treatment response monitoring of several types of cancers, even brain tumors[27, 28, 31, 33].

3.2.2. Circulating cell-free and tumor nucleic acids

Nucleic acids are the component group that contains all forms of DNA and RNA. Circulating nucleic acids can be divided into two categories, a broader group being cell-free circulating nucleic acids (cfNA) and a more specific group being tumor nucleic acids (ctNA). cfNAs originate from nonmalignant somatic tissues, tumors and embryos or fetuses and are released when cells undergo apoptosis, necrosis or via active release[25, 27, 33]. When cfNA is derived from a tumor, it is more specifically referred to as ctNA, with ctDNA generally being present at only 0.1-10% of the total cfDNA[25, 28]. cfNAs can be characterized based on their density, electric charge, length, physical size, and surface molecules[33]. Tumor-specific mutations can act as biomarkers to distinguish cancer patients from healthy individuals[25]. Interestingly, the ctDNA levels and sizes vary subject to tumor burden, tumor stage, and therapeutic response[28].

3.2.3. Extracellular vesicles

EVs are small (30-200 nm), membrane-bound, dish-shaped vesicles secreted by cells and found in almost all body fluids[28, 34]. EVs encompass a variable spectrum of diverse biomolecules, including nucleic acids (e.g., DNA, mRNA, microRNA, long non-coding RNA, etc.), lipids and proteins[28, 34]. EVs have now been established to play critical roles in cell-to-cell communication and cancer development, including angiogenesis, epithelial to mesenchymal transition, immunosuppression, metastasis, and promoting tumor growth. These findings have led to the idea that analyzing tumor-derived EVs and their cargo provide new opportunities for cancer liquid biopsy, highlighting its potential as biomarkers for cancer diagnosis, prognosis, and monitoring therapeutic response[26, 28]. EVs have additional benefits over CTCs and cfNAs, since (a) EVs are present in high amount in biofluids, (b) EVs are secreted by living cells and not only linked to apoptotic or dead tumor cells, and (c) EVs are naturally stable due to their lipid bilayers and thus can withstand even the harsh tumor microenvironment[26]. However, the main challenges for the application of EVs in liquid biopsy remain the standardized and optimized isolation and detection methods, limited sensitivity and specificity, and low purity and time-consuming methods[26].

3.3. Liquid biopsies for cancer applications

Liquid biopsy is a powerful tool for a broad range of cancer applications (Figure 2). In cancer research and care, all liquid biopsy components have shown their potential in enabling risk-free screening of asymptomatic individuals, early diagnosis, tumor staging, real-time monitoring of disease progression, predicting sensitivity and/or resistance to specific therapies, and early detection of residual disease or relapse[24, 28, 33]. Liquid biopsy components, such as ctDNA, enable tumor-specific molecular profiling of the patients to guide targeted therapy for precision medicine. ctDNA allows real-time and long-term monitoring of the treatment effect, enabling possible treatment adjustments and better prognosis[25, 27]. Currently, the European Medicine Agency and the FDA have approved testing for epidermal growth factor receptor mutations using ctDNA for therapy guidance in non-small cell lung cancer patients[35, 36]. Additionally, the prediction of resistance to therapies can be based on monitoring liquid biopsy components, such as CTCs. For example, mutations in the estrogen receptor gene can provide information on the resistance to hormone therapy in breast cancer, while expression of androgen receptor variant 7 in CTCs can predict the resistance to anti-androgen therapy in prostate cancer[31].

Despite the many advantages, the clinical application of liquid biopsies is hampered by limitations such as lack of standardization and isolation procedures, high costs, and lack of sensitivity and specificity[25, 28]. One of the main drawbacks is the stability of the components in the body fluids. The chemical stability and degradation of liquid biopsy components can be affected by physical stress during processing, and storage temperatures and times. Current solutions are storage under strict conditions and the immediate addition of stabilizing reagents. Another development to potentially solve the problem of preserving samples from collection to analysis is Lab On Chips (LOCs). The ability to introducing LOCs that can process, store, or analyze body fluids would represent a major change. However, further development is needed before these can be clinically applicable[30].

In general, there is a need to improve technology, standardize and harmonize laboratory processes from sample collection, through storage to processing (isolation and detection) and further clinical validation of potential biomarkers. If all the challenges currently associated with liquid biopsies could be addressed

through advances in research and technology, the clinical applicability of liquid biopsies would revolutionize cancer research and care[28].

Urine as liquid biopsy

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Urine is non-invasive, it is easy to collect, even at home, without the need for a health care practitioner and has a lower matrix complexity. Moreover, urine is seen as a fluid that reflects the health of the entire body, as the kidneys continuously filter, excrete, and reabsorb substance from all over the body[30, 37]. Therefore, it contains many clinically relevant components such as EVs, circulating nucleic acids, and proteins[28]. Daily, an individual produces a large volume of urine through 6-7 urinations of 400-2000 mL. Urine can be collected indefinitely and repeatedly, enabling serial sampling and multi-omic analysis[28, 30]. This highlights the potential for large-scale screening applications[27, 30]. Urine has been shown to be useful in detecting cancers of both urological and non-urological sources with more or less similar sensitivities to blood[28]. Additionally, urinalysis can reveal diabetes, hypertension, infections, and pregnancy[30]. Despite these benefits, urine's applicability in the clinic remained limited because of the highly variable and dynamic nature of urinary concentration and contents due to differences in age, diet, exercise, fluid intake, gender, health status, medications, type of fraction collected next to time of collection. These factors can complicate the data interpretation and therefore the use of urine in diagnostics[37].

4.1. Urine fractions

Not all urine types and fractions are the same. First a division can be made between full void, first-catch and midstream urine. Full void is defined as the entire urine flush from start to finish. First-catch (first-void or first-pass) urine is typically the first part of urine flush collected at any time of the day. Several studies have shown that for certain disease screenings, first-catch urine samples allow for improved sensitivity, such as for cervical and prostate cancer[38-40]. Midstream urine is collected after discarding the first 10-50 mL. This fraction is often considered the standard for the detection of metabolic diseases and urinary tract infections, as it is known to contain fewer contaminants[41].

Secondly, a urine sample can be kept as whole (full void) or centrifuged to generate urine supernatant and urine sediment. Several studies have investigated and compared these different urine fractions for the detection of urinary biomarkers for various cancers[42-44]. For cervical cancer, different urine fraction were shown to have excellent performance, of which urine sediment demonstrated to have the best accuracy to detect CIN3 using DNA methylation analysis[42]. While, for the detection of endometrial cancer, full void was shown to be the most optimal fraction using methylation analysis[43]. Although, all fractions showed significantly increased methylation levels of all investigated markers in patients compared to controls[43]. Similarly, all three fractions (full void, urine sediment and urine supernatant) were demonstrated to be suitable for urinary methylation analysis for the detection of bladder cancer[44].

4.2. Urinary biomarkers

Urine contains many clinically relevant components such as EVs, circulating nucleic acids, and proteins. Several urinary biomarkers have been described for different cancers, such as nuclear matrix protein 22, TMPRSS2:ERG fusion gene expression and prostate cancer gene 3 for prostate cancer, IQGAP3 and UBE2C cell-free nucleic acids for bladder cancer, non-coding piRNA for renal cell carcinoma, and BLCA-4 for bladder cancer[28, 30]. More evidence and examples of urinary biomarkers and their applicability in cancer research and care can be found in **Chapter 3** of this dissertation.

4.2.1. Trans-renal vs. non-trans-renal urinary biomarkers

Urinary DNA can be divided into two size categories: heterogeneous, high molecular weight DNA (1 kb or higher) and relatively homogeneous, low molecular weight DNA (10-250 bp)[45, 46]. The large size class, cellular DNA, originates from genital tract cells or cell debris shed into urine, while the small size class, cfDNA, mainly originates from glomerular filtration of blood cfDNA[45-48]. Cell-free and circulating nucleic acids in blood are the results of apoptosis and/or necrosis in actively proliferating healthy cells or tumor tissue. Apoptosis can be distinguished from necrosis based on the produced DNA fragment size. Apoptosis produces DNA fragments of 180-200 bp (and multiples of this amount), whereas

necrosis results in much larger fragments[45, 49]. These highly fragmented nucleic acids circulate in the blood as part of super-molecule-complexes, such as nucleosomes, nucleoprotein complexes and membrane-coated microparticles. Active shedding of DNA by cells has also been described[45, 46, 48].

The glomerular filter has been proven to be permeable to DNA molecules[46]. However, the way DNA crosses the renal barrier is not yet fully understood. Since glomerular filtration of blood is limited by the permeability of the basal membrane and slit membranes between podocytes' pedicles, only complexes smaller than 6.4 nm in diameter and with a molecular weight no greater than 70 kDa (the equivalent of 100 bp of DNA), can pass through the renal barrier[45]. The average molecular weight of urinary cfDNA is about 90 kDa (150 bp) and is mostly bound to nucleosomes, which have a diameter larger than the membrane pores. However, it was suggested that the serum amyloid P component is capable of unwinding the nucleosomes[50]. The negatively charged cfDNA might face an additional barrier because of the negative charge of the glomerular basement membrane[46, 48, 50]. Nucleic acids are known to bind to proteins as well, which may have both positive and negative effects on transportation[45]. Additionally, cfDNA might be covered by liposomes, which makes their penetration through the renal barrier possible[50]. Despite the lack of understanding about how DNA crosses the renal barrier, several studies have shown the presence of trans-renal cfDNA in urine for different physiological and pathological conditions, such as pregnancy, cancer, and inflammation[45-47, 50-52]. Recent studies showed that cell-free nucleic acids and tumor DNA are shed into the circulation and pass the renal barrier to be excreted in urine[53, 54]. Additionally, a recent study showed that tumor DNA from urogenital cancers in urine is the result of direct shedding and renal-excreted tumor material[51].

Currently, the presence of trans-renal ctDNA in urine and its potential has been demonstrated in various studies, concerning non-urothelial linked cancers such as breast cancer[55-58], colorectal cancer[59], gastric cancer[60], hepatocellular carcinoma[61], non-small cell lung cancer[62-69] and pancreatic cancer[70].

The presence of proteins in urine is very strictly controlled by the functioning of the kidneys, only proteins with very low molecular weight will pass through. The presence of bigger proteins like albumin leads to the diagnosis of proteinuria or albuminuria, which are chronical kidney misfunction diseases[71]. Cancer-related proteins have mainly been investigated for their applicability in bladder

cancer, for which already two FDA approved tests (NMP22® BC test and NMP22® BladderChek®) exists focusing on nuclear matrix protein 22[72].

4.3. Urine collection, storage, and processing

An important source of variability in biomarker discovery and validation lies in pre-analytical decisions regarding sample collection, storage, and processing. Additionally, some biological variables are important to consider, including impact of comorbidities such as kidney disease, the impact of medications, time of day of urine collection, variation in hydration status, use of diuretics[47].

Several variables such as place of collection (home versus clinic), time of collection, transportation time and conditions (e.g., distance from the laboratory or biobank and cold chain needs), the need for specialized laboratory equipment and personnel make immediate analysis impossible[73, 74]. Therefore, urine must be stored, transported, and processed in such a way that urinary analytes are preserved throughout the process from sample collection to downstream analysis.

4.3.1. Collection methods

There are various differences between urine collection containers and devices, (i) they are usually made of plastics, such as high-density polyethylene or polypropylene, (ii) they can be open or closed, (iii) they can be sterile or unsterile, (iv) they have tube transfer systems or are anatomically compatible. Depending on the analyte of interest, it is important to choose the appropriate urine collection container or device. Of importance is for the collection container or device to not bind the analyte of interest nor disperse microplastic particles in the collected urine. A sterile container or device is considered important for studying bacteriological culture or microbial analytes, while for the collection of a specific urine fraction, a collection device specifically developed to collect that fraction in a standardized way is important[30, 37]. Currently, there are some collection containers and devices commercially available or under development, such as the urine cup (various manufacturers), Colli-Pee® (Novosanis NV, Belgium), Firstburst (DRW, Cambridge, UK), Monovette (Sarstedt, Nümbrecht, Germany), Peezy First Stream (Forte Medical, London, UK), Vacuette (BD, New Jersey, USA) and Uriswab (Copan, Brescia, Italy).

4.3.2. Storage & Preservation methods

The use of preservatives prevents the degradation of analytes of interest in urine during storage. Different preservation methods can be employed for different analytes of interest. For example, measuring environmental contaminants in urine did not require refrigeration or preservatives[75]. While analytes such as ethyl-glucuronide and ethyl-sulphate require refrigeration or preservatives to inhibit their degradation[76]. Furthermore, the addition of antibiotics can prevent bacterial growth and ethylenediaminetetraacetic acid (EDTA) reduces DNA degradation[77]. Many, but not all, cancer studies investigating trans-renal ctDNA used EDTA as preservative. However, the final EDTA concentrations used were variable and possibly led to inter-study variation[47]. One study investigated the integrity and stability of urinary EVs when stored at 4°C or at room temperature with EDTA for up to 8 days. They demonstrated that both methods are suitable for urine storage and downstream urinary EV analysis. Room temperature storage with EDTA offers more flexibility in sample collection and storage and may therefore be a more convenient method[73]. For proteins, several studies have used protease inhibitors, which have been shown to preserve some specific urinary EV proteins, such as NKCC2, but analysis of other proteins, such as CD9 and TSG101, showed that not all EV proteins are suspected of proteolysis in urine[78, 79]. For metabolites, Wang et al. demonstrated that storage at low temperature (4°C) can minimize metabolite changes in urine, while high temperatures (40°C) lead to metabolite changes. Furthermore, they showed that there were no significant changes when urine samples were kept at room temperature (22°C) for up to 24 hours, while there were significant changes when samples were kept for 48 hours. Finally, they demonstrated that thymol was effective in preserving the metabolite stability under all conditions, while boric acid was not[80]. Until now, different analytes of interest require different preservatives, some companies have developed analyte or urine specific preservatives that are commercially available with all their own claims, format, and analyte stability. Some of these preservatives are AssayAssure™ (Thermo Fisher Scientific, Waltham, Massachusetts, Verenigde Staten), Complete™ EDTA-free Protease inhibitor cocktail (Roche, Bazel, Zwitserland), Cult-Ur™ Tablets (Cargille, New Jersey, USA), Stabilur® Tablets (Cargille, New Jersey, USA), UAS™ preservative (Novosanis NV, Wijnegem, Belgium), UCM™ preservative (Novosanis NV, Wijnegem, Belgium), Urine Conditioning Buffer (Zymo, Irvine, California, USA), Urine Preservative (Norgen Biotek Corp., Ontario, Canada) and Urine Preserve (Streck Inc., La Vista, Nebraska, USA).

4.4. Applicability

Urine as liquid biopsy for oncology applications has gained increasing interest over the past three decades and is expected to continue into the current decade (Figure 3). The number of publications (registered on PubMed) for urine and liquid biopsy in the last decade (2010-2019; 268 publications) is more than seven times the number of registered publications in the previous decade (2000-2009; 37 publications). While when looking at urine and oncology, the number of publications in the last decade (2010-2019; 8963 publications) has almost doubled compared to two decades ago (1990-1999; 5221). The fact that interest in urine is increasing worldwide is also shown by the conducted or ongoing clinical studies with urine (Figure 3). Most of the urine-related studies are conducted or ongoing in North America (1816) followed by Europe (807), while South Asia (27) has the least number of studies.

Urine has a lot of potential based on different cancer types and different applications. On the one hand, urine has been examined for different cancer types, not only urological cancers, such as bladder and prostate cancer, but also non-urological cancers, such as breast cancer, gastrointestinal cancer, gynecological cancers, and lung cancer. On the other hand, urine has been investigated as liquid biopsy for cancer screening, monitoring of cancer progression or recurrence, and the efficacy of chemotherapy and radiation therapy[81]. More details and examples of the applicability of urine can be found in **Chapter 3** of this dissertation, where a systematic review of the literature on urine as liquid biopsy for cancer research and care has been performed.

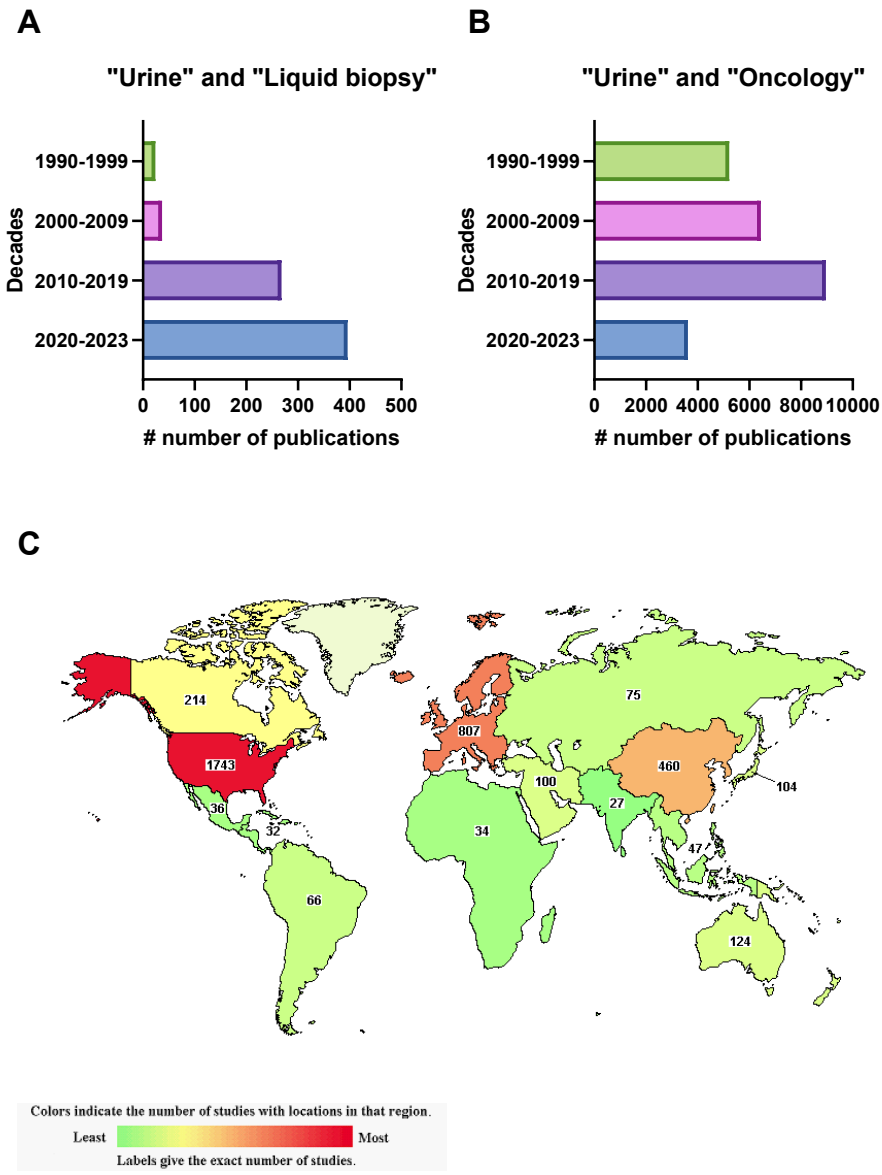

Figure 3: Overview emerging field of urine as liquid biopsy for oncology applications. A general search of PubMed was performed with search terms “urine” and “liquid biopsy” (A) and “urine” and “oncology” (B) to extract the number of publications. A search of ClinicalTrials.gov was performed indicating “cancer” as disease or condition and “urine” as other term to generate an overview of the results on a world map (C).

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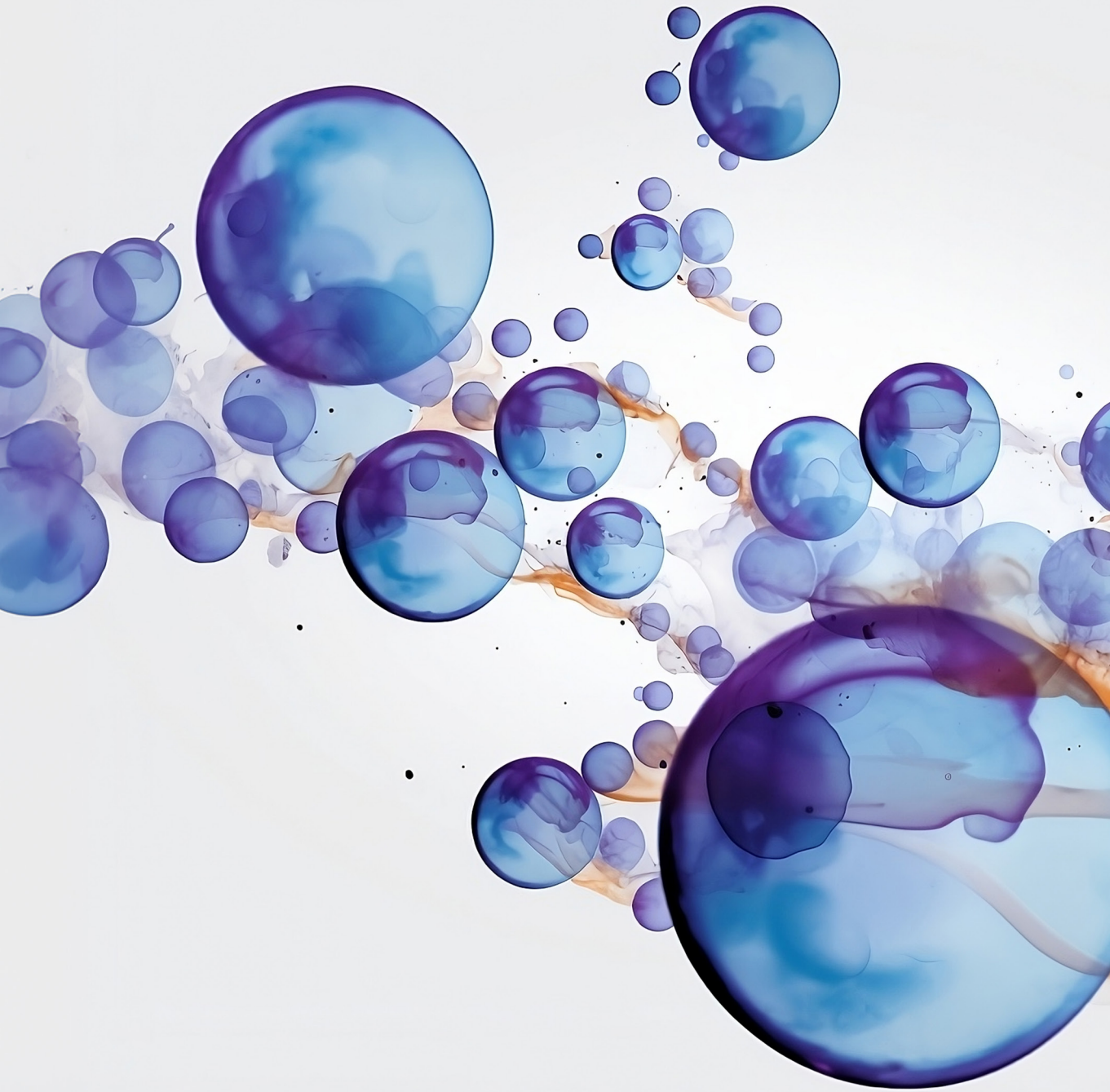
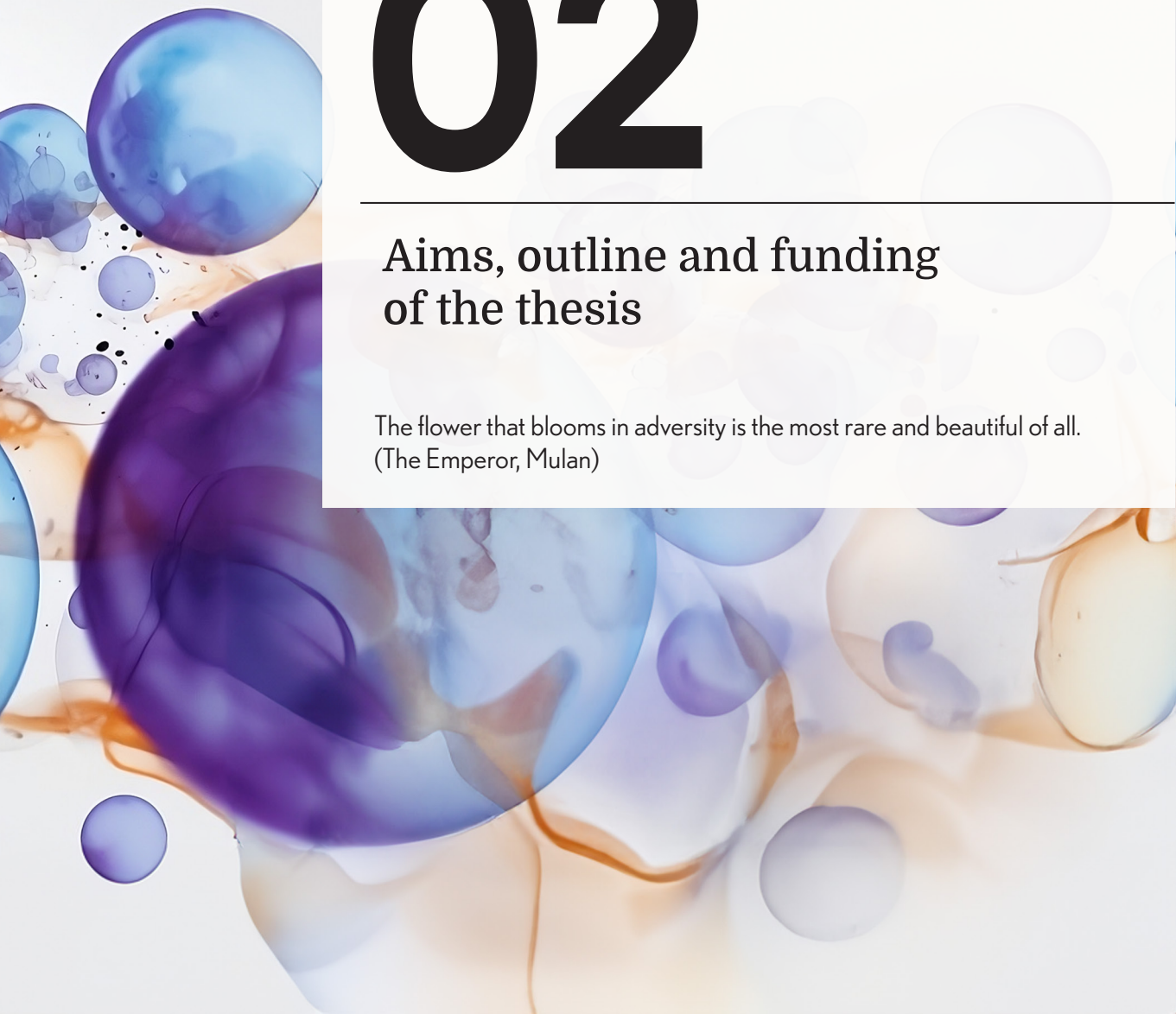


CHAPTER

02

Aims, outline and funding of the thesis

The flower that blooms in adversity is the most rare and beautiful of all.
(The Emperor, Mulan)



Aims and Outline

01

The overall goal of this dissertation was to fully exploit the potential of urine as a liquid biopsy in cancer research and its potential for screening and patient monitoring. Therefore, urine samples were collected from healthy volunteers, pregnant women and breast- and prostate cancer patients to investigate pre-analytical sample collection methods and storage conditions for biomarker detection. Colli-Pee®, a device developed by Novosanis, enables standardized and volumetric collection of first-catch urine allowing for immediate mixing with UAS™, a newly developed preservative by DNA Genotek (Novosanis' sister company). The development of a new generation of Colli-Pee® variants, including preservatives, will further improve its applicability for oncological purposes.

In **Chapter 3**, we provide a comprehensive overview of the current status of urine sampling as liquid biopsy for non-invasive cancer research. Interestingly, we found 924 publications investigating urine for many types of cancers, different analytes and various applications. We critically discuss the reporting of pre-analytical parameters and applied methods for urine sampling. Furthermore, we explain some future considerations and suggest some recommendations to further elaborate the clinical utility of urine in cancer research, detection, and disease monitoring.

In this dissertation, we investigate the potential of urine as liquid biopsy for different cancer types. Two cancer types are addressed (i) prostate cancer as second most common cancer in men and its possible association with urine and (ii) breast cancer as most common cancer in women and the not so obvious association with urine. To further elaborate on the clinical applicability of urine as liquid biopsy, there are some challenges to overcome, including but not limited to: the degradation of interesting urinary analytes, the collection

of the correct fraction of urine using appropriate and user-friendly devices, and the low concentration of urinary analytes of interest. Furthermore, since standardization of urine collection and preservation could assist in elaborating the clinical utility of urine as liquid biopsy, it is important that devices developed for immediate preservation and collection of the correct fractions is easy, hygienic and user-friendly.

Chapter 4 investigates the performance of a recently developed preservative, UAS™ by DNA Genotek (sister company of Novosanis), for the preservation of urinary analytes at room temperature for up to 14 days and after simulated transport conditions for oncology applications. Subsequently, this preservative is included in the Colli-Pee® device for the studies performed in **Chapter 5.**

In **Chapter 5,** we compare the isolation efficiency of four cfDNA isolation methods. Total cfDNA concentration, fragmentation profiles and integrity were determined by Qubit analysis, TapeStation analysis, digital droplet PCR (ddPCR) and real-time quantitative PCR (qPCR). For this optimization study, samples from pregnant women, breast and prostate cancer patients and healthy female and male volunteers were included. In this chapter, we also investigate the presence of trans-renal urinary biomarkers by detecting male genes, such as SRY and DYS14, in women pregnant with a boy.

The focus of **Chapter 6** is to examine the experiences and preferences of the study participants with regard to first-catch urine collection and preservation using the Colli-Pee® UAS™ devices, both for collection during doctor's visit as home-sampling.

Many interesting analytes are present in urine, although, in a low concentration. There are already some solutions to filter or concentrate these analytes of interest in the lab. However, if this filtration or concentration could take place earlier in the process, these analytes could be immediately well-preserved. **Chapter 7** describes the development pathway of a potential new Colli-Pee® variant for the enrichment of urinary analytes of interest.

This dissertation is funded by VLAIO Baekeland (see 2. Study Funding), a mandate intended to support research that, if successful, adds clear economic value to the company holding the grant, in this case Novosanis, a company active in the field of urine sample collection and preservation. Indeed, urine as source of

biomarkers for early detection and monitoring of prostate and breast cancer has great market potential. However, research into validation of biomarkers and optimal urine pre-analytical parameters requires time and investments. The work conducted in this dissertation will ultimately result in: (i) data and insights on biomarkers present in urine as well as optimal conditions for collection, storage and transport of urine which will offer a competitive advantage in the field of non-invasive liquid biopsies (**Chapter 3-6**); (ii) development and validation of novel preservation chemistries for the optimal preservation of different analytes in urine aiding in Novosanis' positioning in the field of at-home collection (**Chapter 4**); (iii) development and validation of new physical design features such as sample enrichment systems applicable in a range of applications (**Chapter 7**); (iv) steps towards establishing collaborations and partnerships for the (co-)development of urinary assays in oncology. Hence, the knowledge and outcomes of this dissertation offer solid scientific evidence and will provide Novosanis with a first mover advantage in the field of non-invasive urinary liquid biopsy sampling and gain market share in the coming years.

Finally, **Chapter 8** discusses the presented results in relation to relevant literature and provides clear insights on future perspectives. Additionally, in the last chapters of this dissertation, a summary (**Chapter 9** (English) & **Chapter 10** (Dutch)), my scientific curriculum vitae (**Chapter 11**) and acknowledgements (**Chapter 12**) can be found.

Study Funding

02

This doctoral thesis was funded by the Flemish Government organization Flanders Innovation and Entrepreneurship (VLAIO). VLAIO aims to bridge the gap between the academia and industry through Baekeland or Innovation mandates. With these mandates, VLAIO supports academic research in an industrial environment. They give individual researchers the opportunity to conduct pre- or postdoctoral research in close collaboration with business activities. These mandates aim to support research that, if successful, adds clear economic value to the company. All this according to the standards for conducting a doctoral thesis and degree.

PART I

Real world data on urine as liquid biopsy

CHAPTER

03

Urine biomarkers in cancer detection: a systematic review of preanalytical parameters and applied methods

Stephanie Jordaens, Karen Zwaenepoel, Wiebren Tjalma, Christophe Deben, Koen Beyers, Vanessa Vankerckhoven, Patrick Pauwels, Alex Vorsters

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Sometimes the right path is not the easiest one.
(Grandmother Willow, Pocahontas)

Abstract

The aim of this review was to explore the status of urine sampling as a liquid biopsy for non-invasive cancer research by reviewing used preanalytical parameters and protocols. We searched two main health sciences databases, PubMed and Web of Science. From all eligible publications (2010–2022), information was extracted regarding: (a) study population characteristics, (b) cancer type, (c) urine pre-analytics, (d) analyte class, (e) isolation method, (f) detection method, (g) comparator used, (h) biomarker type, (i) conclusion, and (j) sensitivity and specificity. The search query identified 7835 records, of which 924 unique publications remained after screening the title, abstract and full text. Our analysis demonstrated that many publications did not report information about the pre-analytical parameters of their urine samples, even though several other studies have shown the importance of standardization of sample handling. Interestingly, it was noted that urine is used for many cancer types and not just cancers originating from the urogenital tract. Many different types of relevant analytes have been shown to be found in urine. Additionally, future considerations and recommendations are discussed: (a) the heterogeneous nature of urine, (b) the need for standardized practice protocols, and (c) the road towards the clinic. Urine is an emerging liquid biopsy with broad applicability in different analytes and several cancer types. However, standard practice protocols for sample handling and processing would help to elaborate the clinical utility of urine in cancer research, detection, and disease monitoring.

Introduction

01

1.1. Urine as a sample type

Liquid biopsy is emerging as a revolutionary tool, not only for diagnosis but also for real-time monitoring of disease progression, especially in cancer research and care, with several significant advantages over tissue biopsy. Tissue biopsy only offers a snapshot of the tumor at a given time and in a specific location, while liquid biopsy may capture intratumor heterogeneity better. Additionally, liquid biopsies can be used as a screening tool for early cancer detection, monitoring of treatment response, and detection of recurrence. Different body fluids have considerable potential as liquid biopsies. They can be obtained using minimally invasive (e.g., blood) or fully non-invasive (e.g., urine) procedures.

Urine as a liquid biopsy for non-invasive cancer diagnosis is gaining interest for different cancer types since it has some additional benefits over blood. As urine is non-invasive, it is easy to collect, even at home, without the need for a health care practitioner. Urine can be collected without limits in volume and repeatability, allowing for serial sampling and multi-omic analysis.

Not all urine fractions are the same. Full void is defined as the entire urine flush from start to finish. First-catch (first-void or first-pass) urine is typically the first part of urine flush collected at any time of the day. Several studies have shown that for certain disease screenings, first-catch urine samples allow for improved sensitivity[1-3]. Midstream urine is collected after discarding the first 10–50 mL. This fraction is often considered the standard for the detection of metabolic diseases and urinary tract infections, as it is known to contain fewer contaminants[4].

1.2. Trans-renal vs. non-trans-renal urinary biomarkers

Urinary DNA can be divided into two size categories: heterogeneous, high molecular weight DNA (1 kb or higher) and relatively homogeneous, low molecular weight DNA (10–250 bp)[5, 6]. The large size class, cellular DNA, originates from genital tract cells or cell debris shed into urine, while the small size class, cell-free DNA (cfDNA), mainly originates from glomerular filtration of blood cfDNA[5-8]. Cell-free and circulating nucleic acids in blood are the results of apoptosis and/or necrosis in actively proliferating healthy cells or tumor tissue. Apoptosis can be distinguished from necrosis based on the produced DNA fragment size. Apoptosis produces DNA fragments of 180 to 200 bp (and multiples of this amount), whereas necrosis results in much larger fragments[5, 9]. These highly fragmented nucleic acids circulate in the blood as part of super-molecule-complexes, such as nucleosomes, nucleoprotein complexes and membrane-coated microparticles. Active shedding of DNA by cells has also been described[5-7].

The glomerular filter has been proven to be permeable to DNA molecules[6]. Despite the lack of understanding about how DNA crosses the renal barrier, several studies have shown the presence of trans-renal cfDNA in urine for different physiological and pathological conditions, such as pregnancy, cancer, and inflammation[5, 6, 8, 10, 11]. Recent studies showed that cell-free nucleic acids and tumor DNA are shed into the circulation and pass the renal barrier to be excreted in urine[12, 13].

1.3. Objectives

Urine has been gaining interest as a non-invasive liquid biopsy for cancer research since it has some additional benefits over blood. Urine as a sample type is non-invasive, easy to collect at home or at the clinic and it can be collected repeatedly without limits in volume allowing for serial sampling and multi-omic analysis. This review aims to provide a comprehensive overview of the current status of urine sampling as liquid biopsy for non-invasive cancer research. Additionally, this review will critically discuss the reporting of pre-analytical parameters and applied methods for urine sampling.

Methods

02

2.1. Search strategy and selection criteria

A review of the literature was performed from January 1, 2010, until January 28, 2022, using two main health and life sciences databases: PubMed and ISI Web of Science (Figure 1). The PubMed database was searched using the following combinations of MeSH terms: '(Extracellular Vesicles OR Exosomes OR Peptides OR Cell-Free Nucleic Acids) AND Neoplasms AND Urine'; 'Early Detection of Cancer AND Urine'; '(Extracellular Vesicles OR Exosomes OR Peptides OR Cell-Free Nucleic Acids) AND Early Detection of Cancer'. In addition, the PubMed database was searched (Title/Abstract) using the following search terms: '(extracellular vesicle OR exosome OR RNA OR DNA) AND (urine OR urinary) AND cancer AND detection. The ISI Web of Science database was searched (Title/ Abstract/ Author Keywords/Keywords Plus) using the following search terms: '(extracellular vesicles OR extracellular vesicle OR exosome OR exosomes OR RNA OR microRNA OR mRNA OR lncRNA OR DNA OR ctDNA OR tumor DNA OR circulating NA OR cfDNA OR cell-free DNA) AND (urine OR urinary) AND (cancer OR cancers OR neoplasms) AND (detection OR assay OR method)'. A prospective protocol, following PRISMA guidelines[14], was registered October 31, 2020 on PROSPERO (CRD42020206241). Studies were eligible for inclusion if they reported on the detection of biomarkers for oncology applications in urine. The search was restricted to English language studies that involved human subjects.

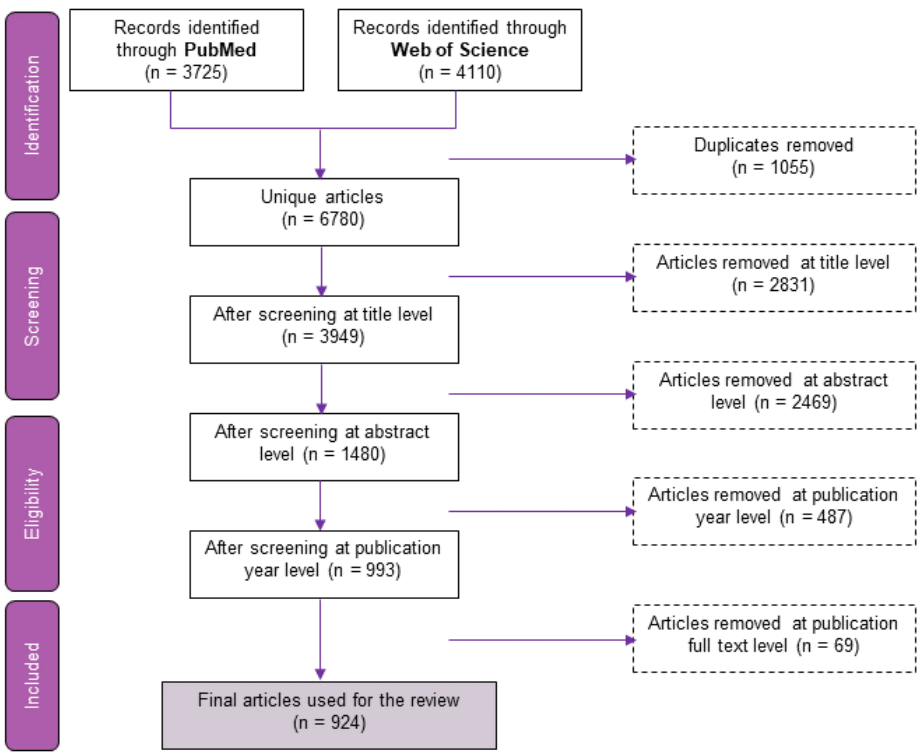

Figure 1: Flow diagram of literature search. The search yielded 6780 unique hits in PubMed and ISI Web of Science imported into Endnote X9. The retrieved records were first screened based on title, abstract, publication year, followed by full-text evaluation. Studies were eligible for inclusion if they reported on the detection of biomarkers for oncology applications in urine, whether these were compared to tissue biopsy or blood sampling. The flow diagram was adapted from the PRISMA flow diagram13.

2.2. Data extraction, synthesis, and presentation of the results

From each eligible article, data related to: (a) study population characteristics, (b) cancer type, (c) urine pre-analytics, (d) analyte, (e) isolation method, (f) detection method, (g) comparator used, (h) biomarker type (Box 1), (i) study conclusion, and (j) sensitivity and specificity were obtained.

BOX 1: Biomarker classes

The definitions for biomarkers were established by the U.S. Food and Drug Administration and the National Institutes of Health as part of their joint Biomarkers, EndpointS, and other Tools resource.

- A **diagnostic biomarker** detects or confirms the presence of a disease or condition of interest or identifies an individual with a subtype of the disease. Such biomarkers may be used not only to identify people with a disease but to redefine the classification of the disease.
- A **monitoring biomarker** can be measured serially to assess the status of a disease or medical condition for evidence of exposure to a medical product or environmental agent, or to detect an effect of a medical product or biological agent.
- A **predictive biomarker** is defined by the finding that the presence or change in the biomarker predicts an individual or group of individuals more likely to experience a favorable or unfavorable effect from the exposure to a medical product or environmental agent.
- A **prognostic biomarker** is used to identify the likelihood of a clinical event, disease recurrence, or disease progression in patients with a disease or medical condition of interest.

It is critically important to have a good distinction between prognostic and predictive biomarkers. Prognostic biomarkers are associated with differential disease outcomes, but predictive biomarkers discriminate those who will respond or not respond to therapy.

Results

03

3.1. Search results

The search query identified 7835 records from PubMed and Web of Science (Figure 1). After excluding 1055 duplicates, the remaining 6780 records were screened, based on title, abstract, year of publication and full text. Studies were excluded if they concerned: (a) non-urine research, (b) other pathology, and (c) urinary biomarker detection in healthy participants. Ultimately, 924 publications were included in the analysis. The literature search showed that urine is used in many types of cancer and not only those cancers linked to the urogenital tract (Figure 2). Specific cancer types of which only one article was found, were excluded. A total of 924 publications were considered for this review (details in Table S1 available online). Given that meta-analysis was not possible due to data heterogeneity, narrative reporting was used.

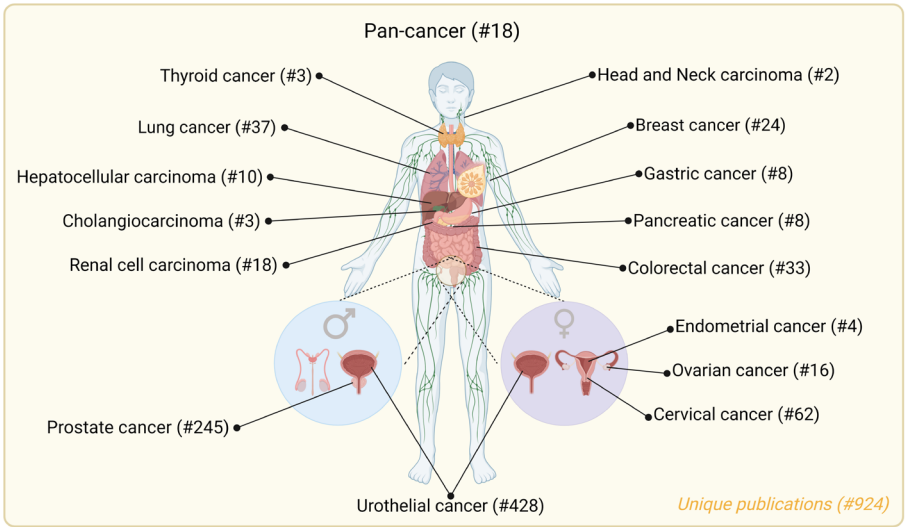

Figure 2: Overview of cancer research in urine. The literature search (924 unique publications) showed that urine is used in many types of cancer and not only the once linked to the urogenital tract. The cancer type is mentioned with the number of publications retrieved in the literature search within brackets. Cancer types of which only one article was found were excluded for further analysis. These cancer types were adrenocortical carcinoma, central nervous system tumors, esophageal cancer, lymphoma, melanoma, neuroblastoma, pheochromocytoma and Wilms tumors. Created in BioRender.com.

3.2. Urine as liquid biopsy

3.2.1. Biomarkers and analytes

Biomarkers can be divided into several classes based on their usage: diagnostic, monitoring, predictive and prognostic (Box 1). Today, urine is mainly used for the detection of diagnostic biomarkers (755) followed by prognostic (83), monitoring (74) and predictive (34) biomarkers (Table 1). Additionally, research demonstrated acceptability and feasibility (7), biomarker discovery (5), and protocol or method development and/or optimization (78).

Many analytes are detected in urine, like cell-free or cellular DNA and RNA, extracellular vesicles (EVs), proteins and metabolites—demonstrating the potential of urine as a multi-omic sample type, covering genomics, epigenomics, transcriptomics, proteomics, metabolomics and metallomics. For more details see section “Potential of urine in different cancers”.

Table 1: Cancer incidences, diagnoses and biomarker types

Cancer type	Incidence (%)	Diagnoses/ year (million)	Publications	Biomarker type			
				Diagnostic (%)	Monitoring (%)	Prognostic (%)	Predictive (%)
Urothelial cancer	3.0	0.6	428	82.2	11.0	11.0	2.1
Prostate cancer	7.3	1.4	245	85.3	1.2	7.3	3.3
Cervical cancer	3.1	0.6	62	85.5	3.2	1.6	0.0
Endometrial cancer	2.2	0.4	4	75.0	0.0	0.0	0.0
Ovarian cancer	1.6	0.3	16	81.3	0.0	12.5	6.25
Vulval cancer	0.2	0.05	0	N/A.	N/A.	N/A.	N/A.
Vaginal cancer	0.1	0.02	0	N/A.	N/A.	N/A.	N/A.
Colorectal cancer	6.0	1.2	33	66.7	15.2	6.1	9.1
Gastric cancer	5.6	1.1	8	100.0	12.5	0.0	0.0
Hepatocellular carcinoma	4.7	0.9	10	60.0	10.0	20.0	0.0
Pancreatic cancer	2.6	0.5	8	100.0	0.0	0.0	0.0
Cholangiocarcinoma	0.6	0.1	3	100.0	33.3	0.0	0.0
Lung cancer	11.4	2.2	37	64.9	16.2	10.8	16.2
Breast cancer	11.7	2.3	24	70.8	20.8	12.5	8.3
Renal cell carcinoma	2.2	0.4	18	83.3	5.6	11.1	5.6
Head and neck carcinoma a	N/A.	0.9	2	50.0	50.0	0.0	50.0
Thyroid cancer	3.0	0.6	3	66.7	33.3	0.0	33.3

Note: The Incidence percentages and Diagnoses/year are estimated for 2020[21]. The publications are the systematic literature review results. The percentages of Biomarker types are calculated based on the total amount of publications of that cancer type. When publications are categorized under more than one type they are counted for each category. a Head and neck carcinoma is a group of cancers; therefore, the incidence percentage is not given, and the diagnoses/year are the sum of all cancers categorized under head and neck cancer.

3.2.2. Preanalytical parameters (urine handling, type, and storage)

Information was extracted about pre-analytical parameters, including time of collection, pre-collection handling, type of urine, preservation methods, collected volume and method, of the 924 publications (Table 2, Table S1). Most publications (73.8%) did not report information about a pre-analytical parameter of their samples, and almost 25.0% did not report any pre-analytical parameter, although multiple studies show the importance of standardization of sample collection, preservation, and storage[4, 5, 15-17]. When mentioned, most publications used ethylenediaminetetraacetic acid (EDTA) or a commercial method for sample preservation. Urine is a suitable matrix for the activity of nucleic acid-hydrolyzing enzymes, since it contains potassium, calcium, magnesium, sodium, and zinc and its pH is between 5.0 and 7.0. Several nucleic acid-hydrolyzing enzymes, including DNase I, DNase II, RNase I, RNase II and phosphodiesterase are present in urine, of which DNase I and DNase II even have a higher activity in urine compared to their activity in blood[5]. Fifty-two studies explored the addition of EDTA for reducing DNA nuclease activity, including the Hernandez-Lopez study[18]. The authors demonstrated significantly better results when using 40 mM EDTA solution compared to no EDTA or 10 mM EDTA solution[18]. Hence, it is important to consider the EDTA concentration when analyzing urinary biomarkers. Additionally, protein stability can be significantly affected by urine collection, preparation, and preservation methods[19]. Of note, 10 publications mentioned only adding a preservative in the lab and 19 publications mentioned the use of commercially available urine collection methods. Depending on the urinary biomarker evaluated, studies either show that urine does not contain the same components at all time of day or in any fraction[1, 20].

Table 2: Overview of specified preanalytical parameters of urine sample collection

TIME OF URINE COLLECTION	- 712 publications did not disclose information (77.1%). - 213 publications did disclose information (23.1%). • 64 publications used first morning urine (30.0%). • 21 publications used second, third, non-first morning urine (9.9%). • 14 publications specified information about fasting (6.6%).
PRE-COLLECTION HANDLING	- 667 publications did not disclose information (72.2%). - 257 publications did disclose information (27.8%). • Pre-collection handling included information on before, during or after physician or surgical actions, including DRE, cystoscopy, surgery, and biopsy.
TYPE OF URINE	- 648 publications did not disclose information (70.1%). - 278 publications did disclose information (30.1%). • 119 publications used first-catch urine (42.8%). • 102 publications used midstream urine (36.7%). • 17 publications used full void urine (6.1%).
PRESERVATION	- 763 publications did not disclose information (82.6%). - 162 publications did disclose information (17.5%). • 63 publications used a commercially available preservative (38.9%). • 54 publications used EDTA, in different concentrations (33.3%). • 10 publications only added a preservative in the lab (6.2%).
COLLECTED VOLUME	- 566 publications did not disclose information (61.3%). - 358 publications did disclose information (38.7%). • Collected volumes ranged from 1 mL to 750 mL.
COLLECTION METHODS	- 740 publications did not disclose information (80.0%). - 185 publications did disclose information (20.0%). • 80 publications used sterile collection methods (43.0%). • 19 publications used specialized commercially available collection methods [incl. Norgen Biotek Corp, Sierra Diagnostics Inc., Novosanis, Biodyne®, Hologic Inc., Exosome Diagnostics, etc.] (10.2%).
COMBINED INFORMATION	- 550 publications did not disclose information on the type of urine, nor the preservation method used (59.5%). - 516 publications did not disclose information on the timing nor the type of urine (55.8%). - 439 publications did not disclose information on the timing nor the type, nor the preservation method used (47.5%). - 334 publications did not disclose information on the timing nor the type, nor the volume of urine collection (36.1%). - 223 publications did not disclose information on any of the pre-analytical parameters mentioned above (24.1%).

Note: The literature search (described in Section2) yielded 924 publications. Information was extracted about preanalytical parameters such as time of collection, pre-collection handling, type of urine, preservation methods, collected volume and collection method. The main percentages are calculated against the total number of publications. When publications mentioned more than one (sub)parameter they are counted for each (sub)parameter. The second bullet percentages are calculated against the defined number above. Abbreviation: DRE, digital rectal examination.

3.3. Potential of urine in different cancers

3.3.1. Urological cancers

Urological cancers originate in the male and female urinary tract organs. The most common urological cancers are prostate cancer (PCa) and bladder cancer (mostly ~ urothelial cancer)[21] (Table 1). Previous literature reviews clearly demonstrate the clinical utility of urine-based biomarkers for urothelial and PCa[22-26]. Several FDA-approved or CLIA tests are available for urothelial and PCa (Table 3). Therefore, only findings about preanalytics will be further discussed in more detail.

3.3.2. Urothelial cancer

Out of the 18.5% of 428 publications in which the type of urine used as liquid biopsy for urothelial cancer was described, 64.6% used midstream urine. Different analytes have been investigated for urothelial cancer, including cells, DNA (cell-free and cellular), exosomes, metabolites, proteins and RNA (cell-free and cellular). Almost 30.0% of the publications used proteins for their research (Table S1).

Table 3: Commercially available urine tests and assays for cancer detection

Cancer	Analyte	Target	Test	Company	Available as
Bladder	Peptides	NMP22	Alere NMP22 BladderChek	Abbott	FDA approved
					CE-marked
	DNA	Aneuploidy for chromosomes 3, 7, 17, and loss of the 9p21 locus	UroVysion	Abbott	FDA approved
					CE-marked
	Proteins	Human complement factor H related protein	BTA stat	Polymedco	FDA approved
					CE-marked
	Proteins	Human complement factor H related protein	BTA Trak*	Bard Diagnostics Sciences, Inc.	FDA approved
					CE-marked
	mRNA	IGFBP5, HOXA13, MDK, CDK1 & CXCR2	CxBladder (Detect, Triage, Monitor)	Pacific Edge Diagnostics	CLIA-approved LDT
	mRNA	UPK1B, IGF2, CRH, ANXA10, ABL1	Xpert® Bladder Cancer Detection	Cepheid	CE-marked
	Tumor cell DNA	FGFR3 & TERT promotor mutations	Uromonitor	IPATIMUP	CE-marked
Prostate	mRNA	HOXC6, DLX1 & PSA (KLK3)	SelectMDx	MDxHealth	CLIA-approved LDT
	Exosomal RNA	ERG, PCA3, SPDEF	ExoDx Prostate (IntelliScore)	Exosome Diagnostics	CLIA-approved LDT
	lncRNA	PCA3	Progensa PCA3	Hologic Inc	FDA approved
	RNA	TMPRSS2-ERG & PCA3, Serum PSA	MyProstate-Score (MPS)	LynxDX	CLIA-approved LDT

Note: A summary of the commercially available urine tests and assays for bladder and prostate cancer. Abbreviations: CE, European Conformity; CLIA, Clinical Laboratory Improvement Amendments; FDA, U.S. Food and Drug Administration; LDT, laboratory-developed test. a Discontinued.

Four studies[27-30] have investigated different urine fractions for the detection of several analytes in urothelial cancer. These studies show that full void urine, containing both cellular and cfDNA, can be used for DNA methylation analysis in patients, with the best diagnostic performance for the biomarker panel GHSR/MA (92.0% sensitivity, 85.0% specificity)[27]. Because researchers first assumed that DNA hypermethylation in urine is a consequence of the direct shedding of bladder tumor cells into the urine, most studies isolated DNA from cell pellets. One study found that epigenetic changes in the noncoding repeat sequence LINE1 were found to be comparable between cfDNA and cellular DNA in urine of patients[30]. However, two studies showed that cfDNA from urine supernatant (74.0%-90.0% sensitivity) was more sensitive for the detection of tumor-derived copy number alternations, loss of heterozygosity, and somatic mutations than cellular DNA from urine sediment (61.0%-68.0% sensitivity)[28, 29]. One study evaluated which urine fraction (full void, urine supernatant or urine sediment) is preferable for the diagnosis of urothelial cancer using methylation markers. This study showed that (a) there was a significant correlation between the DNA methylation levels in each of the fractions and the matched tumor tissue, and (b) the area under curve (AUCs) of the different markers were comparable among fractions. They concluded that both cellular and cfDNA in urine can be used for DNA methylation analysis[31]. Another research group compared microRNAs (miRNAs) in urine supernatant and sediment, showing that supernatant-derived miRNAs seemed more reflective of tumor burden than sediment.

3.3.3. Prostate cancer

Urinary biomarkers for PCa have been investigated in 245 publications in our search. Although information about the type of urine or preservation method was only mentioned in 23.6% of publications, 12.7% used commercially-available preservation methods and RNA was the most commonly used (45.0%; Table S1). The biological connection between the prostate and the urethra via the prostatic ducts forms the foundation for urine as a sample type for PCa detection. However, the precise nature of how urinary PCa cells and cfDNA are released into urine is not fully understood. If the release is largely due to the shedding of prostate cells and cfDNA during proliferation or the exfoliation of PCa cells entering urine by execution of a digital rectal examination (DRE) during prostate massage, one would expect that first-catch urine is the most interesting fraction[26]. Our search showed that 29.0% used first-catch urine and 3.3% used midstream urine for

their research (Table S1). Additionally, it was demonstrated that nucleic acids for the detection of PCa are found in the cellular (sediment), in the extracellular (supernatant) fraction of urine, and within EVs[32].

A DRE is often performed in studies for biomarkers before urine collection to increase the amount of prostate-derived material. The impact of DRE on different analytes has been demonstrated across numerous studies and has been shown to increase the number of detectable biomarkers, facilitating their detection[26, 33-35]. A recent study evaluated the impact of DRE on prostate biomarkers in different urine fractions (full void, cell pellet, and exosomes). They showed that biomarker levels were significantly higher (10-fold) after performing DRE for all tested biomarkers in each urine fraction[33]. However, other studies have shown that urinary biomarkers are sufficiently present without DRE[36-38]. A gene signature derived from normalized PCA3 and ERG RNA expression in exosomes from urine, was predictive for PCa without the need for DRE before urine collection36. Another study showed no significant difference in cellular DNA methylation levels between urine collected before or after DRE37. Interestingly, our search showed that already 59.6% provided information about DRE, of which 83.6% used post-DRE, 17.1% non-DRE and 4.1% pre-DRE urine (Table S1). Together, these findings show that it is unresolved whether a DRE must be performed before urine collection. However, this process adds variability and should be kept in mind and reported. The omission of DRE in the urine collection process would be advantageous for standardization of the collection procedure and more acceptable to patients for routine monitoring.

3.3.4. Gynecological cancers

Gynecological cancers originate in woman's reproductive organs: the vulva, vagina, cervix, endometrium, and ovary. Cervical cancer (CC), endometrial cancer (EC), and ovarian cancer (OC) are the most common gynecological cancers in women, whereas vulvar and vaginal cancers are rare[21] (Table 1). Our search included 82 unique publications on gynecological cancers, including CC, EC, and OC. Our research results did not identify studies on vulvar or vaginal cancers. Most publications investigated Human Papillomavirus (HPV) for CC, followed by proteins, DNA, metabolites, and RNA. Of the 25 publications mentioning the preservation method, 14 mentioned using a commercially available method and 6 used EDTA as a preservative. Interestingly, all studies using a commercially

available preservation and/or collection method investigated HPV as a biomarker for CC (Table S1).

3.3.5. Cervical cancer

A recent updated meta-analysis of urinary HPV in CC research demonstrated that, for the clinical applications of urinary HPV detection, there are four considerations: the usage of (a) first-catch urine and collected with appropriately designed devices, (b) a preservative to avoid degradation during extraction and storage, (c) polymerase chain reaction (PCR)-based assays, and (iv) a sufficient volume of urine[39]. Therefore, we will only discuss the findings on pre-analytics from our search.

Recent evidence confirmed that ctDNA from CC in urine results from natural shedding as well as from glomerular filtration[11]. One group evaluated different urine fractions (full void, urine supernatant, and urine sediment) for high-risk HPV (hrHPV) detection and DNA methylation analysis in two studies. They found (a) strong correlations (r>0.60) between the different urine fractions, (b) a strong agreement between cervical scrapes and urine sediment for both hrHPV detection (kappa 0.85, 95.0% Cl: 0.64-1.00) and DNA methylation (correlation coefficient 0.509-0.717), (c) methylation levels increasing significantly with severity of underlying disease in all fractions, (d) that all markers in all fractions were significantly different between patients and controls, and (e) a high discriminatory power of six DNA methylation markers in urine[40, 41].

Additionally, next to the difference in supernatant and sediment, a distinction can be made between first-catch and midstream. A systematic review and meta-analysis on urinary HPV DNA for the detection of CC showed that (a) urine detection of any HPV had a pooled sensitivity of 87.0% (95.0% Cl: 78.0%-92.0%) and specificity of 94.0% (95.0% Cl: 82.0%-98.0%) and (b) there was a 22-fold increase in overall accuracy when samples were collected as first-catch urine compared to random or midstream urine samples[42]. Another group showed that higher concentrations of hrHPV and human DNA were found in first-catch urine. They additionally showed the need for a preservative to prevent DNA degradation[43-45]. Given these findings, it is important to use a preservation method and refrain from extensively washing the genitals before collecting the most preferred, first-catch urine sample. Our results demonstrated that 60.0%

of the publications used first-catch urine; however, only 11.3% reported that women were instructed to not wash their genitals extensively before collection, and 64.5% didn't specify preservation (Table S1).

Different studies evaluated HPV DNA-based PCR platforms, such as the BD Onclarity HPV assay[46], the Roche cobas 4800 HPV assay[47, 48], and the Abbott RealTime High Risk HPV assay as part of the VALHUDES study (NCT03064087)[49, 50] and CoCoss-Trial[51] on urine samples showing non-inferior results to clinician-collected samples and/or vaginal self-samples. Currently, no HPV high-throughput assay has been CE-marked or FDA-approved for use on first-catch urine, despite the development of collection devices, the identified need for preservation, and the reported preference for urine as sample type in CC screening[47, 52-56].

3.3.6. Endometrial cancer

Currently, endometrial curettage is performed for the diagnosis of EC. In this process, cervicovaginal samples can also be collected. Additionally, urine can contain cfDNA derived from the bloodstream that passed renal filtration. Understanding this, DNA methylation markers for EC were explored in different urine fractions (full void urine, urine sediment, and urine supernatant) to determine the most suitable fraction. A strong to very strong correlation (r=0.77-0.92) was shown among the urine fractions, with the best diagnostic potential in EC for full void[57]. Urine sediment MCM5 has the potential to distinguish EC cases from benign gynecological disease with 87.8% sensitivity and 75.9% specificity[58].

3.3.7. Ovarian cancer

Biologically similar findings are expected for OC as for CC and EC, since the ovaries are connected by the cervix and endometrium via the fallopian tubes. However, no studies from our search investigated the origin of urinary analytes or different urine fractions for OC.
Twelve studies explored the diagnostic and predictive potential of proteins and metabolites in urine for OC[58-69]. Four studies show the potential of DNA methylation profiles, miRNA expression and exosomes in urine for detection[70-73]. Although all these studies showed good performance, almost none specified which urine fraction or preservation method, if any, they used. Currently, there is no screening strategy for OC. However, screening using urinary biomarkers could be considered based on the promising results.

3.3.8. Gastrointestinal cancers

Gastrointestinal cancers refer to malignant conditions of the gastrointestinal tract and accessory organs of digestion, including the esophagus, stomach, intestines, pancreas, liver, and gallbladder. Colorectal cancer (CRC), gastric cancer, hepatocellular carcinoma (HCC), pancreatic cancer, and cholangiocarcinoma came back in our search. In 2020, it was estimated that the combination of these five cancers would lead to 3.8 million yearly diagnoses worldwide21 (Table 1).

Our search yielded a total of 63 unique publications looking into urine and gastrointestinal cancers, including CRC, HCC, pancreatic cancer, gastric cancer, and cholangiocarcinoma. For gastrointestinal cancers research, the type of urine was not specified in 68.3% of the publications, while 20.6% used midstream urine. For preservation, 23.8% researchers mentioned the use of EDTA, while 68.3% didn't mentioned anything about the sample preservation. Some interesting findings were that (a) midstream urine was used for research in 37.5% of gastric and 37.5% of pancreatic cancer publications, (b) half of the publications used EDTA in the case of DNA research, and (c) the most investigated analytes were DNA and metabolites. Highly represented detection techniques for metabolites were mass spectrometry (MS) techniques, high-performance liquid chromatography (HPLC) approaches, and proton nuclear magnetic resonance (H-NMR); moreover for DNA, these were qPCR, digital droplet PCR (ddPCR), and methylation-specific PCR (Table S1).

3.3.9. Colorectal cancer

Various studies investigated urinary ctDNA for CRC detection and monitoring[74-82]. Urinary ctDNA was shown to have 64.0% sensitivity and 100.0% specificity for molecular residual disease detection[74]. Three groups showed that urinary ctDNA carries KRAS mutations similar to tumor tissue, with a sensitivity of 90.7% and specificity of 82.0%; furthermore, this urinary ctDNA has potential for disease monitoring and prognosis, since urinary ctDNA KRAS levels decreased after surgery in patients with bone metastases[75-77]. Moreover, great concordance (89.0%-91.0%) between tissue- and urine-based DNA testing was shown[75, 79, 81]. Other studies investigated DNA methylation levels in urine for CRC detection, with sensitivities between 73.0% and 88.0% and specificities between 60.0% and 86.0%[83-87].

Oxidative stress markers, proteins, and metabolites have been explored for their potential in CRC detection, with promising results so far (sensitivity up to 89.9%, specificity up to 92.0%)[88-102]. Several groups have developed different methods for the detection of oxidative stress markers[90, 91, 94] or volatile organic compounds[103-105] in urine, elucidating their potential for CRC detection.

3.3.10. Hepatocellular carcinoma

Five studies investigated urinary DNA biomarkers for HCC[106-110]. Random forest, a machine learning technique, and a two-step model were demonstrated as possibilities for developing a panel of multiple biomarkers for HCC[108]. The potential of a novel TP53 249T assay and the detection of CTNNB1 mutations in urinary cfDNA as a predictive biomarker for HCC recurrence were demonstrated[109, 110]. Furthermore, the potential of miR-618 (sensitivity, 64.0%; specificity, 68.0%) and miR-650 (sensitivity, 72.0%; specificity, 58.0%) were shown in detecting HCC in hepatitis C virus (HCV)-positive individuals[111]. Additionally, the same group investigated the over-expression of three proteins: DJ-1, chromatin assembly factor-1 (CAF-1), and heat shock protein 60, as a characteristic event among HCC post-HCV-infected patients. They showed that CAF-1 identified HCC among HCV-infected patients with a sensitivity of 66.0% and specificity of 90.0%[112]. Two studies also investigated proteins as a biomarker for HCC[113, 114].

3.3.11. Pancreatic cancer

KRAS mutations could be detected in urine and therefore could lead to a diagnosis of pancreatic cancer[115]. Two studies showed the diagnostic and prognostic potential of miRNAs[116, 117], of which miR-1246 even showed a higher AUC as well as sensitivity and specificity in urine (AUC, 0.90; sensitivity, 90.2%; specificity, 83.3%) compared to serum (AUC, 0.87; sensitivity, 92.3%; specificity, 73.3%)[116]. Additionally, proteins and metabolites have been investigated for their potential in pancreatic cancer detection and monitoring[118-120] and, therefore, analysis techniques have been optimized or developed[121, 122].

3.3.12. Gastric cancer

LINC00365, a long noncoding RNA (lncRNA), and its target are differentially expressed in patients compared to controls and are predicted to be urine-excretory[123]. One study demonstrated the feasibility of urinary miRNAs for

gastric cancer detection[124]. Additionally, the excellent performance (AUC=0.885) of a panel combining urinary miR-6807-5p, miR-6856-5p and serum H. pylori status was demonstrated. This panel could distinguish between healthy controls and stage I gastric cancer (AUC=0.748) and the urinary expression of the miRNAs significantly decreased after curative resection[125]. Proteins and metabolites have been investigated for their diagnostic potential for gastric cancer[126-129]. Moreover, the microbiome and bacterial-derived EVs have been investigated in stool, urine, and serum samples to demonstrate their potential[130].

3.3.13. Cholangiocarcinoma

Proteins[131], nucleosides,[132] and metabolic profiles[133] have been investigated as potential urinary biomarkers for cholangiocarcinoma. Further research is needed to prove their potential.

3.3.14. Lung cancer

Lung cancer is the second most frequently occurring cancer with an estimate of 2.2 million diagnoses worldwide in 2020[21] (Table 1). During our search, publications on lung cancer were divided into non-small-cell lung cancers (NSCLC) and all other lung cancers. Urinary biomarkers for lung cancer were investigated in 37 publications from our search, of which 30.0% used midstream urine. Several analytes were investigated, including different types of DNA, proteins, and metabolites. When DNA was investigated, in 58.8% of the cases, researchers used EDTA as preservative with concentrations between 10mM and 0.5M. The isolation and detection methods used were highly dependent on the analyte of interest (Table S1).

Various studies demonstrated the potential of ctDNA analysis for risk profiling, disease management, or monitoring of NSCLC[134-148]. Good concordance between urinary cfDNA and matched plasma (70.0%) or tissue biopsies (84.0%-93.0%) was shown in patients with all different stages of lung cancer138-143. The potential of cfDNA targeting ALU repeats[134] and LINE1 fragments[135] at different sizes was demonstrated for the diagnosis of lung cancer. Differences in genetic profiles of cfDNA were investigated in sputum (74.0%), plasma (86.0%), and urine (70.0%), a good correlation and complementarity to tissue was demonstrated[148]. Additionally, the combination of plasma, sputum and urine was shown to increase the detection of EGFR or TP53 mutations[136].

Three studies investigated miRNA and lncRNA, showing that both analytes could be useful in NSCLC detection and monitoring[149-151]. The diagnostic potential of proteins[152-157] and metabolites1[58-160] for lung cancer was evidenced in multiple studies. Urinary midkine[157], N1, N12-diacetylspermine[161] and collagen XXII[162] were shown to separately have diagnostic and prognostic potential. Two studies elucidated the effect of oxidative stress on lung cancer pathogenesis[163, 164].

A literature review about urinary biomarkers for early diagnosis of lung cancer, including publications from 2010-2020, was performed by Gasparri et al.[165]. They showed that: (a) there are no clinically available validated urinary biomarkers, (b) many promising research projects have been performed over the past decade, and (c) there is a lack of a standard approach which limits the scientific robustness[165].

3.3.15. Breast cancer

Breast cancer (BCA) is, with an estimated 2.3 million diagnoses in 2020, the most common cancer[21] (Table 1). Our search yielded 24 publications pertaining to urinary biomarkers in BCA, covering all different types of analytes. About 41.6% of the publications were investigating metabolites in urine for the detection of BCA. Despite mentioning the analytes, most researchers didn't disclose information regarding the type of urine, or the preservation method used; however, 33.3% mentioned using midstream urine. The studies investigating urinary metabolites or proteins mostly used solid-phase extraction methods for isolation and forms of MS or HPLC for detection. For urinary DNA, most researchers used a commercially available isolation kit from QIAGEN and ddPCR as detection method. Urinary biomarkers look really promising, with sensitivities between 66.7% and 98.6% and specificities between 66.1% and 100.0% (Table S1).

Three studies have shown a great concordance (97.2%-97.3%) between urinary ctDNA and tissue biopsy [166-168]. Additionally, they showed that ctDNA could be used for routine monitoring for both disease progression and relapse[166-169]. Two independent groups demonstrated that the concentration of 8-Oxo-7,8-Dihydro-2'-Deoxyguanosine, an oxidative DNA damage biomarker, was significantly different between early-stage BCA and non-cancer controls (benign breast diseases and healthy controls)[170, 171]. Another group explored the diagnostic potential of urinary EV miRNAs and found that miRNA panels or profiles could

distinguish between BCA patients and healthy controls with 91.7% to 98.6% sensitivity and 91.7% to 100.0% specificity[172, 173].

Several studies have suggested the potential of urinary proteins and metabolites for research[69,174-182] and optimized and developed techniques for their analysis[183-187]. One study showed that 59 urinary proteins are differentially expressed in patients compared to healthy controls, and of them, 36 clinical stage-specific. They identified a panel of 13 upregulated proteins to be used for detection[174]. Another study demonstrated an improved diagnostic sensitivity (AUC=0.973) when combining serum C15-3 with 1-methyladenosine, 1-methylguanosine, and 8-hydroxy-20-deoxyguanosine[175].
The association of endogenous estrogens and their metabolic pathway with carcinogenesis for estrogen-induced BCA risk was shown[176], while the effect of exemestane treatment on estrogen generation was investigated in patients[177]. A technique was developed to separate and detect six urinary pteridines[183], which was used to demonstrate that isoxanthopterin and xanthopterin were significantly higher in patients than in healthy controls[178]. In 2012, the volatile organic metabolomic (VOM) profile of BCA patients was compared to healthy controls using solid-phase microextraction combined with gas chromatography-MS. Six VOMs were found to be statistically different between patients and healthy controls[179]. In 2019, this analysis technique was optimized and it showed the possibility of identifying endogenous metabolites as a platform to discover potential BCA biomarkers[184]. Additionally in 2019, H-NMR was investigated as a powerful approach to identify urinary metabolomic patterns in BCA patients[180].

3.3.16. Renal cell carcinoma

Renal cell carcinoma (RCC) is more common in men (tenth most common) than in women (fifteenth). Worldwide, 0.4 million new diagnoses were estimated to occur in 2020[21] (Table 1). RCC and urine were the subjects of 18 publications, most of them not specifying which type of urine (72.2%) or which preservation method (88.9%) was used. Several types of analytes were investigated, the majority were RNA (33.0%) and proteins (28.0%). The studies about urinary RNA mostly used commercial kits for isolation and reverse transcription PCR (RT-qPCR), as the detection technique. The studies investigating urinary proteins used various detection techniques ranging, from MS to ELISA (Table S1).

Although it was shown that RCC is a cfDNA-low malignancy[188], other studies showed that significantly higher urinary DNA methylation frequencies were found in RCC patients compared to controls, with 69.0% to 78.0% sensitivity and 69.0% to 80.0% specificity for different applications[189, 190]. The difference between the cell-free RNA (cfRNA) integrity index of RCC patients and controls was investigated, showing that the cfRNA index of GAPDH was significantly lower in urine of patients when compared to controls. However, the small-sized cfRNA fragment of the tumor-related VEGF mRNA was more abundant in cancer patients[191]. Five studies investigated miRNAs for RCC, showing that single or combined miRNAs are higher in patients compared to controls. This supported their diagnostic and prognostic potential since the miRNA levels decrease after surgery or in disease-free patients during follow-up[192-196]. Various groups have developed or optimized techniques to perform urinary proteomic[197-200] or volatile metabolic[201, 202] analysis for RCC. Most studies focused on peptides panels to discriminate between patients and healthy volunteers[198, 199, 203], while one study focused on a single protein, Survivin[197].

3.3.17. Other cancers: Head and neck carcinoma and thyroid cancer

Our search yielded two publications for head and neck carcinoma (HNC) and three publications for thyroid cancer (THCA). In 2020, it was estimated that 0.9 million people would be newly diagnosed with HNC. While, for THCA, the tenth most common cancer, 0.6 million new diagnoses were estimated to occur in 2020[21] (Table 1). Metabolites (40.0%) are the main analytes investigated in these two cancers[204, 205]. Our search showed that none of the publications specified the pre-analytical parameters used (Table S1).

LC-MS proteomic analysis can be used for monitoring treatment by detecting changes in the urinary proteome. Furthermore, it was shown that specific proteins in urine could be followed to differentiate one cancer from another. Retinol-binding protein 4 was shown to discriminate between patients suffering from HNC and patients with THCA[206]. Another study showed the power of urinary modified nucleosides patterns to discriminate between healthy volunteers and HNC patients[204].

Urinary exosomal thyroglobulin was investigated as a substitute for serum thyroglobulin, and it was demonstrated that urinary exosomal thyroglobulin

could be used to eliminate the suspicion of THCA recurrence better than existing serological tests[207]. Another study showed that urinary steroids were upregulated in THCA patients compared to controls. Furthermore, this research investigated the pathogenic differences in THCA according to gender and menopausal conditions[211]. The retention and excretion rates of radioiodine were explored in THCA for treatment follow-up[208].

3.3.18. Pan-cancer

Although all cancers are molecularly distinct, many share common driver and risk factor-associated mutations[209]. Therefore, a separate Pan-cancer category was included in this research. In this research, the Pan-cancer category captures the publications investigating analytes of interest with applications in several cancer types. The most well-known Pan-cancer project is The Cancer Genome Atlas[210].

We classified 18 publications in our Pan-cancer category. However, most didn't disclose any information regarding the type of urine or the preservation method used. Five (27.8%) of 18 studies discussed using urine as the source of metabolites[211-215], four (22.2%) studies reported on proteins[215-218], three (16.7%) on DNA[219-221], three (16.7%) on oxidative stress markers[222-224], two (11.1%) on RNA[225, 226], and two (11.1%) on metals[227, 228] (Table S1). These 18 publications showed some promising diagnostic urinary biomarkers for Pan-cancer applications. However, further research is necessary.

Future considerations and recommendations

04

Urine as a liquid biopsy for cancer biomarker detection is an interesting field in which a lot of research is ongoing. Therefore, we propose below that several aspects should be considered and improved upon to help further advance urine's utility as a non-invasive sample type for cancer detection.

4.1. Variability

Urine is heterogeneous among individuals. Urine is a dynamic body fluid, meaning its composition changes with lifestyle and hydration status, renal pathology, urine volume, and medication use. Therefore, studies involving urine should consider the effect of urine collection timing (e.g., first-morning versus later in the day) and the effect of urine fraction (full void versus first-catch versus midstream) as discussed before (Section 3.3 "Potential of urine in different cancers"). Additionally, since certain potential biomarkers need to cross the renal barrier before ending up in the urine, their concentration can be lower than in plasma[82, 229, 230]. Currently, most studies are difficult to generalize due to the small samples sizes and the lack of well-defined, appropriate control groups. It is strongly recommended to include healthy individuals and/or appropriate patient cases.

4.2. Variability

Many researchers don't disclose all or sufficient information about sample collection, storage, and handling, while several publications have already shown these parameters affect the results. In our analysis, no significant improvement

was observed over the last 10 years for such parameters involving urine type, preservation method, or both (Figure 3). Furthermore, researchers use different ways to normalize their results, adding additional variability in sample processing. Various isolation, extraction, detection, and analysis methods are available and used interchangeably for all different analytes. An interesting innovation in this field is the development of microfluidic methods to aid in the separation and detection of certain analytes[231], with test cases already available for bladder[232], prostate,[233] and gastric[234] cancer.

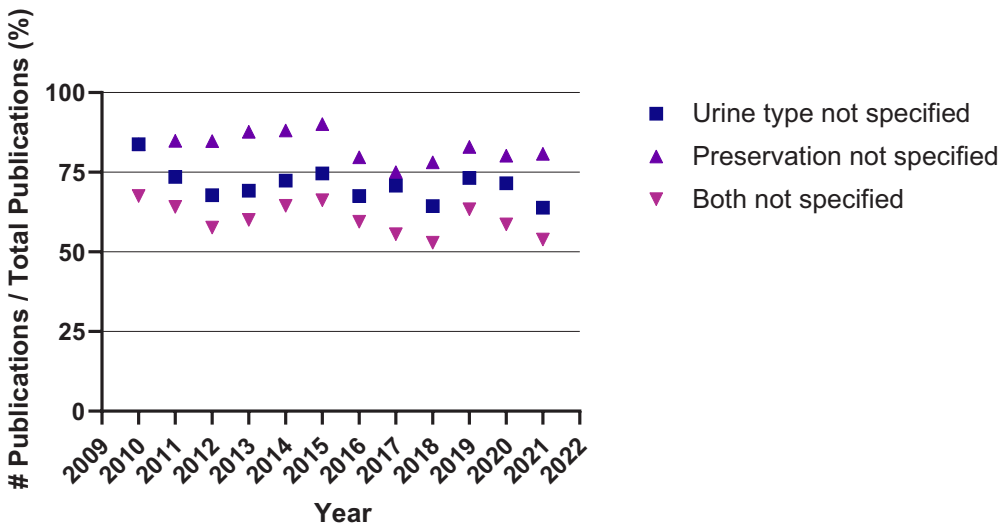

Figure 3: Reporting of preanalytics over time. For every publication in our search, data was extracted on the reporting of preanalytical parameters, including the type of urine and the preservation method used. The percentage of publications not specifying the type of urine, preservation method or either was calculated for each year of publication separately, to show the evolution of reporting these preanalytical parameters over time; 2022was excluded from this graphical representation since there were only two publications with this year of publication.

Researchers should report all information properly and fully, elaborating further on uniform test conditions to yield reproducible and reliable results needed towards optimal protocols for urinary biomarkers. Given that biomarker discovery and validation rely on proper sample handling and processing, we recommend using a standard parameter checklist or template (e.g., see Table 4) when reporting on research using urine as liquid biopsy.

4.3. The road toward the clinic

The Early Detection Research Network (EDRN), established by the National Cancer Institute (NCI), defined a structure to guide the process of biomarker development. They categorized the process into five phases a biomarker needs to pass through to produce a useful and valid population-screening tool[235]. Urine as liquid biopsy is a new and continuously evolving. Therefore, the currently published results are not exhaustive and the discoveries so far cannot yet be applied in daily clinical practice. Most active studies are in phase 1 or 2 and the full process of bringing a potential biomarker to the clinic is not yet complete. For bladder and PCa, several biomarker assays are commercially available as FDA-, EMA- or CLIA-approved methods. For CC major progress has been made, WHO and HRP have even suggested in their new guidelines that self-collected samples, such as urine, can be used when providing HPV DNA testing. Studies have shown that women often feel more comfortable taking their own samples and this could increase the screening participation rate[236]. Still, the road towards the clinic for urine requires the further discovery and validation of potential biomarkers.

Table 4: Parameter checklist, recommendations and templates

Protocol Categories	Examples
Pre-analytical parameters	
Time of urine collection	First morning, second morning, after fasting, …
Pre-collection handling	Urine collection before, during or after cystoscopy, DRE, needle biopsy, surgery, ….
Type of urine	First-catch, midstream, full void, spot urine, …
Urine fraction	Sediment, supernatant, full void, …
Preservation method	Commercially available preservatives with company details, chemical substances with concentration, without preservation, …
Collected volume	The average collected volume, the urine volume used for experiments, …
Collection method	Specific devices, urine cup, …
Research parameters	
Analyte of interest	cfDNA, cfRNA, cellular DNA, EV, lncRNA, metabolites, miRNA, proteins, …
Isolation method	Commercially available kits with company details, in-house protocols explained, …
Detection method	Commercially available assays with company details, in-house methods explained, …
Normalization method	24-hours urine, creatinine ratios, no normalization, …
Comparators	Comparator sample types (e.g., blood, tissue, …)
	Comparator populations (e.g., healthy volunteers, other diseases, disease stages, …)
	Comparator methods (e.g., gold standard of care methods, …)
Recommended parameter template text	
For this study [insert time of urine collection], [insert pre-collection handling], [insert type of urine] urine was collected using [insert collection method including brand names], [insert preservation method including brand names] from [insert comparator populations]. [Insert comparator sample types or methods] were collected as comparator.	
All participants were asked to collect [insert type of urine], [insert pre-collection handling]. The urine samples were collected using [insert collection method], [insert preservation method]. [Insert comparator samples types or methods].	

Note: A checklist of all preanalytical and research parameters recommended to report properly in publications regarding urine as liquid biopsy. Some examples and recommendations are given for each parameter. Additionally, some templates are provided for article writing. Abbreviations: cfDNA, cell-free DNA; cfRNA, cell-free RNA; DRE, digital rectal examination; EV, extracellular vesicles; lncRNA, long noncoding RNA; miRNA, microRNA.

Limitations of the study

05

Our study had some limitations. First, a meta-analysis based on sensitivities and specificities for urinary biomarkers was not performed due to the high variability in cancer types, analyte types, isolation and detection methods, and the lack of reporting of pre-analytical parameters. Secondly, information on the use of normalization methods or the need for them was not extracted from the resulting publications, due to the high variability in analytes and the undetermined need for normalization.

Conclusion

06

Urine is clearly an emerging liquid biopsy with relevance for a broad range of cancers. Additionally, urine can be used as a multi-omic sample type, covering DNA, RNA, proteins, metabolites, and essential metals. However, standard practice protocols for sample collection, storage, and processing need to be determined to elaborate further on the clinical utility of urine for cancer research, early detection, screening for rare cancers, and disease and treatment monitoring.

References

07

All publication information of the 924 publications included this systematic review are mentioned in **Supplementary Table 1**.

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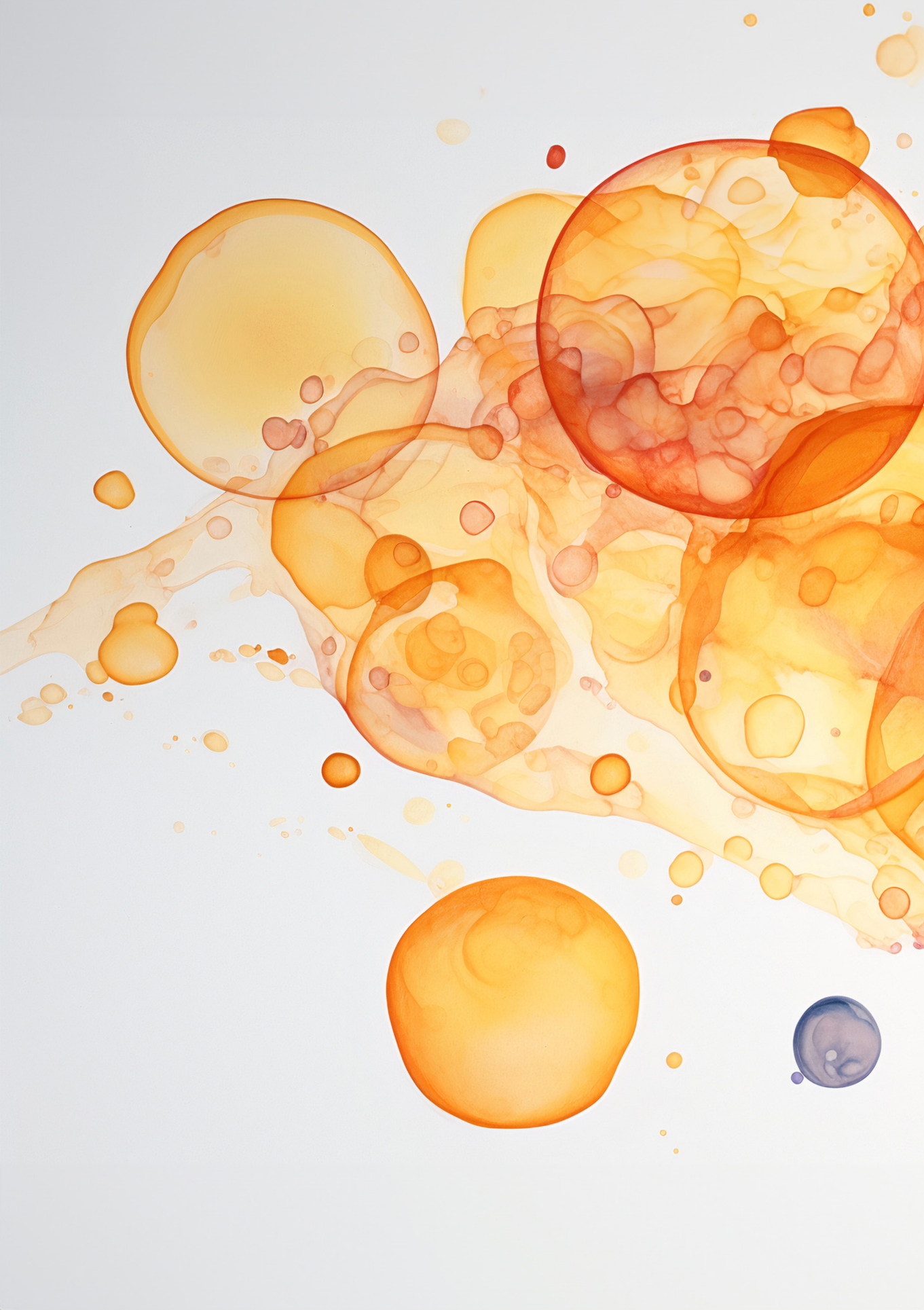

PART II

Urine as liquid biopsy for oncology applications

CHAPTER

04

UAS™ - a urine preservative for oncology applications

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Let it go
(Frozen)

Abstract

Liquid biopsy is a revolutionary tool that is gaining momentum in the field of cancer research. As a body fluid, urine can be used in non-invasive diagnostics for various types of cancer. We investigated the performance of UAS™ as a preservative for urinary analytes. Firstly, the need for urine preservation was investigated using urine samples from healthy volunteers. Secondly, the performance of UAS™ was assessed for cell-free DNA (cfDNA) and host cell integrity during storage at room temperature (RT) and after freeze-thaw cycling. Finally, UAS™ was used in a clinical setting on samples from breast and prostate cancer patients. In the absence of a preservative, urinary cfDNA was degraded, and bacterial overgrowth occurred at RT. In urine samples stored in UAS™, no microbial growth was seen, and cfDNA and cellular integrity were maintained for up to 14 days at RT. After freeze-thaw cycling, the preservation of host cell integrity and cfDNA showed significant improvements when using UAS™ compared to unpreserved urine samples. Additionally, UAS™ was found to be compatible with several commercially available isolation methods.

Introduction

01

Cancer is one of the leading causes of death, with an estimated 19.3 million new cases and 10 million deaths worldwide in 2020[1]. Liquid biopsy is a revolutionary method that has been gaining momentum in the past decades in the field of cancer research and care and has important advantages over tissue biopsy. Liquid biopsy can capture intratumor heterogeneity, while tissue biopsy only offers a snapshot of the tumor at a specific location and at a given time. Different body fluids, including blood, cerebrospinal fluid, saliva, sputum, and urine, have great potential as liquid biopsies[2-5]. Liquid biopsies can be used for cancer screening and therefore (i) have the potential to significantly impact the early detection and prognosis of cancer patients, (ii) will allow for monitoring of treatment response, and (iii) will allow for detection of recurrence[2-5]. Moreover, these liquid biopsies can be obtained using minimally invasive (blood, cerebrospinal fluid) or fully non-invasive procedures (urine, saliva, sputum), offering the possibility to sample more frequently and monitor the patient closely. Lately, urine as a liquid biopsy is gaining interest for non-invasive cancer diagnosis for different cancer types[2-5], including but not limited to urological cancers, breast cancer, and lung cancer[6]. Due to the non-invasive nature of urine collection, it is easy to collect even at home, eliminating the need for collection at the clinic. It can be collected repeatedly and without the volume restrictions often incurred with other liquid biopsy sample types, allowing for serial sampling and multi-omics analysis. Human urine is a complex biological fluid that primarily consists of water, organic and inorganic compounds, bacteria, and multiple cell types, including erythrocytes, leukocytes, prostate cells, renal cells, and urothelial cells[4]. Cellular and cell-free material can be released directly into the urine or via glomerular filtration[7]. Our previously conducted literature review on urinary biomarkers has shown that urine contains several analytes, including DNA, RNA, extracellular vesicles (EVs), proteins, and metabolites[6]. These

analytes make urine an attractive multi-omic sample type, potentially allowing for genomics, epigenomics, transcriptomics, proteomics, metabolomics, and microbiome analysis[2-5].

Urinary cell-free DNA (cfDNA) exists in the extracellular space and can be classified into two categories according to their size: low-molecular-weight (LMW) DNA (10–250 bp) and high-molecular-weight (HMW) DNA (1 kb or higher). LMW DNA can be derived from apoptotic cells in urine or circulation via glomerular filtration, and HMW DNA can originate from necrosis of cells shedding from the urogenital tract directly into the urine. Cellular DNA (in kbs) resides in the urogenital tract cells or debris shedding into the urine[7-11]. Urinary DNA can be investigated for tumor-specific changes, including methylation, microsatellite composition disorders, and point mutations, as well as the presence of viral DNA[9, 12, 13].

Urine is a promising emerging liquid biopsy; however, guidelines around pre-analytical parameters, including collection, preservation, and storage, are not yet well defined. This study investigated the important issue of the need for standardization of preanalytical protocols and the importance of urine preservation for oncological applications in light of a home collection and screening setup. This aligns with earlier studies[12-14], including our recent publication[6]. Here, we investigated the performance of a recently developed preservative, UAS™[15], for the preservation of urinary cfDNA and host cell integrity at RT for up to 14 days and after freeze-thaw cycling for oncology applications. Thereafter, this preservative was used in a clinical setting on samples from breast and prostate cancer patients.

Materials and Methods

02

2.1. Pre-evaluation of the need for urine preservation

Samples were collected under a protocol approved by institutional review board provider Advarra (Columbia, WA, USA), and informed consent was obtained from participants prior to sample collection. Unpreserved, first-void urine samples were obtained from five females and two males using a standard urine collection cup or Colli-Pee® FV-5020 (Novosanis, Wijnegem, Belgium). Samples were transported to the lab on ice packs within 5–6 h of collection. Each urine sample was aliquoted (4.5 mL per aliquot); one aliquot was taken as a baseline sample (T0), and the other was stored at RT (20–26 °C) for seven days (T7). At both time points, the aliquots were centrifuged at 3800× g for 20 min. The obtained supernatants (4 mL) were stored at −80 °C for downstream processing. Total cell-free nucleic acids (cfTNA) were extracted using the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). The extracted cfTNA was analyzed using a 16S bacterial rDNA qPCR and a human β-globin DNA qPCR assay (see below). Additionally, the sample DNA profile was investigated using the High-Sensitivity D5000 DNA ScreenTape (Agilent, Santa Clara, CA, USA) on the Agilent 4150 TapeStation system.

2.2. Evaluation of urine storage conditions with UAS™ preservative

2.2.1. Sample collection

Samples were collected under a protocol approved by institutional review board provider Advarra (Columbia, WA, USA), and informed consent was obtained from participants prior to sample collection. First-void urine samples were obtained from 42 females and 42 males using Colli-Pee® FV-5040 (Novosanis, Wijnegem, Belgium) without preservatives. The urine samples were collected between 10 a.m. and 1 p.m., and donors were instructed to wait a minimum of one hour after their last urination.

2.2.2. Experimental set-up

Three female or male samples were combined to form female-pooled (FP) or male-pooled (MP) urine samples, respectively. A total of 28 (14 FP and 14 MP) pooled urine samples were generated. A total of 14 pooled urine samples (7 FP and 7 MP) were used for RT (20–26 °C) and freeze-thaw cycling testing (Figure 1). After pooling, each pooled sample was split into two aliquots (55 mL total), with the UAS™ preservative being added to the first aliquot (38.5 mL urine with 16.5 mL UAS™ preservative), while the other was left as is without preservative addition (neat, 55 mL unpreserved urine).

1. UAS™ preservative performance during storage at room temperature

UAS™ preserved (55 mL) and neat (55 mL) pooled urine samples were aliquoted separately into three aliquots (18 mL per aliquot). One aliquot of each pooled sample was processed immediately at baseline (T0), while the remaining aliquots were stored at RT (20–26 °C) for 7 (T7) or 14 days (T14) before processing (Figure 1).

2. UAS™ preservative performance after freeze-thaw cycling

UAS™ preserved (55 mL) and neat (55 mL) pooled urine samples were separated into two aliquots (20 mL per aliquot). One aliquot of each pooled sample was processed immediately at baseline (T0), while the other aliquot underwent 3 freeze-thaw cycles (−20 °C to +40 °C) with a minimum of 3 h at each temperature per cycle before processing (Figure 1).

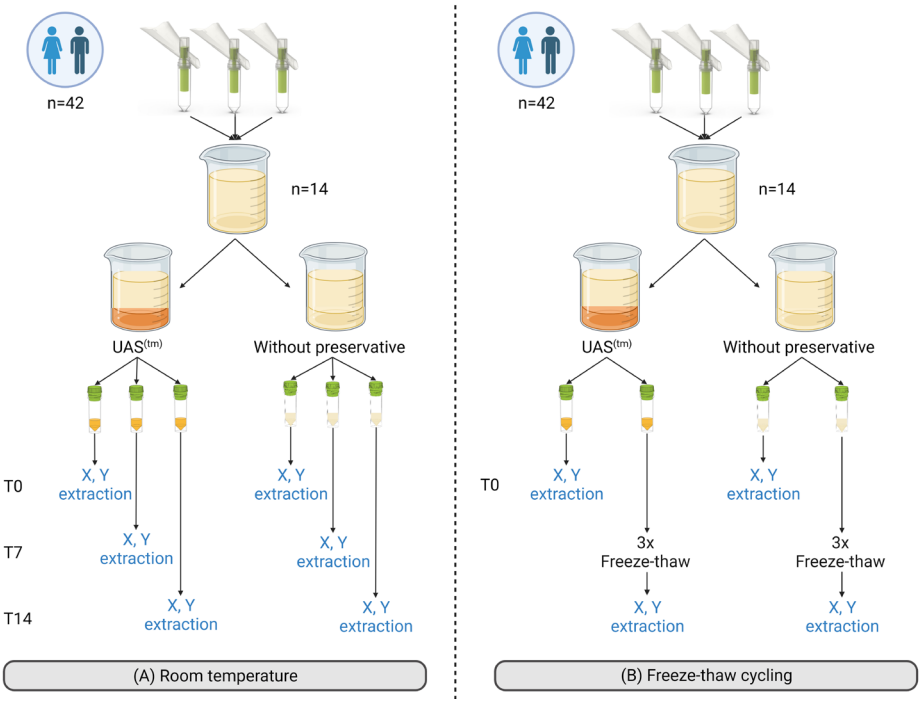

Figure 1: Experimental set-up to evaluate UAS™ preservative performance under different storage conditions—Sample collection and pooling were done similarly for the room temperature experiment (A) or the freeze-thaw experiment (B); urine samples were obtained from 21 females and 21 males using Colli-Pee® FV-5040 (Novosanis) without preservative. After collection, three female or male samples were combined to form female pooled (FP) or male pooled (MP) urine samples, respectively. A total of 14 (7 FP and 7 MP) pooled urine samples were generated and used for the experiment. Abbreviations: no addition, neat; T0, day 0 or baseline; T7, 7 days; T14, 14 days; X, cell-free total nucleic acid extraction; Y, cellular pellet extraction. Created with BioRender.com.

2.2.3. Extractions

Sample processing

After the specified conditions, each aliquot was centrifuged at 3000× g for 10 min. The resulting supernatant was filtered through a 0.8 µm filter into a new 50-mL conical tube and stored at −80 °C for downstream extraction. The resulting pellet was resuspended in 100 µL of PBS, transferred to a 1.5 mL Eppendorf tube, and stored at 80 °C for downstream extraction.

Cell-free total nucleic acid extraction

Samples were extracted in batches such that all corresponding aliquots (baseline, time points, or post-freeze-thaw cycling) from a given pooled sample were

extracted at the same time. Samples were removed from the −80 °C freezer and thawed in a 37 °C water bath for approximately 15 min. cfTNA was extracted using the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). For the urine samples containing UAS™ preservative, a 3 mL input volume was used, while for the neat samples, 2.1 mL of urine supplemented with 0.9 mL of PBS was used as an input volume (to ensure equal urine volumes in extraction). The cfTNA extractions were aliquoted in 25 µL aliquots for cell-free DNA analysis.

Cellular pellet extraction

Samples were extracted in batches such that all corresponding pellets (baseline, time points, or post-freeze-thaw cycling) from a given pooled sample were extracted at the same time. Samples were removed from the −80 °C freezer and thawed at RT. The cellular pellets were extracted using the PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany).

2.2.4. PCR analysis

The extracted analytes were analyzed using the qPCR assays outlined in Table 1. All samples (baseline, time points, or post-freeze-thaw cycling) from a given target analyte and pooled urine samples were quantified on the same qPCR run. All samples, standards, and no template controls (NTC) were run in duplicate. For the 16S bacterial rDNA qPCR and TS143 qPCR assays, all FP samples were diluted 1:10 in nuclease-free water prior to the assay.

The human β-globin qPCR (cfDNA) reactions were performed using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, California, USA), while the 16S bacterial rDNA qPCR and TS143 qPCR reactions were performed on a Rotorgene RG-3000A/RG-6000 (Corbett), with the conditions described in Table 2. The average CT value for each sample was determined by taking the average of the qPCR replicates.

Table 1: Overview of qPCR assays for the different targets – Summary of the different qPCR and RT-qPCR assays that were used for the analysis of the different analytes. The human β-globin qPCR assay was used to determine cfDNA stability, the 16S bacterial rDNA qPCR for microbial growth prevention, and the TS143 qPCR for host cell integrity. The commercially available products used are from Bio-Rad (Hercules, CA, USA) or Thermo-Fisher Scientific (Waltham, MA, USA). Abbreviations: ACTB, beta-actin; cfDNA, cell-free DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TS143, thymidylate synthase gene with an expected qPCR product size of 143 bp

PCR assay	Master mix	Forward primer	Reverse primer
Human β-globin qPCR[16]	2X iTaq Universal SYBR Master mix (Bio-Rad)	5′-ACA CAA CTG TGT TCA CTA GC-3′	5′-CAA CTT CAT CCA CGT TCA CC-3′
16S qPCR	Taq DNA Polymerase + Syto 9 based in-house master mix	5'-ATT ACC GCG GCT GCT GG-3'	5'-CCT ACG GGA GGC AGC AG-3'
TS143 qPCR		5'-GCC CTC TGC CAG TTC TA-3'	5'-GCC CTC TGC CAG TTC TA-3'

Table 2: Overview of the cycling conditions of each PCR assay – Overview of the amplification conditions used for each qPCR and RT-qPCR assay (see Table 1). Abbreviations: 16S, 16S bacterial rDNA; TS143, thymidylate synthase gene with an expected qPCR product of 143 bp.

Steps	Human β-globin qPCR			16S qPCR			TS143 qPCR		
Initial heat activation	5 min	95°C	1x	2 min	95°C	1x	5 min	95°C	1x
Denaturation	20 sec	95°C	45x	30 sec	95°C	35x	20 sec	95°C	35x
Annealing/ Extension	30 sec	56°C		20 sec	55°C		20 sec	55°C	
				20 sec	72°C		30 sec	72°C	

2.3. UAS™ preservative performance on clinical samples during collection and storage

2.3.1. Sample collection

This study is part of URODETECT (ClinicalTrials.gov identifier: NCT05453604) and was approved by the Ethical Committee of the Antwerp University Hospital (UZA) (EC filing: 20/10/115). Random midday, first-void urine samples were obtained at the Antwerp University Hospital and the University of Antwerp

from four healthy female volunteers (HVFs), six healthy male volunteers (HVMs), seventeen pregnant women (PRW), five breast cancer patients (BCP), and five prostate cancer patients (PCP) after signing informed consents. All participants collected a first-void urine sample using Colli-Pee® UAS™ FV-5040 (Novosanis, Wijnegem, Belgium).

2.3.2. Experimental set-up

Each collected sample was immediately centrifuged at 3000× g for 10 min, and the supernatants were divided into six aliquots (2 × 12 mL, 4 × 4 mL; Figure 2). From each sample, two aliquots were taken as technical replicates to account for method variance.

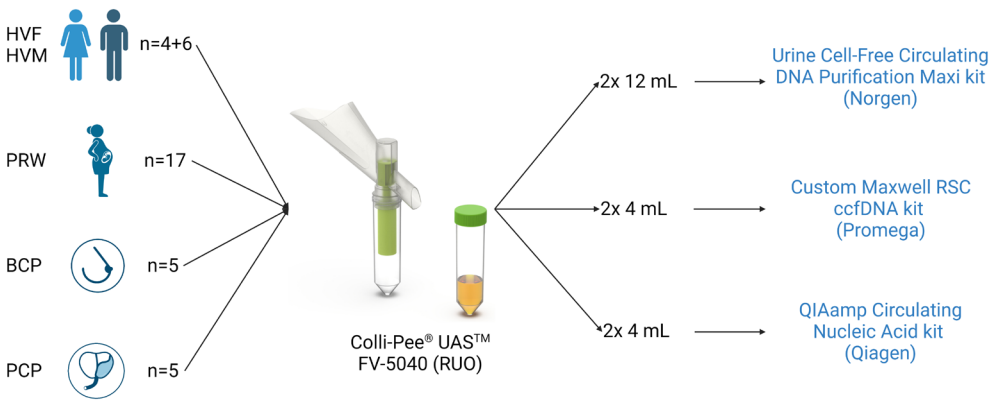

Figure 2: Experimental set-up of clinical study comparing different isolation methods—Abbreviations: BCP, breast cancer patients; HVF, healthy volunteer female; HVM, healthy volunteer male; PCP, prostate cancer patients; PRW, pregnant women. Created with BioRender.com.

2.3.2. Cell-free DNA extractions

Three commercially available cfDNA isolation kits were investigated: (i) the Urine Cell-Free Circulating DNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada), which is column-based and developed to purify and concentrate high-quality, high-purity, and inhibitor-free cell-free circulating DNA. (ii) the Maxwell® RSC Circulating DNA Purification Kit (Promega, Madison, WI, USA), which is magnetic beads-based and developed to purify fragmented DNA from human plasma with a protocol adapted for urine. (iii) the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), which is column- and vacuum-based

and developed to purify and concentrate free-circulating DNA, RNA, miRNA, and viral nucleic acids from human plasma, serum, urine, or other cell-free body fluids.

2.3.4. Analysis

The DNA concentration of each sample was determined using the Qubit® dsDNA High Sensitivity Assay Kit (Thermo-Fisher Scientific, Waltham, MA, USA). Additionally, the sample DNA profile and cfDNA percentage were investigated using the Cell-Free DNA ScreenTape (Agilent, Santa Clara, CA, USA) for the Agilent 4150 TapeStation system. The DNA profile and cfDNA percentage were analyzed using Agilent TapeStation analysis software 4.1.1. cfDNA fragments are represented by a region ranging from 40–450 bp.

2.4. Statistical analysis

Data is expressed as mean ± SEM unless otherwise indicated. All analyses were performed using GraphPad Prism (version 9.4.1, GraphPad Software Inc., La Jolla, CA, USA). All datasets were first explored for normality and outliers before the appropriate statistical test was applied. Statistical testing was performed using Welch's T-test, Kolmogorov-Smirnov test, Kruskal-Wallis, Welch's ANOVA test with a Dunnett's T3 multiple testing correction, and a Factorial ANOVA for the factors "preservative" and "time" with a Tukey multiple testing correction, respectively. Data were considered significant at $p < 0.05$. Applied statistical analyses are indicated in the figure legend.

Results

03

3.1. Pre-evaluation of the need for urine preservation

Unpreserved urine samples stored at RT for 7 days showed a prominent decrease in human cfDNA (mean ΔCt = 6.58) and a clear increase in bacterial cfDNA (mean ΔCt = −2.83). Statistically significant differences were noticed for both human cfDNA ($p < 0.0001$; Figure 3A) and bacterial cfDNA ($p = 0.0367$; Figure 3B) after 7 days of storage at RT compared to baseline (Figure 3C). Overall, these results demonstrate bacterial growth in unpreserved urine as well as the loss of human cfDNA over time.

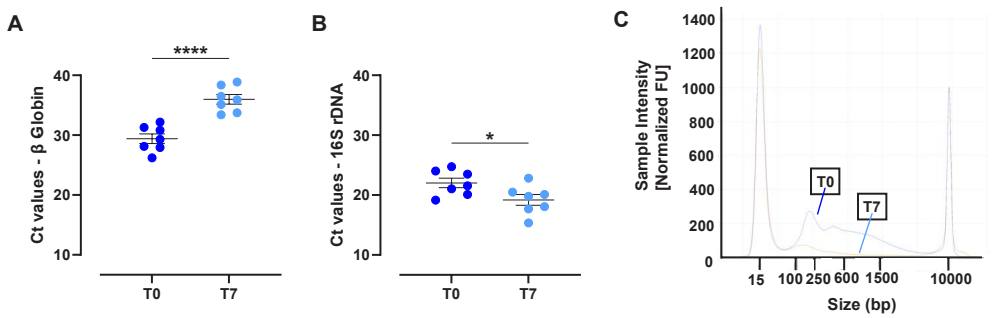

Figure 3: Pre-evaluation of the need for urine preservation—(A) human cell-free DNA presence; (B) bacterial growth; and (C) representative cell-free DNA profile of a female donor at baseline or after 7 days of storage at room temperature (20–26 °C). All experiments were performed with n = 7. For statistical analysis, Welch's T-test was used (A,B). Statistical significance levels for comparison: * $p \leq 0.03$; **** $p \leq 0.0001$. Data are presented as mean ± SEM. Abbreviations: 16S rDNA, 16S bacterial DNA; T0, baseline; T7, 7 days

3.2. Evaluation of the UAS™ preservative for urine storage

3.2.1. Storage at room temperature for up to 14 days

Different analytes were investigated to assess the overall performance of the UAS™ preservative for the storage of urine at RT for up to 7 and 14 days. Investigation into microbial growth prevention showed a statistically significant difference between samples preserved with UAS™ (ΔCt = 0.30) and neat samples (ΔCt = −7.64) after 7 days ($p < 0.0002$; Figure 4A). In addition, a comparison of cfDNA and host cell integrity indicated statistically significant differences between UAS™ preserved urine samples and neat samples after 7 days (cfDNA UAS™, ΔCt = 0.18; cfDNA neat, ΔCt = 8.25; host cell UAS™, ΔCt = 0.07; host cell neat, ΔCt = 6.05; $p < 0.0001$; Figure 4B) and 14 days storage at RT (cfDNA UAS™, ΔCt = −0.01; cfDNA neat, ΔCt = 9.61; host cell UAS™, ΔCt = 0.33; host cell neat, ΔCt = 7.68; $p < 0.0001$; Figure 4C). Moreover, the quality of cfDNA was checked by TapeStation analysis of cell-free nucleic acids extracted from a set of representative urine samples from Figure 4 stored for 7 and 14 days post-collection. No differences in fragmentation profiles were found between timepoints. Taken together, these results show an overall statistically significant inhibition of microbial growth and the preservation of cfDNA and host cell integrity when UAS™ is used as a preservative compared to neat urine samples stored at RT for up to 14 days.

3.2.2. Impact of simulated shipping conditions

Different analytes were investigated to assess the performance of UAS™ preservative as a urine preservative during simulated shipping conditions by freeze-thaw cycling. Three freeze-thaw cycles (−20 °C to +40 °C) were performed on each sample. A statistically significant difference ($p < 0.0001$) was observed between UAS™ preserved (ΔCt = 0.32) and neat (ΔCt = 7.49) urine samples related to cfDNA preservation (Figure 4D). Additionally, statistically significant differences between UAS™ preserved and neat urine samples for host cell integrity (UAS™, ΔCt = 0.31; neat, ΔCt = 6.68; $p < 0.0001$; Figure 4E) were observed. Taken together, these results show an overall statistically significant improvement in the preservation of cfDNA and host cell integrity when UAS™ is used as a preservative compared to neat urine samples after freeze-thaw cycling.

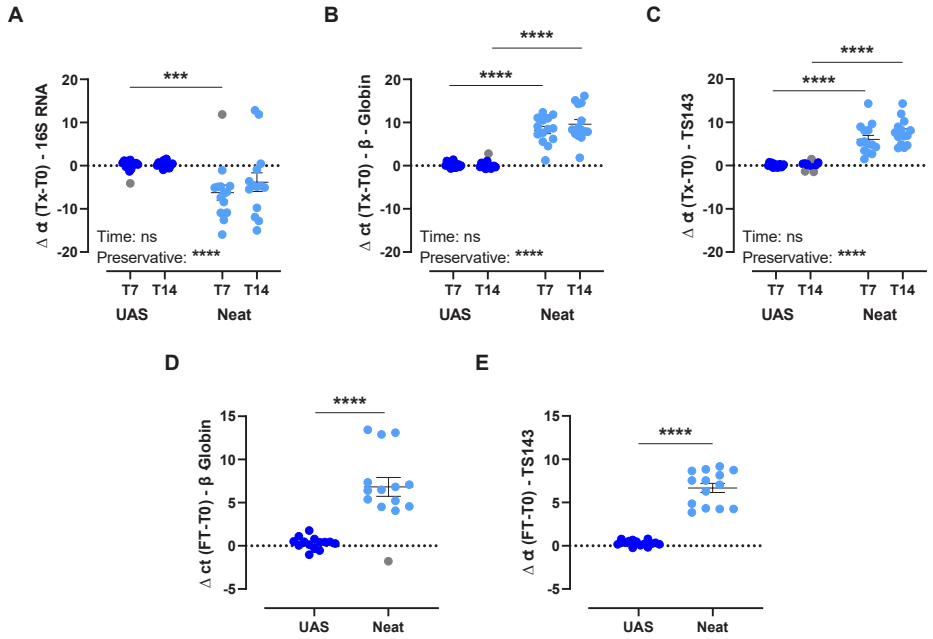

Figure 4: Performance of UAS™ preservative under different storage conditions—(A) microbial growth prevention; (B) cell-free DNA stability; and (C) host cellular integrity after 7 and 14 days of storage at room temperature (RT, 20–26 °C). All RT experiments were performed with n = 14 urine pools. Outliers were examined, and if identified, they were shaded in gray in the relevant graphs and removed from the statistical analysis: (A) two, (B) one, and (C) three outliers. Factorial ANOVA with a Tukey post-hoc test was performed for the factors "preservative" and "time" (A–C). The factor "preservative" was statistically significant in (A) $p < 0.0001$, (B) $p < 0.0001$, and (C) $p < 0.0001$. (D) cell-free DNA stability and (E) host cell integrity after freeze-thaw (FT) cycling (3×). All FT cycling experiments were performed with n = 14 urine pools. Outliers were investigated and, if identified, removed: (D) one outlier was removed from the statistical analysis and graphical representation. The Kolmogorov-Smirnov test (D) and Welch's T-test (E) were performed. For all experiments, statistical significance levels for comparison are: *** $p < 0.0002$; **** $p < 0.0001$. Data are presented as mean ± SEM. Abbreviations: FT, freeze/thaw cycling; RT, room temperature; T0, baseline; T7, 7 days; T14, 14 days; TX, T7 or T14.

3.2.3. Evaluation of first-void urine preserved with UAS™ and compatibility with three commercially available isolation methods for urinary cfDNA

The DNA concentration of the different samples ranged from 0.00 ng DNA/mL urine to 236.53 ng DNA/mL urine without statistically significant differences (Figure 5A); however, lower averages were found for the male samples (2.30 ± 2.33 ng DNA/mL urine for healthy male volunteers and 4.63 ± 3.66 ng DNA/mL urine for prostate cancer patients) compared to the female samples (98.43 ± 58.05 ng DNA/mL urine for healthy female volunteers, 33.28 ± 19.02 ng DNA/mL urine for pregnant women, and 31.04 ± 15.87 ng DNA/mL urine for breast cancer patients).

Statistically significant differences were demonstrated between the Norgen isolation kit and the Promega ($p < 0.03$) and Qiagen ($p < 0.0001$) isolation kits, indicating that a higher amount of DNA per start volume of urine was obtained using the Promega and Qiagen isolation kits (Figure 5B). It is important to note that for Norgen, the starting volume of urine was higher, allowing fewer isolations to obtain more DNA. Furthermore, the cfDNA percentage of all participant types averaged around 20%, with no statistically significant differences between the participant types (Figure 5C). Additionally, no profound differences in sample DNA profiles were found between the urine samples of all different participant types or the isolation methods (Figure S1). Overall, these results suggest that all three commercially available isolation methods performed comparably based on DNA profiles and cfDNA percentages for the isolation of urinary cfDNA. Additionally, the UAS™ preservative is extraction-agnostic given its compatibility with three isolation methods based on different mechanisms.

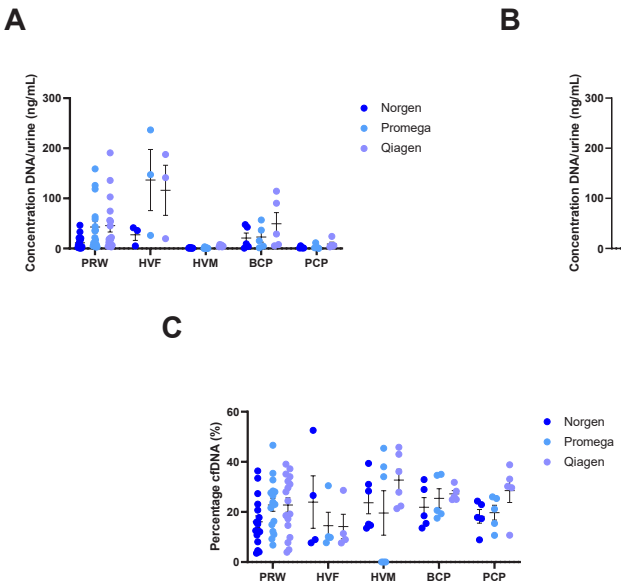

Figure 5: The UAS™ preservative is compatible with extraction methods based on different mechanisms as assed using clinical samples—(A) DNA concentration of each participant group and colored per isolation method; (B) DNA concentrations combined per isolation method; and (C) percentage cell-free DNA of each participant group and colored per isolation method. The isolations were performed with three different isolation methods (Norgen, Promega, and Qiagen) from healthy (HVF n = 4; HVM n = 6; PRW n = 17) and clinical (BCP n = 5; PCP n = 5) urine samples. Welch's ANOVA test with Dunnett's T3 correction (A) and Kruskal-Wallis tests (B,C) were performed. For all experiments, statistical significance levels for comparisons are: *, $p < 0.03$; and ****, $p < 0.0001$. Data are presented as mean ± SEM. Abbreviations: BCP, breast cancer patients; HVF, healthy female volunteers; HVM, healthy male volunteers; Norgen, Urine Cell-Free Circulating DNA Purification Kit; PCP, prostate cancer patients; Promega, Maxwell® RSC Circulating DNA Purification Kit; PRW, pregnant women; Qiagen, QIAamp® Circulating Nucleic Acid Kit.

Discussion

04

Urine is a promising emerging liquid biopsy; however, guidelines around pre-analytical parameters, including collection, preservation, and storage, are not yet well defined or standardized. Therefore, a novel preservative, UAS™, has been designed to prevent chemical and enzymatic degradation of nucleic acid and cellular lysis, as well as microbial growth. The performance of this recently developed and patented preservative15, UAS™, was assessed in the current study for the preservation of urinary cfDNA and host cell integrity. First, a small pilot study was designed using urine from healthy volunteers to simulate a baseline experiment and demonstrate the importance of urine preservation. Thereafter, two studies were performed to demonstrate the preservation of urinary cfDNA and host cell integrity at RT for up to 14 days and after simulated transport conditions. Finally, the UAS™ preservative was used to collect samples from a patient cohort to show the applicability of UAS™ preservative in a clinical setting with individual samples. Together, these three different studies highlight the preservative value of UAS™, and its performance under various expected sample handling conditions, and its potential in a clinical setting. This work demonstrates the potential of Colli-Pee® UAS™ in addressing the important issue of standardization of preanalytical workflows by allowing volumetric urine collection combined with optimized preservation, storage, and transportation conditions, thereby improving downstream processes. Given the expected heterogeneity in analyte concentrations between healthy and clinical samples, it would be valuable for future studies to further evaluate UAS™ preservative performance with large clinical cohorts.

4.1. Importance of urinary analyte preservation

Standardization of the pre-analytical parameters for urine processing, such as urine collection, storage, and handling, is critical for the clinical usefulness of urinary biomarkers[17-19]. All these parameters may lead to water evaporation, host cellular lysis, nuclease activity, bacterial growth and/or contamination, and potentially changes in urine composition[20, 21]. Preventing bacterial growth is important as it can cause the urine sample to reach higher turbidity, making the extraction and detection of specific analytes more difficult[22]. Preservation of host cell integrity is important to avoid host cell lysis and release of their cellular contents, including DNA, thereby preventing an accurate assessment of cfDNA. Urine contains potassium, calcium, magnesium, sodium, and zinc and has a pH between 5.0 and 7.0, providing a suitable environment for nuclease activity. DNase I, one of these DNA-hydrolyzing enzymes, has a more than 100-fold higher activity in urine compared to its activity in blood. DNase II is also present in urine, although its activity is 30 times lower than that of DNase I. On the other hand, RNA-hydrolyzing enzymes, including RNase I, RNase II, and phosphodiesterase I, are also present in urine. All these nucleic acid-hydrolyzing enzymes jeopardize the preservation of DNA and RNA fragments[9]. Our experiments confirmed prior findings that accurate analysis of urinary analytes requires prevention of microbial growth and that urinary analytes should be protected against nucleases and chemical degradation[4, 5, 9, 12-14, 23, 24].

The addition of preserving agents, like ethylenediaminetetraacetic acid (EDTA), has been demonstrated to improve the stability of urinary analytes upon storage[4, 13, 17-20, 24-26]. EDTA is a well-established chelating agent that binds the ions required for DNase activity. Consequently, adding EDTA inhibits nucleic acid hydrolyzing enzymatic activity and reduces DNA degradation[23]. EDTA is a frequently used preservative; however, it can only protect DNA[17-19, 26]. Other preservatives have been frequently used for other analytes, e.g., guanidine thiocyanate preservatives for RNA[17] and boric acid or thymol preservatives for metabolites[20]. Currently, it appears that each analyte requires a different preservative. However, from a collection and processing point of view, the need for multiple reagents is not convenient for the continuously evolving multi-omics scene, emphasizing the need to validate an efficient preservative in urine that can preserve multiple analytes. Additionally, preservatives allow the storage of urine prior to analysis, whether due to home collection, sample shipment, or biobanking. The benefit of home collection for participants or patients is the comfort and privacy of a home environment to provide a sample without having to travel or visit a clinic; this

in turn increases donor compliance and allows for easier recruitment of larger cohorts. The UAS™ preservative has been developed for preservation at room temperature, and the Colli-Pee® device has been developed for the standardized volumetric collection and immediate preservation of urine while being user-friendly and suited for home collection.

4.2. Performance of UAS™ preservative

The performance of UAS™ preservative as a urinary analyte preservative was examined in different experiments on the preservation of cfDNA and host cell integrity after (i) storage at room temperature for up to 14 days and (ii) freeze-thaw cycling. The room temperature experiment was designed to investigate the performance of UAS™ preservative under standard sample collection, storage, and handling conditions, while the freeze-thaw experiment was designed to be representative of transport conditions that will occur when samples are sent to the laboratory. First, UAS™ preservative was shown to prevent microbial growth and preserve cfDNA and host cell integrity, as urine samples preserved with UAS™ preservative showed statistically significantly improved results than neat urine samples after up to 14 days at room temperature and after freeze-thaw cycling (Figure 4). In this study, human cellular integrity was determined indirectly by the concentration of human DNA extracted from urine cellular pellets, and no direct measurements such as microscopy, flow cytometry, or fluorescent dyes were used. During sample storage, human cell lysis would compromise the availability of the cellular pellet after the centrifugation step, which would ultimately result in the loss of cellular DNA (leading to a change in the TS143 qPCR ΔCt). These results further add to the applicability of the UAS™ preservative in biobanking and batch extraction of urinary analytes from patients' samples received in clinics at varying times. Another note about these experiments and results is the pooling of urine samples from different gender-equal individuals, as done in previous studies as well[20, 27]. The experimental design combines urine samples from three female or male individuals to form one female or male, respectively, pooled urine sample, which removes the individualistic character of the samples. For these experiments, however, the benefits of pooling outweighed the need for the individualistic character of a particular sample. Since the experiments were performed using healthy volunteers, the individualistic nature of the urine samples was secondary to the pooling, which allowed for (i) an experimental design with higher volumes so that each condition was measured with the same pooled sample, (ii) a broad representation of the performance of UAS™

preservative on urine, and (iii) an evaluation independent of the parameters influencing a subject's urine sample. For the clinical trial experiment, the samples were not pooled and kept as individual samples to maintain the uniqueness of each sample type since there were different cancers examined.

4.3. Clinical applicability of first-void urine preserved with UAS™

In the URODETECT study, three commercially available isolation methods were tested and compared for their performance in isolating cfDNA from urine collected from different participant groups with Colli-Pee® UAS™. This experiment led to two interesting conclusions: (i) cfDNA targets as urinary biomarkers and (ii) the compatibility of UAS™ preservative with different isolation methods. Firstly, cfDNA was investigated in different study populations: healthy female and male volunteers, pregnant women, and breast and prostate cancer patients. There were some differences in the DNA concentration between these groups; however, the percentage of cfDNA was relatively similar. This result emphasizes the ability to compare a healthy population to a diseased population and thereby the opportunity to explore cfDNA targets as biomarkers for prenatal and cancer diagnosis, screening, and monitoring of disease progression and recurrence[28]. Secondly, three isolation methods were examined: the Urine Cell-Free Circulating DNA Purification Maxi Kit[29], the QIAamp® Circulating Nucleic Acid Kit[30], and the Maxwell® RSC Circulating DNA Purification Kit[31], which are all based on different isolation mechanisms. Since our results show comparable performance for all three different isolation methods, it was demonstrated that UAS™, as a preservative, is compatible with and does not interfere with silicon-carbide technology, silica-membrane vacuum technology, or paramagnetic particle technology.

4.4. Strengths, limitations and future research

The strengths of our study are the test set-ups for investigating both the storage of urine at room temperature and under simulated transport conditions. This allowed assessing the performance of UAS™ for its preservation capabilities of urinary cfDNA and host cell integrity under all these conditions and preventing bacterial overgrowth. This is important for its applicability for at-home collection and shipment of samples to the laboratory. To enhance the clinical utility of urine for cancer research, it is considered a strength to have a volumetric urine

collection device, Colli-Pee®, allowing for standardization and immediate mixing of urine with the UAS™ preservative to improve pre-analytical conditions and downstream processing.

Our study also has some limitations. First, the UAS™ performance studies were conducted on healthy volunteers only. Secondly, the study conducted in a clinical setting was on a small cohort of patient samples, and no targeted downstream analysis, such as PCR or NGS, was performed. The latter has important implications since Qubit analysis may overestimate the DNA concentration after isolation. Especially for the Qiagen isolation kit, because in this protocol carrier RNA is added, which can influence the Qubit DNA measurements.

Due to these strengths and limitations, future research is necessary to further elaborate on the potential of Colli-Pee® UAS™ for the standardized and volumetric collection and preservation of first-void urine. In future research, it is warranted to (i) use patient samples to investigate the overall performance of UAS™; (ii) include larger patient cohorts to properly investigate the differences between groups; (iii) perform targeted downstream analysis; and (iv) investigate the potential of UAS™ for other urinary analytes such as extracellular vesicles, RNA, and proteins. Additionally, research is ongoing to investigate further improvements to the preservative: urine ratio, including (i) reducing the amount of liquid preservative in the collector tube and (ii) exploring possibilities for the creation of a powder/solid version of the preservative using lyophilization, spray drying, and other technologies. If further elucidated, the potential of Colli-Pee® UAS™ could enable individuals to collect a urine sample in the comfort of their own home and send the sample to the laboratory, all in a more standardized manner. That would all help to increase cancer screening coverage and provide more comfort to cancer patients during their treatment and disease follow-up.

Conclusion

05

Urine as a liquid biopsy has the potential to become a game changer in personalized cancer care, allowing for its use for primary and secondary prevention, diagnosis, treatment response measurement, and detection of recurrence. Besides the need for clinical validity and utility, there is also an urgent need for analytical validity. The current study clearly tackled the important issue of lack of standardization of preanalytical workflows and demonstrated the potential of Colli-Pee® UAS™ as a suitable urine collection and preservation method allowing for standardization in collecting, preserving, and analyzing. Specifically, the UAS™ preservative allows for accurate and clinically translatable analysis of urinary analytes, prevention of bacterial growth and analyte degradation, as well as preservation of host cell integrity and cfDNA during pre-analytical sample storage and handling, which is deemed critical. The UAS™ preservative also showed statistically significant improvement in the preservation of host cell integrity and cfDNA during freeze-thaw cycling. The agnostic nature of the UAS™ preservative was demonstrated by its compatibility with commercially available isolation methods based on different mechanisms using clinical samples. Additionally, the study demonstrates the applicability of the first-void at-home urine collection device Colli-Pee® UAS™ by Novosanis as a standardized, volumetric tool to collect urine and immediately preserve urinary analytes for oncology applications.

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CHAPTER

05

Standardization of urine pre-analytical workflow: cell-free DNA extraction methods and Colli-Pee® UAS™ urine collection devices

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(Manuscript in preparation)

Even miracles take a little time.
(Fairy Godmother, Cinderella)

Abstract

Background: Circulating cell-free DNA (cfDNA) has been used as an informative biomarker in cancer detection and screening, infectious disease detection and screening, organ transplantation monitoring and, prenatal screening. Since cfDNA and more specifically circulating tumor cfDNA (ctDNA) are believed to reflect genetic abnormalities found in tumor tissue, they can be used as liquid biopsy. Urine cfDNA analysis has some clear advantages in clinical application compared to other cfDNA sources (e.g., plasma) because it is non-invasive and allows for self-sampling at home. Despite its potential, no standardized protocols for pre-analytical parameters or isolation are available, which are critical to maximize reproducibility.

Methods: We investigated the effect of different cfDNA extraction methods and different Colli-Pee® UAS™ device variants on the concentration of cfDNA and cellular genomic DNA (gDNA) contamination. Urine samples from healthy volunteers, pregnant women and breast and prostate cancer patients were collected and processed according to specific experimental set-ups.

Results: No statistically significant differences were found between the four urinary cfDNA extraction methods with regard to DNA concentrations, short (50-450bp) and long (>451bp) fragment concentrations and ratios, and DNA profiles. The main differences were seen between female and male individuals, where female individuals have higher DNA concentrations than male individuals. Furthermore, no statistically significant differences were observed between the three Colli-Pee® UAS™ (Novosanis NV, Wijnegem, Belgium) volume variants (FV-5010, collecting approx. 10mL; FV-5020, collecting approx. 20 mL; FV-5040, collecting approx. 40 mL).

Conclusion: The current study addressed the important issue of the lack of standardization of preanalytical workflows by exploring differences between cfDNA extraction methods and differences in urine volume collected with Colli-Pee® UAS™ devices. We demonstrated that all four cfDNA extraction methods performed similarly in this study and that no differences were observed between the thee volume variants of Colli-Pee® UAS™ (Novosanis NV), indicating their potential to enhance the standardization and potential of urine as liquid biopsy for cancer research.

Introduction

01

Circulating DNA fragments released from cells of healthy and diseased tissues are referred to as circulating cell-free DNA (cfDNA)[1, 2]. Circulating cfDNA has been used as an informative biomarker in cancer detection and screening[3-8], infectious disease detection and screening[9-11], organ transplantation monitoring[12-14] and, prenatal screening[3, 15-18]. The portion of circulating cfDNA that is derived from tumor cells is referred to as circulating cell-free tumor DNA (ctDNA)[1, 2]. Since cfDNA and more specifically ctDNA reflect genetic abnormalities found in tumor tissue, cfDNA can be used as liquid biopsy[19]. Furthermore, DNA molecules have been shown to be able to pass the glomerular filtration and therefore cfDNA can be detected in urine samples[20]. Urine cfDNA analysis has a greater advantage in clinical application compared to samples from other cfDNA sources (e.g., plasma, saliva) because urine collection is non-invasive and more convenient, allows for self-sampling without the need for a healthcare practitioner and even home-sampling[1, 21, 22].

Circulating cfDNA is highly fragmented with a size distribution of ~130-200bp, with identified prominent cfDNA fragment lengths of 167bp and multiples thereof. This fragment length corresponds to the size of nuclease-cleaved nucleosomes[22, 23]. When DNA is released outside the cell, nuclease cleavage occurs, degrading the linker DNA between adjacent nucleosomes, while the DNA around the histones will be protected from degradation[22]. Urinary cfDNA can be divided into two types: (1) trans-renal tumor DNA (trtDNA), which enters the urine via glomerular filtration of plasma and is limited in size; (2) cfDNA originating from tumor cells that come directly from the urinary tract, which don't undergo glomerular filtration and can be larger in size[1]. It is clear that intact non-malignant cells from the urinary tract can secrete long genomic DNA (gDNA) fragments when cell lysis, necrosis, apoptosis occurs. This leads to a

dilution of the low concentration ctDNA in the samples, which will inevitably complicate the accurate detection of biomarkers[21].

The three main steps in a liquid biopsy workflow are: (i) biofluid collection (including collection, storage and handling), (ii) isolation or extraction of biomarkers and, (iii) analysis or detection of biomarkers. Each of these steps brings possible variation and thus offers opportunities for optimization and standardization[23]. Despite the great potential of urine as liquid biopsy, no standardized protocols for pre-analytical parameters are available[24], which is surprising as it has been shown that most of the test errors are due to problems during the pre-analytical phase[25, 26]. Therefore, it is of utmost importance to use appropriate pre-analytical conditions, such as optimized collection and preservation methods and, storage and transport temperatures, to achieve accurate detection of urinary biomarkers[1, 21, 22, 27]. Additionally, the optimization of cfDNA extraction, including sample preparation steps, concentration steps and the extraction step, towards methods capable of efficiently capturing short, diluted DNA fragments is critical to maximize the clinical sensitivity and reproducibility[28].

In this study, all urine samples were collected using Colli-Pee® UAS™ devices (Novosanis NV, Wijnegem, Belgium), standardized volumetric devices that allow immediate mixing with a preservative, to standardize the collection and preservation of first-catch urine. The aim of the study was to investigate which urinary cfDNA extraction method is the most suitable in combination with the Colli-Pee® UAS™ devices for cancer and prenatal screening. Therefore, we compared four different urinary cfDNA extraction methods: Urine Cell-Free Circulating DNA Purification Kit (Norgen Biotek Corp., Ontario, Canada), the Maxwell® RSC Circulating DNA Purification Kit (Promega, Madison, Wisconsin, USA), the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and, the Revolution System® (nRichDX, Irvine, California, USA). Additionally, we compared the three different Colli-Pee® UAS™ device formats: Colli-Pee® UAS™ FV-5010, Colli-Pee® UAS™ FV-5020 and Colli-Pee® UAS™ FV-5040 (Novosanis NV, Wijnegem, Belgium).

Methods

02

2.1. Sample collection

The URODETECT WP1 study is a prospective study, which aimed to investigate the potential of urine as liquid biopsy for cancer detection (ClinicalTrials.gov identifiers: NCT05453604). This study was approved by the Ethical Committee of the University Hospital of Antwerp (UZA) (Dossier No 20/10/115). Samples were obtained from 9 healthy female volunteers (HVFs), 11 healthy male volunteers (HVMs), 26 pregnant women (PRW), 9 breast cancer patients (BCP) and 6 prostate cancer patients (PCP), after signing informed consents. All participants had to be at least 18 years old and were allowed to participate in multiple experiments. Sample collection and patient inclusions took place from June 2020 till February 2023. Samples were collected using Colli-Pee®, a first-void urine collector developed by Novosanis NV (Wijnegem, Belgium). The Colli-Pee® devices were prefilled with UAS™ preservative for the preservation of analytes of interest. Different parameters were evaluated in this study, therefore the study set-up and sample size per experiment is different and will be described below.

2.2. Experimental set-ups

2.2.1. Comparison of extraction kits

In the first experiment (Figure 1A), three commercially available cfDNA extraction kits were compared: (i) the Urine Cell-Free Circulating DNA Purification Kit

(Norgen Biotek Corp., Ontario, Canada), (ii) the Maxwell® RSC Circulating DNA Purification Kit (Promega, Madison, Wisconsin, USA), (iii) the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). In this experiment 17 pregnant women, 4 healthy female volunteers, 6 healthy male volunteers, 5 breast cancer patients and 5 prostate cancer patients were included. The second experiment (Figure 1B) was performed to compare the QIAamp® Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) with the Revolution System® (nRichDX, Irvine, California, USA). In this experiment 5 pregnant women were included. All participants collected a first-catch urine sample using Colli-Pee® UAS™ FV-5040 (Novosanis, Wijnegem, Belgium), which collects a total volume of approximately 40 mL. The collected sample was immediately centrifuged for 10 minutes at 4000g and the supernatant was divided into aliquots (first experiment: 2x 12 mL for Norgen extractions, 4x 4 mL for Promega and Qiagen extractions; second experiment 1x 4 mL for Qiagen extractions and 20 mL for nRichDX extractions). In the first experiment, each sample was taken in duplicate as technical replicates to account for method variance.

2.2.2. Comparison of Colli-Pee® UAS™ variants collecting different volumes

The third experiment (Figure 1C) was performed to compare Colli-Pee® UAS™ FV-5010, Colli-Pee® UAS™ FV-5020 and Colli-Pee® UAS™ FV-5040 (Novosanis, Wijnegem, Belgium), collecting all different volumes. FV-5010 collects approximately 10mL, FV-5020 approximately 20mL and FV-5040 approximately 40mL. In this experiment 4 pregnant women, 5 healthy female volunteers, 5 healthy male volunteers, 6 breast cancer patients and 3 prostate cancer patients were included. Every participant collected urine samples using all three device variants within a timeframe of 2 days. After arrival at the laboratory, the collected samples were centrifuged for 10 minutes at 4000g and the supernatant was divided in two aliquots (2x 10 mL), except for the samples collected with Colli-Pee® UAS™ FV-5010 since these only collect 10mL these were kept as one aliquot.

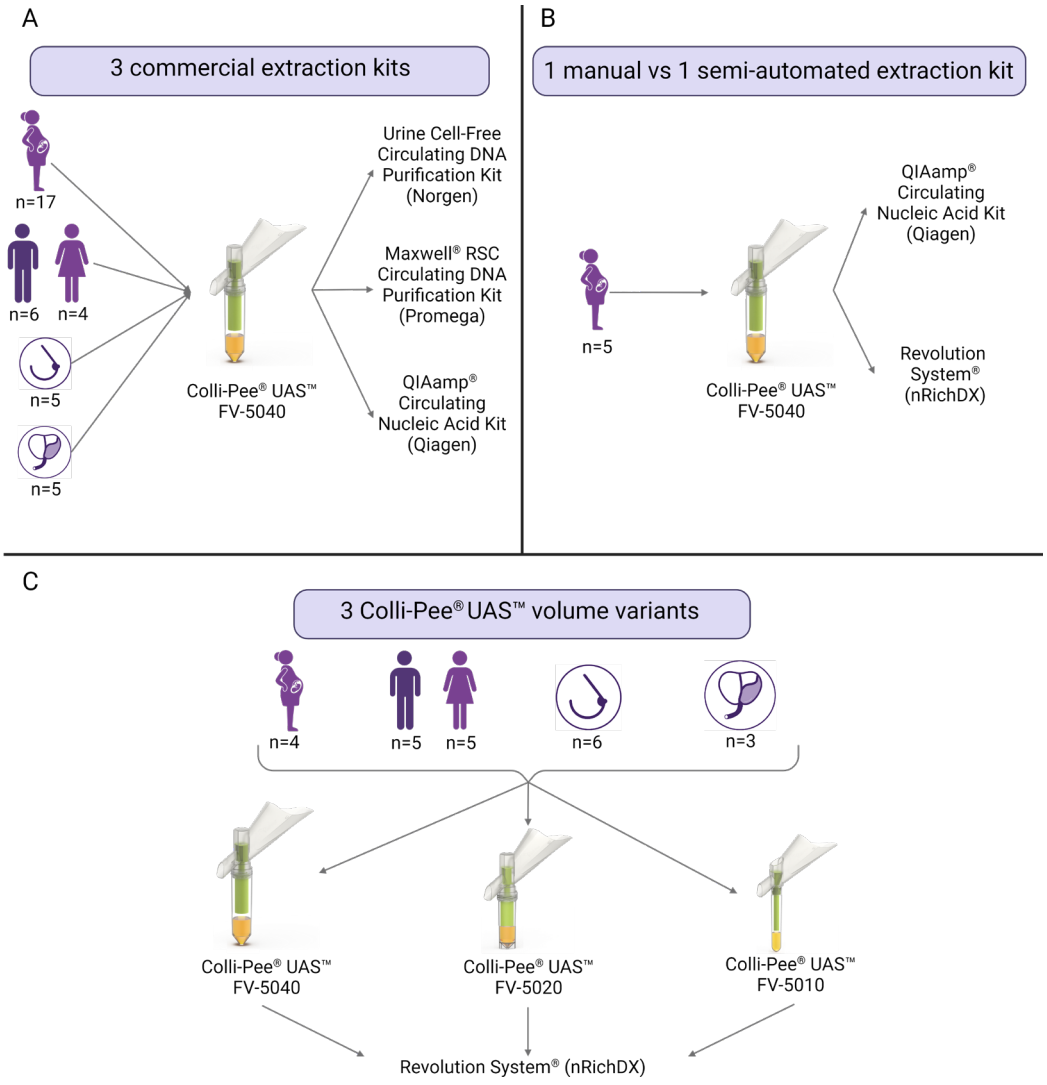

Figure 1: Experimental overview of the study. For this study three commercial available cfDNA extraction kits were compared from Colli-Pee® UAS™ FV-5040 collected first-catch urine samples of pregnant women, healthy female and male volunteers, breast and prostate cancer patients (A), a commercially available manual cfDNA extraction kit and commercially available semi-automated cfDNA extraction system were compared from Colli-Pee® UAS™ FV-5040 collected first-catch urine samples of pregnant women (B) and, three Colli-Pee® UAS™ variants collecting different volumes of urine were compared from urine collected with the different variants of pregnant women, healthy female and male volunteers, breast and prostate cancer patients. For all three experiments, the urine samples were immediately centrifuged upon arrival at the laboratory for 10 minutes at 4000g and the supernatant was divided in the determined about of aliquots. Abbreviations: cfDNA, cell-free DNA; n, sample size.

2.3. Cell-free DNA extraction

The Urine Cell-Free Circulating DNA Purification Kit (Norgen Biotek Corp., Ontario, Canada) is column-based and developed to purify and concentrate high quality, high purity and inhibitor-free cell-free circulating DNA. CfDNA extraction was performed according to the manufacturer's protocol and cfDNA was eluted in a volume of 50 µL. The Maxwell® RSC Circulating DNA Purification Kit (Promega, Madison, Wisconsin, USA) is magnetic beads-based and developed to purify fragmented DNA from human plasma, but with an adapted protocol for urine. CfDNA extraction was performed according to the manufacturer's protocol and cfDNA was eluted in a volume of 40 µL. The QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) is column- and vacuum-based and developed to purify and concentrate free-circulating DNA, RNA, miRNA, and viral nucleic acids from human plasma, serum, urine or other cell-free body fluids. CfDNA extraction was performed according to the manufacturer's protocol and cfDNA was eluted in a volume of 50 µL. The Revolution system® (nRichDX, Irvine, California, USA) is a semi-automated sample preparation system designed to purify cfDNA from 1-20 mL biological fluid. CfDNA extraction was performed according to the manufacturer's protocol and cfDNA was eluted in a volume of 50 µL. After extraction, the samples were stored at -20°C until further analysis.

2.4. Analysis

All urine samples, were analyzed using three techniques assessing either the total DNA concentration or the length of the DNA fragments (long fragments are defined as fragments with sizes >451bp and short fragments as fragments with sizes 50-450bp). The DNA concentration of each sample was determined using the Qubit® dsDNA High Sensitivity Assay kit (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol. All samples were tested in triplicate using 2 µL. Additionally, the sample DNA profile and cfDNA percentage were investigated using the Cell-free DNA ScreenTape (Agilent, Santa Clara, California, USA) for the Agilent 4150 TapeStation system, according to manufacturer's protocol. DNA profile and cfDNA percentage were analyzed using Agilent TapeStation analysis software 4.1.1. Short fragments (~ cfDNA) were represented by the region 40-450 bp.
Digital droplet PCR (ddPCR) analysis was performed using the QX200 ddPCR equipment (dHsaCP2500472, Bio-Rad, Hercules, California, USA). All samples

were analyzed with the ddPCR Copy Number SRY assay (Bio-Rad, Hercules, California, USA) and as references B2M (dHsaCPE5053101, Bio-Rad, Hercules, California, USA) and GAPDH (dHsaCPE5031597, Bio-Rad, Hercules, California, USA) were used. PCR master mixtures were produced by 2x ddPCR Supermix for Probes – no dUTP (Bio-Rad), ddPCR SRY assay, ddPCR B2M assay, and ddPCR GAPDH assay. Data analysis was performed with the QuantaSoft version 1.0.596.0525 (Bio-Rad, Hercules, California, USA).

2.5. Statistical analysis

Data was expressed as mean ± SEM unless otherwise indicated. All analyses were performed using GraphPad Prism (version 9.5.0, GraphPad Software Inc., La Jolla, California, USA). All datasets were first explored for normality and outliers before the appropriate statistical test was applied. Statistical testing was performed using Paired T-test, Wilcoxon matched-pairs signed rank test, Kruskal-Wallis with Dunn's correction for multiple testing, and a Factorial ANOVA for different factors depending on analysis 'participant type', 'extraction method' and 'measurement method', with a Tukey multiple testing correction respectively. Data were considered significant at $p < 0.05$. Applied statistical analyses are indicated in the figure legend.

Results

03

3.1. Study population

Between the 25th of June 2020 and the 16th of February 2023, 61 participants were enrolled in the URODETECT study. There were 44 women and 17 men, with participant's ages ranging from 20 to 83 years, with a median age of 41 years. It was noticed that most of the participants were between 30 and 39 years old. A total of 9 breast cancer patients (14.75%), 9 healthy female volunteers (14.75%), 11 healthy male volunteers (18.03%), 26 pregnant women (42.62%) and 6 prostate cancer patients (9.84%) provided their urine samples for this study. The characteristics of the participant are compiled in Table 1.

Table 1: Characteristics of the participants enrolled in the study (N=61)

Characteristics	Number [1]	Percentage (%)
Age [2]	**61**	**100**
18-29 years	12	19.67
30-39 years	26	42.67
40-49 years	5	8.20
50-59 years	7	11.48
60-69 years	7	11.48
70-79 years	3	4.92
80-... years	1	1.64

Characteristics	Number [†]	Percentage (%)
Gender identity	**61**	**100**
Woman	44	72.13
Man	17	27.87
Participant type	**61**	**100**
Breast cancer patients	9	14.75
Healthy female volunteers	9	14.75
Healthy male volunteers	11	18.03
Pregnant women	26	42.62
Prostate cancer patients	6	9.84

[†] Participants were allowed to collect multiple samples for the different pilot studies; [‡] the age of the first sample collection was used for participants who collected multiple samples.

3.2. Comparison of three commercially available extraction kits (Norgen, Promega and, Qiagen)

Using the recommended maximum input volume for Promega and Qiagen and the intermediate volume for Norgen (and standard elution volume) as stated by the kit manufacturer. It was shown that the three extraction methods were not statistically significantly different when comparing the DNA concentration measured by Qubit and Tapestation (Figure 2A). However, it is shown that the DNA concentration was slightly higher in the samples isolated with Qiagen (mean = 2.397 ng/µL) than Promega (mean = 2.003 ng/µL) and Norgen (mean = 2.043 ng/µL). Compared to TapeStation, which is known as a more sensitive method, Qubit shows an overestimation of the DNA concentration. When looking into more detail and comparing the different participant types per extraction method nine statistically significant differences were found, of which all were between participant types within a certain extraction method (Figure 2C; Figure 3A-C; Figure S1A-C). Generally, it was observed that female samples contain a higher DNA concentration than male samples (Figure 2C; Figure 3A-C). However, additional statistically significant differences were observed for Promega between healthy female volunteers (avg: 7.2267 ng/µL) and breast cancer patients (avg: 1.4383 ng/µL, p=0.0108) or pregnant women (avg: 2.1219 ng/µL, p=0.0072; Figure 3B) and, for Qiagen between healthy female volunteers (avg: 8.0383 ng/µL) and pregnant women (avg: 2.4054 ng/µL; p=0.0015; Figure 3C).

Additionally, when comparing the DNA content normalized for the start volume of urine for all three extraction methods (Norgen, 12 mL; Promega, 4 mL; Qiagen, 4 mL), it was seen that Norgen (mean: 10.9869 ng/mL) was statistically significantly outperformed by Promega (mean: 40.4235 ng/mL; p=0.0357) and Qiagen (mean: 40.1625 ng/mL; p=0.0302; Figure 2B,D). When looking into more detail and comparing the different participant types per extraction method nine statistically significant differences were found, of which only two (Figure 2D) were between extraction methods and the remaining seven (Figure 3D-F) were between participant types within a certain extraction method. When looking at the participant types, it was seen that Norgen was statistically significantly outperformed by Promega (p=0.0011) and Qiagen (p=0.0058) mainly for the samples from healthy female volunteers (Figure 2D). Generally, it was observed that Qiagen showed a higher DNA content for almost all participant types compared to Norgen and Promega and, that female samples contain a higher DNA content than male samples (Figure 2D; Figure 3D-F; Figure S1D-F). There were no additional statistically significant differences found for the Norgen extraction (Figure 3D). However, for Promega additional statistically significant differences were found between healthy female volunteers (avg: 108.4 ng/mL) and breast cancer patients (avg: 21.5739 ng/mL, p<0.0001) or pregnant women (avg: 31.8285 ng/mL, p<0.0001; Figure 3E) and, for Qiagen between healthy female volunteers (avg: 100.479 ng/mL) and pregnant women (avg: 30.0671 ng/mL, p=0.0003; Figure 3F).

Since interest was sparked in short fragments of DNA, the DNA fragmentation profile of all samples was further investigated. For this study, short fragments were defined as fragments with sizes between 50-450bp, and long fragments as fragments of sizes higher than 451bp. Here, as well it was demonstrated that female samples (healthy female volunteers, pregnant women and, breast cancer patients) showed statistically significantly higher short and long fragment concentration than male samples (healthy male volunteers and prostate cancer patients) (Figure 4). The DNA profiles determined using TapeStation Analysis Software showed no profound differences between the different extraction methods nor the different participant types (Figure S2).

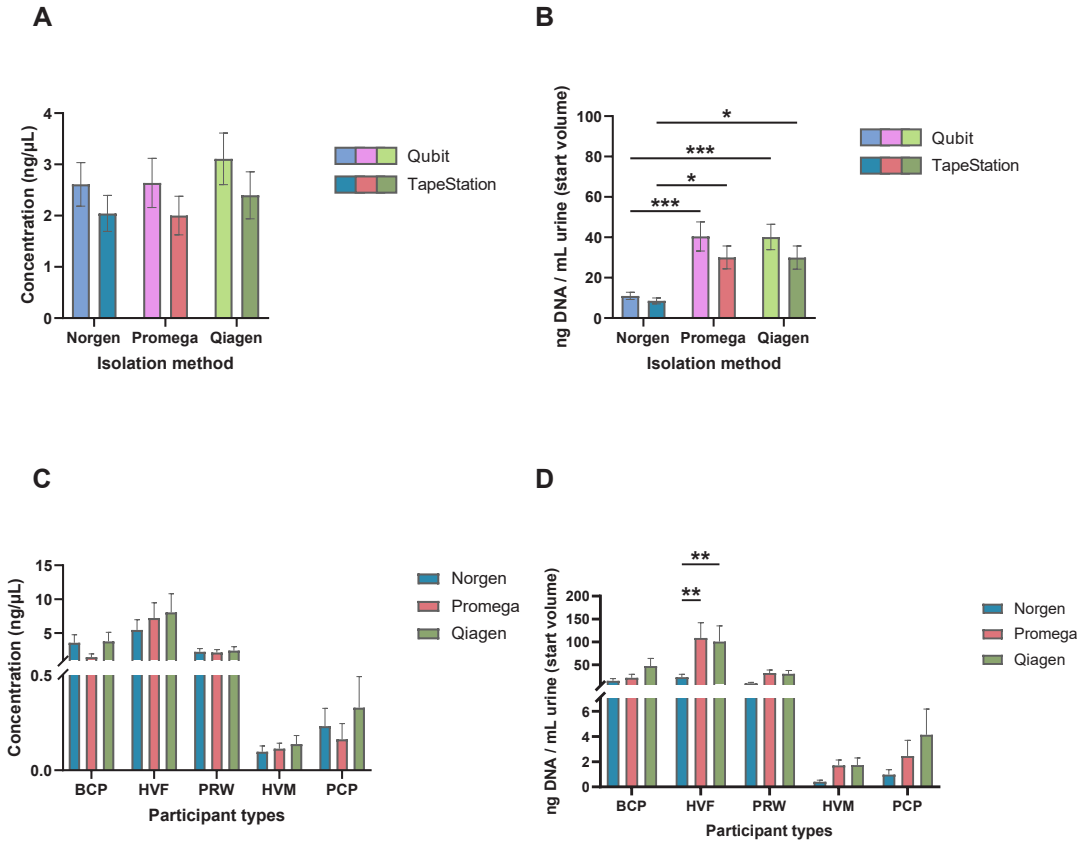

Figure 2: Comparison of three commercially available extraction kits (Norgen, Promega, Qiagen) based on DNA concentration and DNA content. The DNA concentrations per extraction method (A), the amount of DNA normalized against the urine start volume per extraction method (B), the DNA concentrations per participant type according to extraction method measured using TapeStation (C), the amount of DNA normalized against the urine start volume per participant type according to extraction method measure using TapeStation (D). This experiment was performed with five participant types: BCP (n=5), HVF (n=4), HVM (n=6), PRW (n=17) and, PCP (n=5). Outliers were examined and if identified, they were removed from statistical analysis: the samples from one HVF participant were removed from all (A-D). Factorial ANOVA, with Tukey correction for multiple testing for the factors participant type and extraction method was performed (A-D). The factor 'Participant type' was statistically significant in (C) $p<0.0001$ and (D), $p<0.0001$. The factor 'extraction method' was statistically significant in (D), $p<0.0001$. The interaction of 'Participant type' and 'extraction method' was statistically significant in (D), $p=0.0085$. The detailed overview of statistically significant differences for the participant types per extraction method are shown in Figure. For all experiments, statistical significance levels for comparison: * p = 0.0332 ; ** = p 0.0021; *** = p 0.0002; **** p < 0.0001. Data are presented as mean ± SEM. Abbreviations: BCP, breast cancer patients; HVF, healthy female volunteers; HVM, healthy male volunteers; Norgen, Urine Cell-Free Circulating DNA Purification Kit; PCP, prostate cancer patients; Promega, Maxwell® RSC Circulating DNA Purification Kit; PRW, pregnant women; Qiagen, QIAamp® Circulating Nucleic Acid Kit.

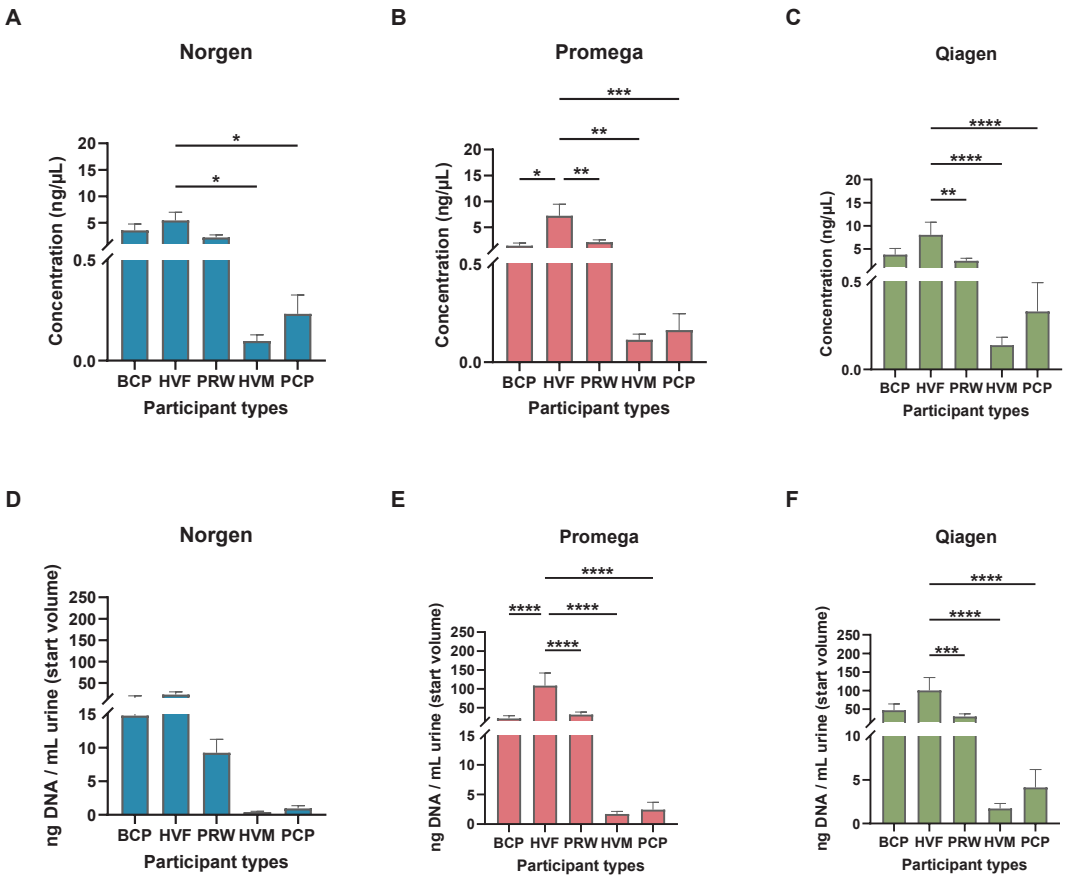

Figure 3: Comparison of DNA concentrations and DNA content per extraction method (Norgen, Promega, Qiagen) and participant type measured using TapeStation. The DNA concentration per participant type for Norgen (A), Promega (B), Qiagen (C), the amount of DNA normalized against the urine start volume per participant type for Norgen (D), Promega (E), Qiagen (F). This experiment was performed with five participant types: BCP (n=5), HVF (n=4), HVM (n=6), PRW (n=17) and, PCP (n=5). Outliers were examined and if identified, they were removed from statistical analysis: the samples from one HVF participant were removed from all (A-F). Factorial ANOVA, with Tukey correction for multiple testing for the factors 'participant type' and 'extraction method' was performed (A-F). The factor 'participant type' was significant ($p<0.00001$) for all (A-F). For all experiments, statistical significance levels for comparison: * $p = 0.0332$; ** $p = 0.0021$; *** $p = 0.0002$; **** $p < 0.0001$. Data are presented as mean ± SEM. Abbreviations: BCP, breast cancer patients; HVF, healthy female volunteers; HVM, healthy male volunteers; Norgen, Urine Cell-Free Circulating DNA Purification Kit; PCP, prostate cancer patients; Promega, Maxwell® RSC Circulating DNA Purification Kit; PRW, pregnant women; Qiagen, QIAamp® Circulating Nucleic Acid Kit.

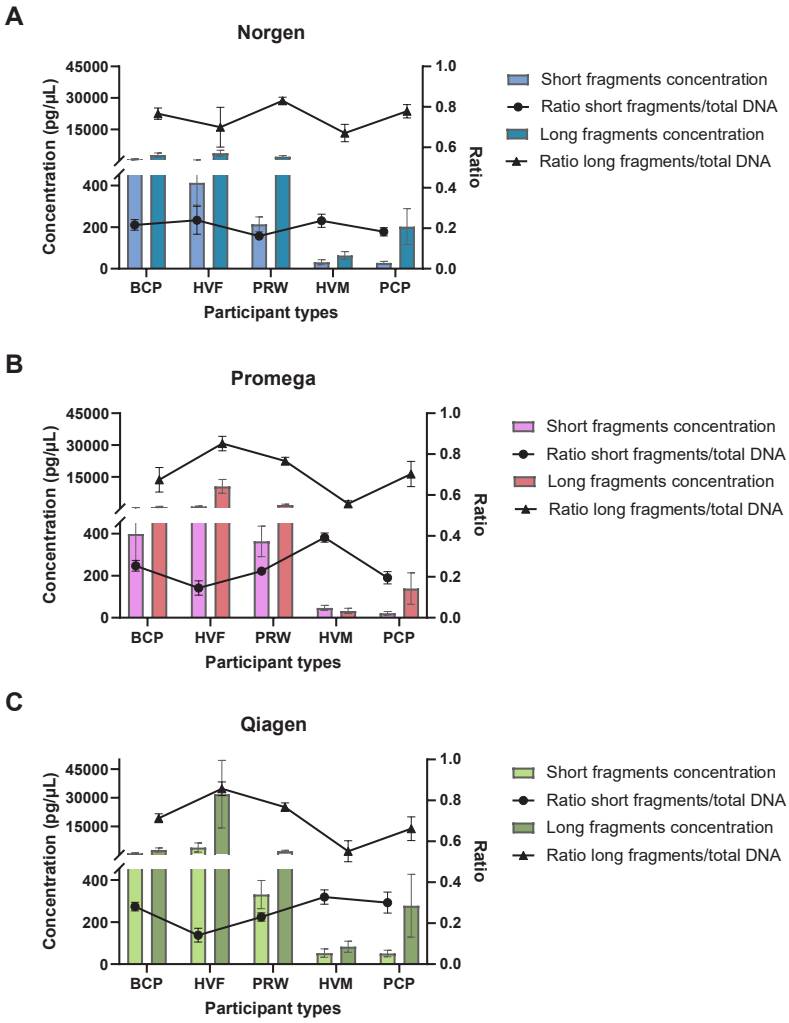

Figure 4: Comparison of three commercially available extraction kits (Norgen, Promega, Qiagen) based on DNA fragmentation profiles. The concentrations and ratios of short and long fragments for Norgen (A), Promega (B), Qiagen (C). This experiment was performed with five participant types: BCP (n=5), HVF (n=4), HVM (n=6), PRW (n=17) and, PCP (n=5). For statistical analysis, Kruskal-Wallis test with Dunn's correction for multiple testing was performed (A-C). For all experiments, statistical significance levels for comparison: * p = 0.0332 ; ** p = 0.0021; *** p = 0.0002; **** p < 0.0001. Data are presented as mean ± SEM. Abbreviations: BCP, breast cancer patients; HVF, healthy female volunteers; HVM, healthy male volunteers; long fragments, fragments with sizes >451bp; Norgen, Urine Cell-Free Circulating DNA Purification Kit; PCP, prostate cancer patients; Promega, Maxwell® RSC Circulating DNA Purification Kit; PRW, pregnant women; Qiagen, QIAamp® Circulating Nucleic Acid Kit; short fragments, fragments with sizes 50-450bp.

3.3. Comparison of commercially available manual extraction kit (Qiagen) and semi-automated system (nRichDX).

The Qiagen starting volume of urine was 4 mL, while for nRichDX a starting volume of 20 mL was used. No statistically significant differences between the Qiagen and nRichDX extraction method were observed when looking at DNA concentrations (Qiagen avg: 1.6256 ng/µL; nRichDX avg: 10.1622 ng/µL; Figure 5A), DNA content normalized against the start volume of urine (Qiagen avg: 10.3195 ng/mL; nRichDX avg: 25.4055 ng/mL; Figure 5C) and the percentage of short fragments in the samples (Figure 5D). Although, a statistically significant difference was found between Qiagen and nRichDX when looking at the concentration of short fragments in the samples (Qiagen avg: 264.84 pg/µL; nRichDX avg: 1281.8 pg/µL; p=0.0239; Figure 5B). However, no statistically significant difference was seen when comparing the ratio of short fragments over the total DNA, the concentration of long fragments and, the ratio of long fragments over the total DNA in the samples (Figure 5B). The DNA profiles determined using TapeStation Analysis Software led to the general remark that since the concentration of nRichDX samples was higher, higher sample intensity (normalized FU) value were observed. However, when looking to the trend for Qiagen and nRichDX no profound differences were seen (Figure S3).

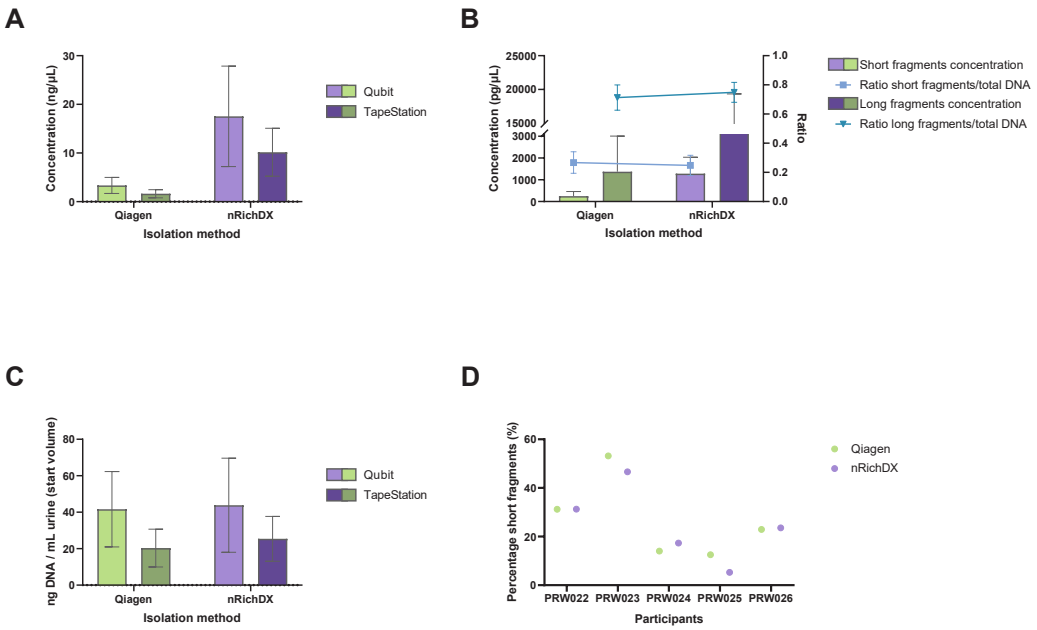

Figure 5: Comparison of commercially available manual extraction kit (Qiagen) and semi-automated system (nRichDX) using samples from pregnant women (n=5). The DNA concentration per extraction method and measurement method (A), The amount of DNA normalized against the urine start volume per extraction method and measurement method (C), The concentrations and ratios of short and long fragments per extraction method (B), the percentage of short fragments per extraction method per sample (D). For statistical analysis, 2-way ANOVA with Tukey correction for multiple testing (A, C), paired T-test (B), Wilcoxon matched-pairs signed rank test (B, D) were performed. Statistical significance levels for comparison: * p = 0.0332 ; ** p = 0.0021; *** p = 0.0002; **** p = < 0.0001. Data are presented as mean ± SEM (A-C) and, individual values (D). Abbreviations: BCP, breast cancer patients; HVF, healthy female volunteers; HVM, healthy male volunteers; nRichDX, Revolution System®; long fragments, fragments with sizes >451bp; PCP, prostate cancer patients; PRW, pregnant women; Qiagen, QIAamp® Circulating Nucleic Acid Kit; short fragments, fragments with sizes 50-450bp

3.3. Comparison of Colli-Pee® UAS™ variants collecting different volumes.

For the third experiment, three Colli-Pee® UAS™ device variants collecting different volumes (Colli-Pee® UAS™ FV-5010, Colli-Pee® UAS™ FV-5020 and Colli-Pee® UAS™ FV-5040 (Novosanis, Wijnegem, Belgium), were compared.
No statistically significant differences were found between the three Colli-Pee® UAS™ variants collecting different volumes when looking at DNA concentrations (FV-5010 avg: 12.692 ng/µL; FV-5020 avg: 8.494 ng/µL; FV-5040 avg: 11.213 ng/µL; Figure 6A) and amount of DNA normalized against the start volume of urine (FV-5010 avg: 31.73 ng/µL; FV-5020 avg: 21.23 ng/µL; FV-5040 avg: 28.03 ng/µL; Figure 6B). When looking into more detail and comparing the different participant types per Colli-Pee® UAS™ variant no statistically significant differences were found (Figure 6C, D; Figure 7A-F; Figure S4A-F). Generally, it was observed that Colli-Pee® UAS™ FV-5010 (concentration avg: 12.692 ng/µL; DNA per start volume avg: 31.73 ng/mL) showed a higher DNA content for almost all participant types compared to Colli-Pee® UAS™ FV-5020 (concentration average: 8.494 ng/µL; DNA per start volume avg: 21.234 ng/mL) and Colli-Pee® UAS™ FV-5040 (concentration avg: 11.213 ng/µL; DNA per start volume avg: 28.032 ng/mL; Figure 6A, B). Additionally, here as well it was observed that female samples contain a higher DNA content than male samples (Figure 6C, D; Figure 7A-F; Figure S4A-F).

For this experiment the DNA fragmentation profiles, determined using TapeStation Analysis Software, of all samples were investigated to determine the concentration of short (size of 50-450bp) and long (size > 451bp) fragments and

the ratio of short and long fragments. No major differences were found for the short and long fragment concentrations nor ratios for the different Colli-Pee® UAS™ variants or participant types, except for the fact that the concentration of both short and long fragments was lower for prostate cancer patients than for the other participant types (Figure 8). The DNA profiles determined using TapeStation Analysis Software showed no profound differences (except for concentration based differences) between the different Colli-Pee® UAS™ variants nor the different participant types (Figure S5). All samples, regardless of Colli-Pee® UAS™ variant or participant type, showed a peak between 150-300bp (Figure S5).

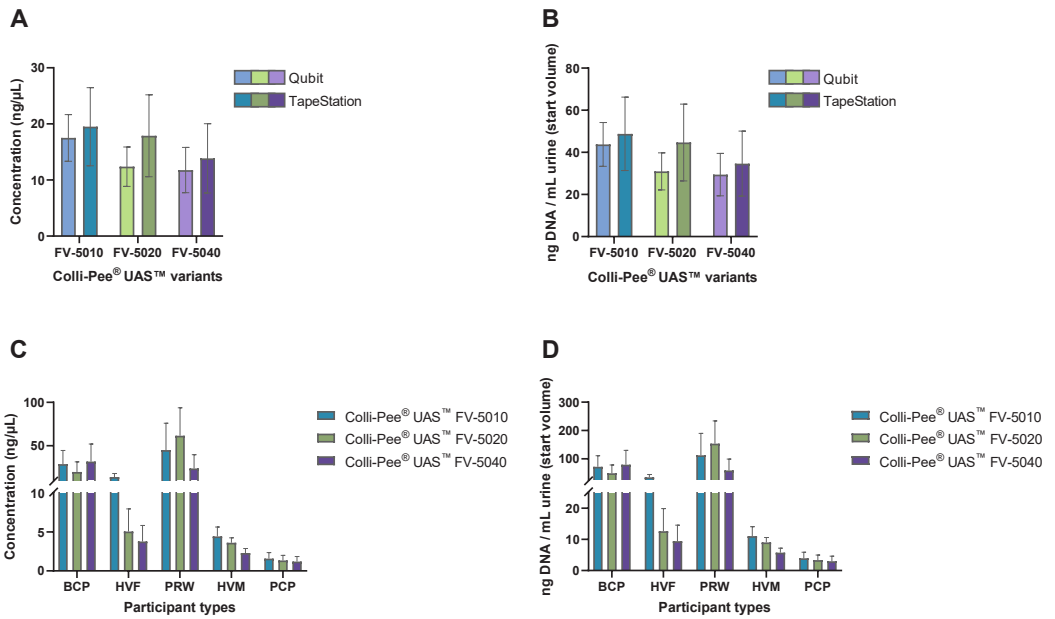

Figure 6: Comparison of Colli-Pee® UAS™ variants collecting different volumes based on DNA concentration and DNA content. The DNA concentrations per Colli-Pee® UAS™ variant (A), the amount of DNA normalized against the urine start volume per Colli-Pee® UAS™ variant (B), the DNA concentrations per participant type according to Colli-Pee® UAS™ measured using TapeStation (C), the amount of DNA normalized against the urine start volume per participant type according to Colli-Pee® UAS™ measure using TapeStation (D). This experiment was performed with five participant types: BCP (n=6), HVF (n=5), HVM (n=5), PRW (n=4) and, PCP (n=3). Factorial ANOVA, with Tukey correction for multiple testing for the factors 'participant type' and 'Colli-Pee® UAS™ variant' was performed (A-D). The factor 'Participant type' was statistically significant in (C) p=0.0042 and (D), p=0.0042. The detailed overview of differences for the participant types per Colli-Pee® UAS™ variant are shown in Figure. For all experiments, statistical significance levels for comparison: * p = 0.0332 ; ** = p 0.0021; *** = p 0.0002; **** p < 0.0001. Data are presented as mean ± SEM. Abbreviations: BCP, breast cancer patients; Colli-Pee® UAS™ FV-5010, collecting approximately 10 mL first-void urine; Colli-Pee® UAS™ FV-5020, collecting approximately 20 mL first-void urine; Colli-Pee® UAS™ FV-5040, collecting approximately 40 mL first-void urine; HVF, healthy female volunteers; HVM, healthy male volunteers; PCP, prostate cancer patients; PRW, pregnant women.

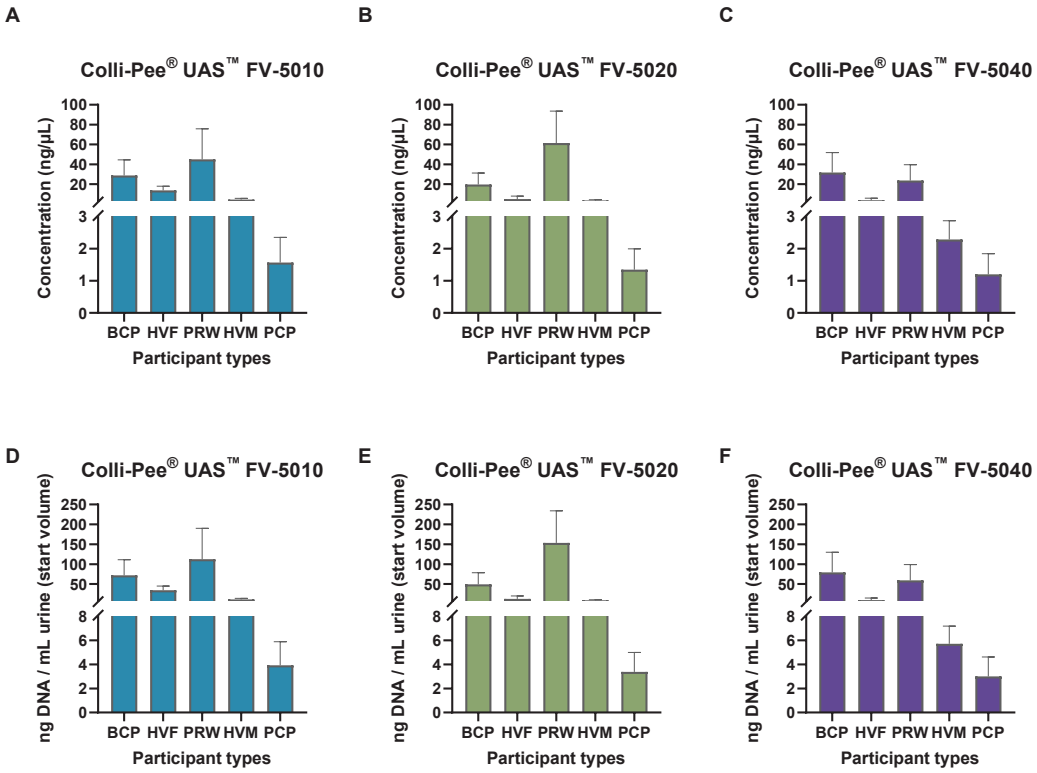

Figure 7: Comparison of DNA concentrations and DNA content per Colli-Pee® UAS™ variant and participant type measured using TapeStation. The DNA concentration per participant type for Colli-Pee® UAS™ FV-5010 (A), Colli-Pee® UAS™ FV-5020 (B), Colli-Pee® UAS™ FV-5040 (C), the amount of DNA normalized against the urine start volume per participant type for Colli-Pee® UAS™ FV-5010 (D), Colli-Pee® UAS™ FV-5020 (E), Colli-Pee® UAS™ FV-5040 (F). This experiment was performed with five participant types: BCP (n=6), HVF (n=5), HVM (n=5), PRW (n=4) and, PCP (n=3). Factorial ANOVA, with Tukey correction for multiple testing for the factors 'participant type' and 'Colli-Pee® UAS™ variant' was performed (A-F). The factor 'participant type' was significant (p=0.0042) for all (A-F). For all experiments, statistical significance levels for comparison: * p = 0.0332 ; ** p = 0.0021; *** p = 0.0002; **** p < 0.0001. Data are presented as mean ± SEM. Abbreviations: BCP, breast cancer patients; Colli-Pee® UAS™ FV-5010, collecting approximately 10 mL first-void urine; Colli-Pee® UAS™ FV-5020, collecting approximately 20 mL first-void urine; Colli-Pee® UAS™ FV-5040, collecting approximately 40 mL first-void urine; HVF, healthy female volunteers; HVM, healthy male volunteers; PCP, prostate cancer patients; PRW, pregnant women.

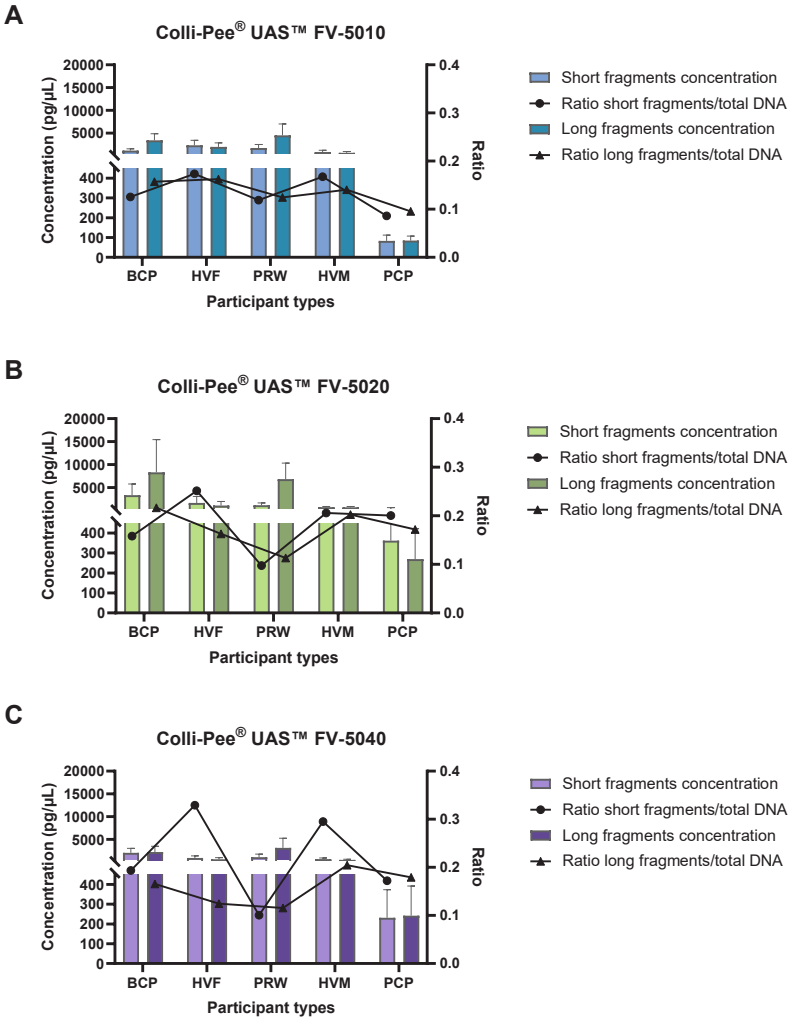

Figure 8: Comparison of Colli-Pee® UAS™ variants collecting different volumes based on DNA fragmentation profiles. The concentrations and ratios of short and long fragments for Colli-Pee® UAS™ FV-5010 (A), Colli-Pee® UAS™ FV-5020 (B), Colli-Pee® UAS™ FV-5040 (C). This experiment was performed with five participant types: BCP (n=6), HVF (n=5), HVM (n=5), PRW (n=4) and, PCP (n=3). For statistical analysis, Kruskal-Wallis test with Dunn's correction for multiple testing was performed (A-C). For all experiments, statistical significance levels for comparison: * p = 0.0332 ; ** p = 0.0021; *** p = 0.0002; **** p < 0.0001. Data are presented as mean ± SEM. Abbreviations: BCP, breast cancer patients; Colli-Pee® UAS™ FV-5010, collecting approximately 10 mL first-void urine; Colli-Pee® UAS™ FV-5020, collecting approximately 20 mL first-void urine; Colli-Pee® UAS™ FV-5040, collecting approximately 40 mL first-void urine; HVF, healthy female volunteers; HVM, healthy male volunteers; long fragments, fragments with sizes >451bp; PCP, prostate cancer patients; PRW, pregnant women; short fragments, fragments with sizes 50-450bp.

3.5. Detection trans-renal DNA

For the overlapping experiment, the presence of trans-renal DNA was measured by detecting the male gene SRY using ddPCR in women pregnant with male fetus. First, ddPCR measurements were performed directly on the eluates of the isolates, however these measurements did not yield useful results. Therefore, a whole-genome amplification (WGA) was performed on the eluates before the ddPCR measurements were performed. This procedure was seen as successful and promising based on runs of healthy female and male volunteer samples and various dilutions after WGA. The post-WGA dilutions were necessary to dilute the ddPCR inhibitors of the WGA procedure. After this optimization, samples from the pregnant women from all three experiments underwent WGA for 20h, after which they were diluted 1/50. The results of all three experiments are summarized in Table 2. It was demonstrated that to achieve an assay sensitivity of 0.5% the limit of detection was 1600 reference droplets and that measurements would be reliable when at least 4800 reference droplets are measured. Most results were at the limit of detection or did not reach the 4800 reference droplets and were therefore uninformative. Only five samples from the different experiments were reliable, but showed negative results despite the women giving birth to a boy. It is clear that this ddPCR SRY procedure needs more optimization before conclusions can be drawn regarding the detection of trans-renal DNA in urine.

Table 2: Overview results trans-renal DNA detection by ddPCR SRY assay after Whole-Genome Amplification. Legend conclusion: the chosen sensitivity was 0.5%, therefore a minimum of 1600 reference droplets are required (otherwise the measurement is indicated as LOD) and when at least 4800 reference droplets are found the measurement is trustworthy (otherwise it is indicated as non-informative). Abbreviations: Colli-Pee® UAS™ FV-5010, collecting approximately 10 mL first-void urine; Colli-Pee® UAS™ FV-5020, collecting approximately 20 mL first-void urine; Colli-Pee® UAS™ FV-5040, collecting approximately 40 mL first-void urine; LOD, limit of detection based; NI, non-informative; PRW, pregnant women.

Participant	Gender baby	Variable	Target droplets	Reference droplets	Conclusion
Comparison of three commercially available extraction kits (Norgen, Promega and, Qiagen)					
PRWUcfN001Aa	Boy	Norgen	0	544	LOD, NI
PRWUcfP001Aa	Boy	Promega	0	498	LOD, NI
PRWUcfQ001Aa	Boy	Qiagen	0	559	LOD, NI
PRWUcfN003Aa	Boy	Norgen	0	1.584	LOD, NI
PRWUcfP003Aa	Boy	Promega	0	256	LOD, NI
PRWUcfQ003Aa	Boy	Qiagen	0	1.430	LOD, NI
PRWUcfN004Aa	Boy	Norgen	0	852	LOD, NI
PRWUcfP004Aa	Boy	Promega	0	321	LOD, NI
PRWUcfQ004Aa	Boy	Qiagen	0	299	LOD, NI
PRWUcfN006Aa	Boy	Norgen	1	424	LOD, NI
PRWUcfP006Aa	Boy	Promega	0	27	LOD, NI
PRWUcfQ006Aa	Boy	Qiagen	0	262	LOD, NI
PRWUcfN007Aa	Boy	Norgen	0	1.343	LOD, NI
PRWUcfP007Aa	Boy	Promega	0	303	LOD, NI
PRWUcfQ007Aa	Boy	Qiagen	0	928	LOD, NI
PRWUcfN008Aa	Boy	Norgen	0	3.092	NI
PRWUcfP008Aa	Boy	Promega	0	902	LOD, NI
PRWUcfQ008Aa	Boy	Qiagen	0	1.977	NI
PRWUcfN014Aa	Boy	Norgen	0	1.360	LOD, NI
PRWUcfP014Aa	Boy	Promega	0	1.024	LOD, NI
PRWUcfQ014Aa	Boy	Qiagen	0	844	LOD, NI
PRWUcfN015Aa	Boy	Norgen	0	1.407	LOD, NI
PRWUcfP015Aa	Boy	Promega	0	1.692	NI
PRWUcfQ015Aa	Boy	Qiagen	0	342	LOD, NI
PRWUcfN017Aa	Boy	Norgen	0	4.395	NI
PRWUcfP017Aa	Boy	Promega	0	1.024	LOD, NI
PRWUcfQ017Aa	Boy	Qiagen	0	8.500	Negative
PRWUcfN018Aa	Boy	Norgen	0	736	LOD, NI
PRWUcfP018Aa	Boy	Promega	0	4.191	NI
PRWUcfQ018Aa	Boy	Qiagen	0	3.118	NI

Participant	Gender baby	Variable	Target droplets	Reference droplets	Conclusion
PRWUcfN005Aa	Girl	Norgen	0	225	LOD, NI
PRWUcfP005Aa	Girl	Promega	0	1.101	LOD, NI
PRWUcfQ005Aa	Girl	Qiagen	0	1.048	LOD, NI
PRWUcfN013Aa	Girl	Norgen	0	165	LOD, NI
PRWUcfP013Aa	Girl	Promega	1	172	LOD, NI
PRWUcfQ013Aa	Girl	Qiagen	0	702	LOD, NI
PRWUcfN016Aa	Girl	Norgen	0	1.724	NI
PRWUcfP016Aa	Girl	Promega	0	723	LOD, NI
PRWUcfQ016Aa	Girl	Qiagen	0	193	LOD, NI
PRWUcfN012Aa	Unknown	Norgen	0	476	LOD, NI
PRWUcfP012Aa	Unknown	Promega	0	58	LOD, NI
PRWUcfQ012Aa	Unknown	Qiagen	0	266	LOD, NI
Comparison of commercially available manual extraction kit (Qiagen) and semi-automated system (nRichDX)					
PRW022N	Boy	nRichDX	1	622	LOD, NI
PRW022Q	Boy	Qiagen	0	1.666	NI
PRW023N	Boy	nRichDX	0	338	LOD, NI
PRW023Q	Boy	Qiagen	0	634	LOD, NI
PRW024N	Boy	nRichDX	0	408	LOD, NI
PRW024Q	Boy	Qiagen	0	2.957	NI
PRW025N	Boy	nRichDX	0	5.976	Negative
PRW025Q	Boy	Qiagen	0	1.497	LOD, NI
PRW026N	Boy	nRichDX	0	619	LOD, NI
PRW026Q	Boy	Qiagen	1	1.790	NI
Comparison of Colli-Pee® UAS™ variants collecting different volumes					
PRWUCP1001	Boy	FV-5010	0	4.713	NI
PRWUCP2001	Boy	FV-5020	0	5.323	Negative
PRWUCP3001	Boy	FV-5040	1	3.398	NI
PRWUCP1002	Boy	FV-5010	1	2.645	NI
PRWUCP2002	Boy	FV-5020	0	1.495	LOD, NI
PRWUCP3002	Boy	FV-5040	0	4.818	Negative
PRWUCP1019	Boy	FV-5010	1	2.083	NI
PRWUCP2019	Boy	FV-5020	0	3.075	NI
PRWUCP3019	Boy	FV-5040	0	1.541	LOD, NI
PRWUCP1020	Boy	FV-5010	0	4.079	NI
PRWUCP2020	Boy	FV-5020	0	6.134	Negative
PRWUCP3020	Boy	FV-5040	0	3.562	NI

Discussion

04

Despite the great potential of urine as liquid biopsy, no standardized protocols for pre-analytical parameters are available[24], which is surprising as it has been shown that most of the test errors are due to problems during the pre-analytical phase[25, 26]. Therefore, it is of utmost importance to use appropriate pre-analytical conditions, such as optimized collection and preservation methods and, storage and transport temperatures, to achieve accurate detection of urinary biomarkers[1, 21, 22, 27]. Additionally, the optimization of cfDNA isolation, including sample preparation steps, concentration steps and the extraction step, towards methods capable of efficiently capturing short, diluted DNA fragments is critical to maximize the clinical sensitivity and reproducibility[28]. In this study, we compared four different urinary cfDNA extraction methods, Urine Cell-Free Circulating DNA Purification Kit (Norgen Biotek Corp., Ontario, Canada); the Maxwell® RSC Circulating DNA Purification Kit (Promega, Madison, Wisconsin, USA); the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and, the Revolution System® (nRichDX, Irvine, California, USA). Additionally, we compared the three different Colli-Pee® UAS™ device formats: Colli-Pee® UAS™ FV-5010, Colli-Pee® UAS™ FV-5020 and Colli-Pee® UAS™ FV-5040 (Novosanis NV, Wijnegem, Belgium).

4.1. cfDNA extraction method

There are several DNA extraction methods on the market, each with their own advantages and limitations. Recently, different research groups have explored cfDNA extraction methods for urine[28-30]. Since not much research has been done on urinary extraction methods in combination with preservatives, four different urinary cfDNA extraction methods, Urine Cell-Free Circulating DNA Purification Kit (Norgen Biotek Corp., Ontario, Canada); the Maxwell® RSC Circulating DNA

Purification Kit (Promega, Madison, Wisconsin, USA); the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and, the Revolution System® (nRichDX, Irvine, California, USA) were compared.

Our first results revealed that no statistically significant differences were found between the Norgen, Promega and Qiagen extraction methods based on DNA concentrations, short (50-450bp) or long (>451bp) fragment concentrations, short or long fragment ratios and the DNA profiles. Although, the differences were not statistically significant, the DNA concentration was slightly higher in the samples isolated with Qiagen than Promega and Norgen. However, Norgen was statistically significantly outperformed by Promega and Qiagen when comparing the DNA content normalized to the starting volume of urine for all three extraction methods (Norgen, 12 mL; Promega, 4 mL; Qiagen, 4 mL). This can be explained by the starting volumes, Norgen's starting volume was higher than the other two. However, normalizing against these volumes assumes that when a certain volume is taken, a certain amount of DNA will be recovered. While using the same amount of urine volume for Qiagen and Promega requires three times as many extractions, the eluates must be combined and followed by a concentration step (to evaporate the excess of elution buffer), compared to Norgen, allowing up to 30 mL of urine in a single extraction.

Based on the first results, Qiagen slightly outperformed the other two and was therefore compared to nRichDX's recently launched automated Revolution system®. This comparison revealed that there were no statistically significant differences between the Qiagen and nRichDX extraction method when looking at DNA concentrations, DNA content normalized against the start volume of urine, the percentage of short fragments in the samples, the ratio of short fragments over the total DNA, the concentration of long fragments and, the ratio of long fragments over the total DNA in the samples. However, a statistically significant difference was found between Qiagen and nRichDX when looking at the concentration of short fragments in the samples. The DNA profiles determined using TapeStation Analysis Software led to the general observation that higher sample intensity values were observed due to the higher concentration of nRichDX samples. However, when looking to the trends in the DNA profiles for Qiagen and nRichDX no profound differences were seen.

Our data systematically confirms previous findings that the cfDNA concentration is higher in urine samples of female individuals than male individuals[21]. The

difference in overall DNA concentration between female and male individuals is probably due to the collection of first-void urine in this study. First-void urine from female individuals is expected to contain the genital and vaginal secretions that were accumulated between the urethra and small labia. While first-void urine of male individuals is expected to mainly contain trans-renally cleared cfDNA and potentially some gDNA coming from the bladder and prostate.

Table 2 summarizes the qualitative and clinically relevant characteristics of the urinary cfDNA extraction methods compared in this study. There were some clear differences between the four extraction methods, such as (i) the Norgen and Qiagen methods are based on spin columns, while the Promega and nRichDX methods are based on magnetic beads, (ii) the Norgen and nRichDX methods allow higher starting volumes (up to 30 mL and 20mL, respectively) than the Promega and Qiagen methods (4 mL) and, (iii) the Norgen, Qiagen and nRichDX method consist of a pre-binding lysis step, while the Promega method does not include a lysis step. This latter difference may have certain consequences, as the lysis step could imply that nucleic acids attached to proteins or entrapped in vesicles and/or exosomes are also captured during extraction. Furthermore, there are also some clinically relevant differences, such as (i) the hands-on time required to perform a single extraction run, (ii) the number of transfers directly related to the probability of errors, (iii) the cost per sample and, (iv) the need for additional equipment beyond standard laboratory equipment. The hands-on time was observed to be similar for Norgen, Promega and nRichDX (25-30 min) and longer for Qiagen (55 min). Norgen and Promega require 3 transfers and Qiagen 2 transfers all of which can be categorized as medium risk of errors, while nRichDX only requires 1 transfer and therefore gets a low risk score for errors. Looking at the cost per sample, it became clear that Promega was the economical method, followed by Norgen and Qiagen. For nRichDX, the cost per sample depended on the starting volume of urine, using small volumes is cheaper than using larger volumes. Of note, only Norgen offers an extraction procedure that does not require specialized equipment.

Based on our experimental results and the qualitative and clinically relevant characteristics, we performed the follow-up experiments with the nRichDX extraction method. The decision was made based on the potential for high-throughput, short hands-on time, allowing high starting volumes of urine in a single extraction and low number of sample transfers and thus low risk of errors. These are all important factors when considering bringing a standardized urine liquid biopsy workflow to the clinic.

Table 2: Comparison of cfDNA extraction kits used in this study

Parameters	Norgen	Promega	Qiagen	nRichDX
cfDNA extraction kit	Urine Cell-Free Circulating DNA Purification Kit	Maxwell® RSC Circulating DNA Purification Kit	QIAamp® Circulating Nucleic Acid Kit	Revolution™ cfDNA Max 20XL Kit
Kit type	Spin column	Magnetic beads, Semi-automated	Spin column	Magnetic beads, semi-automated
Lysis step before cfDNA capture	Yes	No	Yes	Yes
Urine volume (mL)	Up to 30	2-Apr	1-Apr	Up to 20
Elution volume (µL)	50-100	75	20-150	25-100
Extractions per run	Unlimited****	16	24	12
Protocol steps per run	12	6	17	12
Total run time (min)*	60	80	120	180
Hands-on time (min)*	25	30	55	30
Number of transfers	3	3	2	1
Chance for errors	Medium	Medium	Medium	Low
Cost per sample**	Medium	Low	Medium	Volume dependent (1-5 mL, Low; 6-10 mL, Medium; 11-15 mL, Medium; 16-20 mL, High)
Additional equipment***	N/A.	Maxwell® RSC Instrument	Vacuum pump	Revolution system®
Additional equipment cost (€)	N/A.	29567.00	388.00	15000.00

* For a full run; sample extraction time only, not including downstream analysis; ** Sample extraction cost only, not including downstream analysis. (scale for prices per sample: low, < €25; medium, €25-75 ; high, > €75); *** All special additional equipment, outside standard laboratory equipment such as centrifuges; **** Not automated or equipment dependent.

4.2. Colli-Pee® UAS™ volume variants

Currently, the most commonly used method of collecting urine is a urine collection cup. However, these cups are not designed to collect the first fraction of urine, while this fraction has been shown to contain high concentrations of certain biomarkers[31-34]. Colli-Pee® is a commercially available device that collects first-void urine in a standardized and volumetric manner, without the individual having to interrupt the urine flow. Furthermore, Colli-Pee® can already be combined with two commercially available preservatives, namely the UCM® and UAS™ preservatives, to enable immediate preservation of the urinary analytes for shipment and storage. As mentioned above, the standardization of the liquid biopsy workflow is of utmost importance to further elaborate on the potential of urine as liquid biopsy. Previously, different Colli-Pee® UCM® devices collecting different first-void urine volumes (Colli-Pee® UCM® FV-5004, collecting approximately 4 mL; Colli-Pee® UCM® FV-5010, collecting approximately 10 mL; Colli-Pee® UCM® FV-5020, collecting approximately 20 mL) have been evaluated for human endpoints and HVP DNA testing[29].

They showed limited differences between the Colli-Pee® UCM® variants for the human endpoints[29], supporting the potential of all three variants as promising analytical and clinical accuracy results are available for Colli-Pee® UCM® FV-5020[33, 35-38]. Furthermore, Téblick et al. suggested that collecting as little as 10 mL could provide an increased sample quality for the detection of HVP DNA and they suggested that 4 mL of first-void urine (approximately 2.33 mL when the preservative is taken into account) is not sufficient to wash away all secretions that have accumulated between the urethra and small labia[29]. These were already some promising results in terms of volume optimization. However, there are some differences between the preservative-to-sample ratio between the UCM® preservative (1:3) and the UAS™ preservative (2:5) Colli-Pee® device variants. Moreover, the Colli-Pee® UAS™ device is also available in a larger volume variant, being Colli-Pee® UAS™ FV-5040, which collects approximately 40 mL of urine.

Therefore, the different Colli-Pee® UAS™ variants were evaluated for oncology applications and trans-renal biomarker detection. No statistically significant differences were found between the three Colli-Pee® UAS™ variants collecting different volumes when looking at DNA concentrations and amount of DNA normalized to the starting volume of urine nor for the short or long fragment concentrations or ratios. Although, in general, Colli-Pee® UAS™ FV-5010 was

observed to have a higher DNA content for almost all participant types compared to Colli-Pee® UAS™ FV-5020 and Colli-Pee® UAS™ FV-5040. This could be explained by the fact that in the Colli-Pee® FV-5010 devices the wash away of all secretions that have accumulated between the urethra and small labia is more concentrated then in the Colli-Pee® FV-5020 and FV-5040 devices.

4.3. Detection of trans-renal DNA

The isolated cfDNA can be amplified and detected by established methods, including polymerase chain reaction (PCR), real-time PCR (qPCR), digital droplet PCR (ddPCR), and next generation sequencing (NGS). The frequency of detecting cfDNA mutations or trans-renal biomarkers in urine often depends on the detection method and the source of the sample. Recent studies have shown the feasibility to detect cfDNA mutations and biomarkers of molecular residual disease in urine[39-41]. Most of them performed custom isolation/extraction/concentration workflows to enhance the concentration of short DNA fragments. Here, the aim was to investigate the presence of trans-renal DNA in the isolated cfDNA by detecting male genes such as DYS14 and SRY in women pregnant with a boy. First, literature searches were performed to investigate promising primers for DYS14 and SRY. The most promising combinations of primers and probes for short fragments were chosen and ordered to design a proprietary ddPCR assay. These designed primers and probes did not perform as expected on healthy volunteer samples. Several optimization attempts were made before this option was abandoned. Second, a ddPCR copy number variation (CNV) assay for SRY (Bio-Rad, Hercules, California, USA) was ordered. This assay would amplify and detect an amplicon of 69bp, which was slightly larger than the proprietary designed assays. The manufacturer validated this assay, but did not list on their validation documentation on which samples the validation was performed. They did mention that the assay specificity was 95%[42]. Additionally, a recent publication showed promising results for this SRY ddPCR assay to determine the gender of the baby in plasma samples of pregnant women[43]. All this information combined made the Bio-Rad SRY ddPCR CNV assay a promising second option for detecting trans-renal cfDNA in urine. The experiments with healthy volunteers for this Bio-Rad SRY ddPCR CNV assay were promising, with male samples positive and female samples negative. Even mixtures of female and male samples tested positive up to a certain sensitivity. After different optimization steps, the study samples were run. However, when running the samples of the

pregnant women, all samples were found to be negative, uninformative or at the limit of detection. As a third option, a DNA amplification step was added by WGA using random primers before the ddPCR reaction. The purpose of this WGA was to increase the amount of input molecules for the ddPCR reaction, as the concentration of male DNA fragments was expected to be too low to be detected. For the healthy volunteers samples, the WGA clearly showed that more positive droplets were detected with WGA than without WGA. Again, several optimization steps were performed before running the study samples. However, the samples of the pregnant women were again negative, uninformative or at the limit of detection. After all attempts, the samples and budget became exhausted and further research was no longer possible. Therefore, despite all efforts and optimizations, we could not draw any conclusions about the detection of transrenal DNA in urine using ddPCR.

Other downstream analysis techniques that we can look further into for future studies are: the minimally-invasive prenatal test (NIPT) or other next-generation sequencing (NGS) based assays on urine samples. Currently, NIPT is performed on blood samples and used to determine the baby's risk of certain genetic disorders and its gender. NGS can further be applied in several ways: (i) clinical exosome sequencing (CES), which includes genes associated with a known clinical association with disease; (ii) whole exome sequencing (WES), which includes protein coding segments from all known genes representing approximately 1-2% of the genome and; (iii) whole genome sequencing (WGS), which additionally includes regulatory genomic sequences, introns and other non-coding sequences.

4.4. Limitations of the study

The main limitation of this study that should be acknowledged is the relatively small sample size, which warrants caution when interpreting the results.

Conclusion

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Urine is an emerging liquid biopsy with broad applicability in cancer research. It has the potential to become a game changers in personalized cancer care allowing for primary and secondary prevention, diagnosis, treatment response monitoring and detection of recurrence. However, standard practice protocols for sample collection, storage, and processing need to be determined to elaborate further on the clinical utility of urine. The current study clearly tackled the important issue of lack of standardization of preanalytical workflows by exploring differences between cfDNA extraction methods and differences in urine volume collected with Colli-Pee® UAS™ devices. The four different urinary cfDNA extraction methods compared in this study, Urine Cell-Free Circulating DNA Purification Kit (Norgen Biotek Corp., Ontario, Canada); the Maxwell® RSC Circulating DNA Purification Kit (Promega, Madison, Wisconsin, USA); the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and, the Revolution System® (nRichDX, Irvine, California, USA), performed comparably with regard to DNA concentrations, short (50-450bp) and long (>451bp) fragment concentrations and ratios, and DNA profiles. The main differences were seen between female and male individuals, where female individuals have higher DNA concentrations than male individuals, possibly linked to the collection of first-void urine. First-void urine from female individuals is expected to contain the genital and vaginal secretions that were accumulated between the urethra and small labia. Furthermore, no statistically significant differences were observed between the three Colli-Pee® UAS™ (Novosanis NV, Wijnegem, Belgium) volume variants (Colli-Pee® UAS™ FV-5010, collecting approximately 10mL; Colli-Pee® UAS™ FV-5020, collecting approximately 20 mL; Colli-Pee® UAS™ FV-5040, collecting approximately 40 mL), indicating their potential in cancer research.

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CHAPTER

06

Usability

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In every job that must be done, there is an element of fun.
You find the fun and snap! the job's a game
(Mary Poppins)

Abstract

First-void urine as sample type has shown great potential for cancer detection, screening and monitoring. It is an emerging liquid biopsy with broad applicability in different analytes and cancers. The aim of this feasibility study was to investigate the experiences of healthy and diseased cohorts of first-void urine collection and preservation using the Colli-Pee® UAS™ devices (Novosanis NV), both for collection during doctors visit as well as home-sampling. Questionnaires from 98 participants enrolled in the URODETECT studies were analyzed. Participants reported that Colli-Pee® was clear (90%) and comfortable (68%) to use. Most respondents (82%) rated their general experience of Colli-Pee® as easy to very easy, while only 2% indicated it was difficult. Participants also reported their intention to use Colli-Pee®again (87%) or to recommend Colli-Pee® to their friends and/or family (88%). A subset of participants (n=60) collected urine samples at home using Colli-Pee® UAS™ devices and returned their samples to the laboratory. Most participants responses were positive (very easy, 35%, easy 40%) about the entire self-sampling process. Urine collection, at home or in the clinic, using Colli-Pee® was clearly accepted by all participants. Furthermore, the self-sampling could offer the possibility to increase at home cancer screening coverage and provide more comfort for individuals.

Introduction

01

In past decades, liquid biopsy samples have gained momentum in several disease fields. In cancer research, liquid biopsy has some advantages over tissue biopsy as it may better capture intratumor heterogeneity and, can be used as a screening tool for early cancer detection, monitoring of treatment response, and detection of recurrence[1-4]. Different body fluids have considerable potential as liquid biopsies for different diseases, like blood which is considered minimally invasive or urine which is considered non-invasive[3]. Urine has added advantages over blood as it is easy to collect, allows for at home sampling, and there is no need for a health care practitioner. Furthermore, urine, allows for serial sampling, multiple tests can be performed on the same sample, and it offers possibilities for multi-omic analysis[5, 6].

Urine as liquid biopsy has shown potential to improve the detection and monitoring of cancers such as prostate cancer[7, 8] and breast cancer[9-11]. The predicted global cancer burden is expected to increase significantly. By 2040, 30.2 million new cancer cases are estimated, compared to 19.3 million cases reported worldwide in 2020[12]. Early detection of cancer can increase the chances of survival and improve the overall quality of life of a patient. Therefore, research into screening methods and sample types enabling screening gained interest in the last two decades. Urine has been proposed as a promising self-sampling method for disease screening. However, guidelines around pre-analytical parameters including collection, preservation, and storage are not yet defined, despite the fact that several studies have already showed the need for urine preservation for oncology applications[13-15]. Several commercial products are already available for nucleic acid preservation, intended for preserving either DNA and/or RNA. Therefore, Novosanis (Wijnegem, Belgium) and DNA Genotek (Ottawa, Canada) developed a urinary preservative that prevents the lysis of bacterial and human

cells, thereby blocking the release of unwanted nucleic acids into the biological sample[16-18]. Currently, the UAS™ preservative (DNA Genotek, Ottawa, Canada) is validated for preventing bacterial growth, preserving cfDNA and maintaining the integrity of host cells for a minimum of 7 days at room temperature[19]. The Colli-Pee® devices (Novosanis NV, Wijnegem, Belgium) on the other hand are developed for the standardized and volumetric collection of first-void (first-catch or first-pass) urine, typically the first part of urine flush collected at any time of the day[20, 21]. When the UAS™ preservative is integrated into the Colli-Pee® devices, these enable standardized volumetric collection and immediate preservation of first-void urine for home-based sampling.

The aim of this feasibility study was to investigate the experiences and preferences of healthy and diseased cohorts for the collection and preservation of first-void (urine using the Colli-Pee® UAS™ devices (Novosanis NV, Wijnegem, Belgium)[20-22], both for collection during a doctor's visit as well as home-sampling. This study did not aim to perform any downstream analysis on the collected urine samples.

Methods

02

2.1. Study population

The questionnaires to assess experiences and preferences were collected from participants in the URODETECT studies. These are prospective studies, aimed to investigate the potential of urine as liquid biopsy for cancer detection (ClinicalTrials.gov identifiers: NCT05453604, NCT05454371, NCT05453591). Study NCT05453604 recruited healthy female and male adult volunteers, breast and prostate cancer patients, and pregnant women, while study NCT05454391 and NCT0545371 recruited healthy female adult volunteers and breast cancer patients or healthy male adult volunteers and prostate cancer patients, respectively. Healthy female and male adult volunteers, breast and prostate cancer patients, and pregnant women were recruited between June 2020 and December 2022 at the Antwerp University Hospital (Belgium) and the University of Antwerp (Belgium). All participants understood Dutch and provided written informed consent.

Participants received a package with a participant information form, a consent form, Colli-Pee® UAS™ device(s) for the collection and preservation of first-void urine (Novosanis, Wijnegem, Belgium), instructions for use (IFU) for the devices, and a questionnaire to be completed online or on paper. Generally, the participant received one Colli-Pee® UAS™ FV-5040 device. Only the subset of participants (n=60) recruited for the home-sampling part received three variants of the device, Colli-Pee® UAS™ FV-5010 (collecting approximately 10 mL), Colli-Pee® UAS™ FV-5020 (collecting approximately 20 mL) and Colli-Pee® UAS™ FV-5040 (collecting approximately 40 mL).

Participants were asked to collect a urine sample using the Colli-Pee® UAS™ device and subsequently complete the questionnaire. The samples, the questionnaire, and the written informed consent were either provided to the nursing staff or mailed to the laboratory of surgical pathology of the Antwerp University Hospital. After arrival in the laboratory, samples were stored to allow for testing on specific urinary biomarkers (not part of this paper). The collected samples were not analyzed as part of this manuscript, as we only aimed to assess usability and acceptability. Ethical approval for the URODETECT studies was provided by the Ethical Committee of the Antwerp University Hospital (no 20/10/115 and B3002021000030).

2.2. Questionnaires

Questionnaires were created in Qualtrics® (Seattle, Washington, USA) online survey software, allowing the possibility to provide a paper or an online questionnaire to the study participants. The questionnaires consisted of a maximum of three categories: general information, Colli-Pee® performance, and home-sampling. These categories were further divided into eight sections: general – collection, general – sexual, general – health information, before collection – preparation of Colli-Pee®, during collection – performance of Colli-Pee®, after collection – disassembly of Colli-Pee®, feedback on overall usage of Colli-Pee®, and home-sampling. The questionnaire had open, multiple choice and rating questions. Rating responses were gathered with a scale ranging from zero, the most negative result, to 100, the most positive result. For the assessment questions, if a given step received a score between 80 and 100, it was classified as in full agreement with the proposed statement. Not always all participants answered all questions, however the number of received answers is clearly indicated throughout the manuscript. The original Dutch questionnaire was translated to English and added as Appendix 1.

2.3. Statistical analysis

Online questionnaire responses were automatically captured in the Qualtrics® online survey software. Questionnaire responses of paper forms were manually entered in the Qualtrics® online survey software, to create one database with all responses.

The questions with open answers were manually analyzed by reading the responses and group them based on similar terms (e.g. for question H2 the terms postal, packaging, shipment were combined in one group). Contingency for Fisher and chi-square tests were used to analyze the categorial variables: the population characteristics (gender, education, profession), the replies to the yes-no, multiple choice and the home-sampling questions. While frequency distribution analyses were performed for the analysis of the continuous variables: population characteristics (age, BMI) and the assessment questions (scores between 0 and 100). All analyses were conducted with GraphPad Prism (version 9.4.1, GraphPad Software Inc., La Jolla, California, USA).

Results

03

3.1. Participant characteristics

Between the 25th of June 2020 and the 31st of December 2022, 100 participants were enrolled in the URODETECT studies. Questionnaires were obtained and analyzed from a total of 98 participants: 59 women and 39 men. The participant's ages ranged from 20 to 83 years old, with a median age of 36 years. Most (84%) of the participants were between 20 and 60 years. Additionally, other relevant characteristics were investigated, including BMI, education, and profession. Most respondents had a higher level of educational (college, university or PhD; 76%). A total of eight breast cancer patients (8%), twenty-eight healthy female volunteers (29%), thirty-one healthy male volunteers (32%), twenty-three pregnant women (23%) and eight prostate cancer patients (8%) completed the questionnaires. Overall participant characteristics are compiled in Table 1.

Table 1: Characteristics of the participants enrolled in the study (N=98)

Characteristics	Number	Percentage
Age	96 [‡]	100%
18-29 years	24	25%
30-39 years	32	33%
40-49 years	10	10%
50-59 years	15	16%
60-69 years	9	9%
70-... years	6	6%

Characteristics	Number	Percentage
Gender identity	98	100%
Woman	59	60%
Man	39	40%
Body Mass Index [weight/ (2x length)]	98	100%
Underweight (<18.5)	15	15%
Healthy weight (18.5-25.0)	64	65%
Overweight (25-30)	15	15%
Obesities (>30)	4	4%
Education	97 ǂ	100%
High school	23	24%
College – University - PhD	74	76%
Profession	98	100%
White collar	50	51%
Working in healthcare sector	14	14%
Governmental official	6	6%
Retired	9	9%
House husband/wife	3	3%
Worker	2	2%
Unemployed	3	3%
Other	11	11%
Participant type	98	100%
Breast cancer patients	8	8%
Healthy female volunteers	28	29%
Healthy male volunteers	31	32%
Pregnant women	23	23%
Prostate cancer patients	8	8%

ɬ, two missing values; ǂ, one missing value.

3.2. Evaluation of Colli-Pee® UAS™ use

Most participants (72%) reported to have used the IFU, while 26% reported not doing so and 2% did not complete the question (Table 2). Approximately 91% responded to the question about the clarity of the IFU, of these 87% indicated that the IFU was sufficient to use Colli-Pee® UAS™ (Figure 1A). Although only

71 participants indicated to have read the IFU, 89 participants indicated it to be clear which can be either explained by misinterpretation of the question or only having a visual check of the IFU. A total of 89% rated the assembly of the collector tube on Colli-Pee® UAS™ as easy (Figure 1B). Additionally, participants reported that it was clear (91%) and quick (94%) to assemble the Colli-Pee® UAS™ device before use (Figure 1C, D).

Table 2: Overall use Colli-Pee® (N=98)

No.	Question	No	Yes	Missing
B2.	Have you used the instructions for use?	25 (26%)	71 (72%)	2 (2%)
D2.	Was the Colli-Pee® held against the body while urinating?	38 (38%)	57 (58%)	3 (3%)
D4.	Did you spill urine?	72 (73%)	25 (25%)	1 (1%)
A1.	Was urine spilled while disconnecting the tube?	79 (80%)	18 (18%)	1 (1%)
F1.	Did you ever collect a urine sample before?	17 (17%)	81 (82%)	0 (0%)
F2.	Would you use Colli-Pee® again for urine collection?	9 (9%)	86 (87%)	3 (3%)
F3.	Would you recommend Colli-Pee® to others?	8 (8%)	87 (88%)	3 (3%)
F4.	Did you have the impression that urine collection was done correctly?	2 (2%)	95 (96%)	1 (1%)

Evaluating the use of Colli-Pee® UAS™, approximately 58% reported that Colli-Pee® was held against the body during urination, while 38% did not (Table 2). Most participants (90%) scored Colli-Pee® clear in use (i.e., participants clearly understood how to use the device and take a self-sample), and 68% scored Colli-Pee® comfortable to use (Figure 1E, F). Looking at spillage, the majority of respondents did not experience any spillage of urine during (73%) and after (80%) collection (Table 2). Spillage was clearly correlated to the female gender ($p=0.0101$) as this was reported by 20 women out of 25 participants who reported spilling. Approximately 40% stated 'moving the Colli-Pee®during collection' as a reason for spillage occurring during collection. While for spillage occurring after collection, most respondents indicated that urine droplets came from the floater (39%) or housing (44%) of Colli-Pee®. No respondent reported a device

malfunction as the reason for the urine spillage (data not shown).
The majority of participants scored detaching and subsequent capping of the tube as easy (detaching, 91%; capping, 97%) (Figure 1G, H). Finally, the majority of respondents (82%) rated their general experience of Colli-Pee® as easy to very easy to handle, while only 2% indicated it was difficult (Figure 1I).

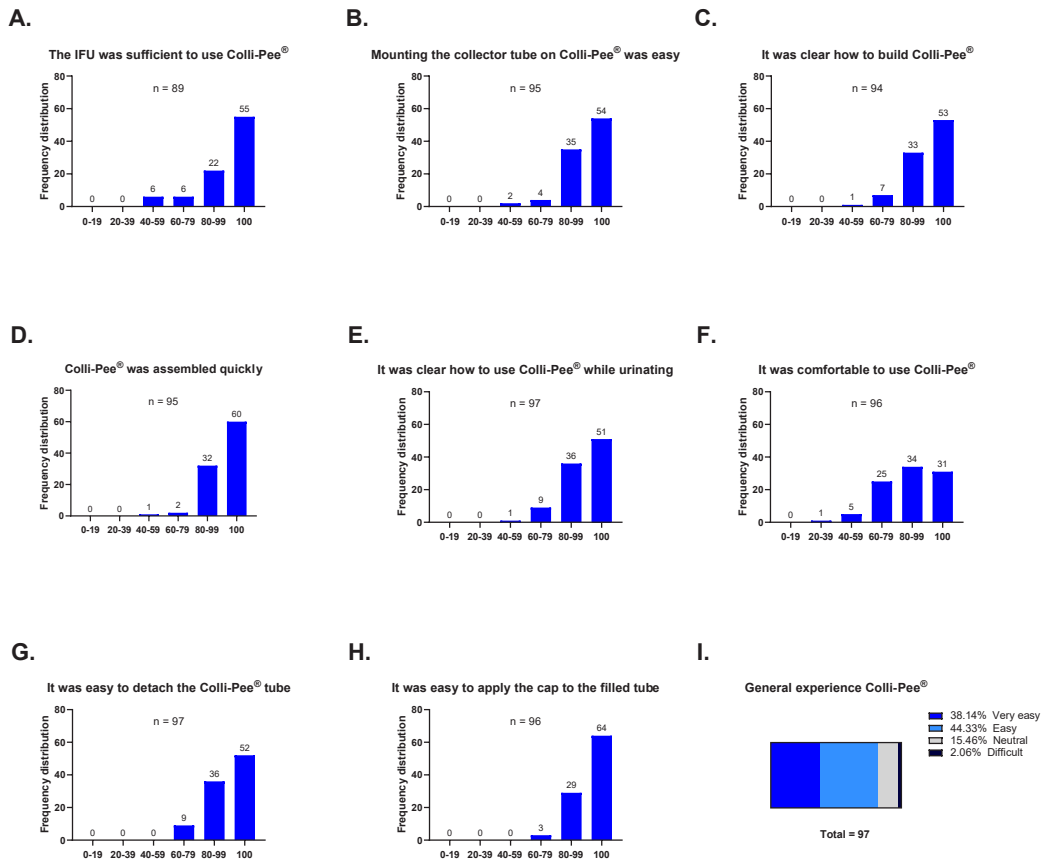

Figure 1: Performance Colli-Pee® UAS™ as first-void urine collection and preservation device (N = 98) – Bar graphs represent scores on steps before collection (A, B, C, D), during collection (E, F) and after collection (G, H) and the general experience on using Colli-Pee® UAS™ (I). Not every participant completed every question, the number of participants completing a particular question is indicated in each graph. (A) Although only 71 participants indicated to have read the IFU, 89 participants indicated it to be clear which can be either explained by misinterpretation of the question or only having a visual check of the IFU. Data is presented as frequency distribution, the number of scores received within a certain range (A-H), or as percentage (I).

3.3. Participant preferences and future considerations

The pie charts in Figure 2 show the preferred method of urine collection method (A) and the choice of urinating position for urine collection (B). Prior experience with urine collection (e.g., urine cup) was reported by 82% of participants (Table 2). A total of 71% of these would choose to collect a urine sample using Colli-Pee® and 29% would use a urine cup. There were no significant correlations found between the preferred method and gender, education, BMI, or spillage (data not shown). However, there was a significant correlation between the preferred method and age (p=0.016), with a higher portion of younger participants preferring a urine cup (26-37 years: 70%). Interestingly, seven of these young participants were pregnant women (44%). Furthermore, the two most used urinating positions to collect urine using Colli-Pee® were normal sitting (51%) and standing upright (43%).

Participants also reported their intention to use Colli-Pee® again in the future (87%) or to recommend Colli-Pee® to their friends and/or family (88%). Additionally, 96% indicated that they had the impression that urine collection was performed correctly using Colli-Pee® (Table 2).

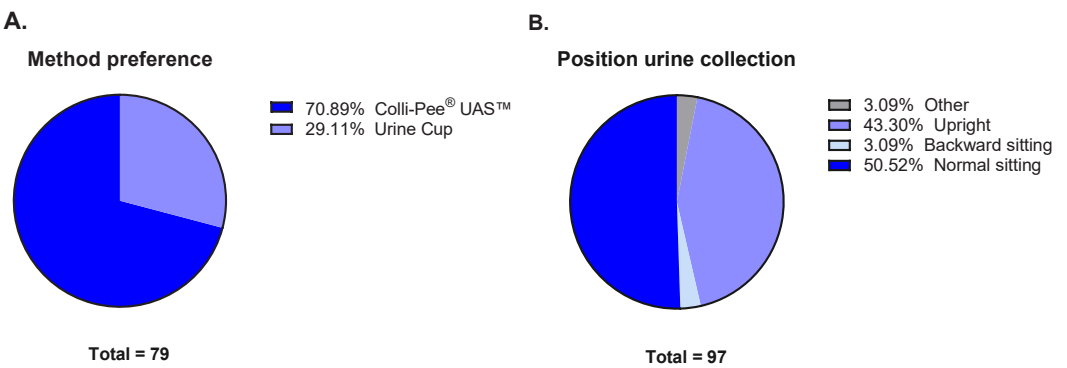

Figure 2: Preferences of the participants (N=98) – Method preference (A) and preference in position of urine collection (B). Only participants who have collected a urine sample before were asked to complete the question on which method they preferred. There were 81 participants who indicated they collected a urine sample before (A, Table 2).

3.4. Home-sampling experiences

A subset of all study participants (n =60) were requested to collect first-void urine samples at home using Colli-Pee® UAS™ device variants and return their samples back to the laboratory via postal mail. These participants were then asked to complete a specific questions on home-sampling and sending samples via postal mail, as well as to freely provide any further feedback. The responses to questions H1a-H1c are summarized in Table 3. Most participants were positive (very easy, 35%; easy, 40%) about the entire process of collecting urine samples at home and sending these back to the laboratory. The home-sampling experience was scored very positively (very easy, 40%; easy, 38%), while postal mailing of the samples was rated slightly lower (very easy, 21%; easy, 30%). Participants reporting 'rather difficult' were limited for the full process (3%), sending the samples (7%) and home-sampling (2%). None of the participants rated any of these questions as difficult or very difficult. About 19 participants (32%) provided additional feedback. Most feedback was related to the size of the envelope or box, which did not fit well or easily in official postal mailboxes in Flanders, Belgium.

Table 3: Experience of participants regarding home-sampling and sending samples, N=60 [n (%)]

No.	H1a	H1b	H1c
Question	The complete process (collecting at and sending 1-4 samples from home).	Sending the envelope/ box with tubes by post.	Collecting urine at home with Colli-Pee®.
Very easy	21 (35%)	21 (35%)	24 (40%)
Easy	24 (40%)	18 (30%)	23 (38%)
Rather easy	9 (15%)	11 (18%)	11 (18%)
Neutral	4 (7%)	6 (10%)	1 (2%)
Rather difficult	2 (3%)	4 (7%)	1 (2%)
Difficult	0 (0%)	0 (0%)	0 (0%)
Very difficult	0 (0%)	0 (0%)	0 (0%)

Discussion

04

Urine sampling offers many advantages as it is easy, non-invasive, and suitable for at home collection. Home-sampling allows individuals to use the comfort and privacy of their home environment to collect a sample without the need to visit a health care institution. Furthermore, home-sampling reduces the pressure on healthcare workers and protective materials required for clinic-based testing[23]. However, the implementation of home-sampling has been slow in clinical trials and in routine clinical diagnostics. Concerns have been raised on the quality and stability of the collected samples, as well as comparability of results to standard sampling methods[24]. To maximize the potential of self-sampling and at home collection, the entire process (i.e., from collection to return of samples to the laboratory) requires optimization. Therefore, sampling devices are needed to allow for maximal ease-of-use. Additionally, trends in digital health require more efficient and reliable communication channels to be developed and used, to provide psychological support to a patient, especially when envisioning virtual patient-doctor meetings or when a test result needs to be communicated[23].

Colli-Pee® UAS™ devices are developed for self-sampling of first-void urine and contain the UAS™ preservative, which allows the storage of first-void urine prior to analysis, whether due to home collection, sample shipment or biobanking. This study shows that first-void urine collection using Colli-Pee® UAS™ is considered easy to very easy even in a home-based setting. Participants reported that Colli-Pee® UAS™ was clear and comfortable in use. We noticed that 26% did not use the IFU before collecting the urine sample. Some patients and pregnant women did ask verbally how the device worked upon receipt at the clinic, which could have impacted their assessment of usability in the questionnaire as they received more information compared to those that collected at home. Most participants

preferred urine collection using Colli-Pee® UAS™ over urine collection using a regular urine cup. We noticed a significant correlation between the preferred method to collect a urine sample and age (p=0.016), with a higher portion of younger participants preferring a urine cup (26-37 years: 70%). Interestingly, of these younger participants, seven were pregnant women (44%). Based on verbal feedback, some participants were unsure about aiming the Colli-Pee® device towards the toilet because they lost sight of the device. Similar verbal feedback has been given in a study using Colli-Pee® devices with participants with a higher BMI (unpublished data). When self-sampling at home, most participants were very positive about their experience.

Other devices have been developed to collect of first-void urine, such as FirstBurst (DRW, Cambridge, UK)[25] and Peezy First Stream (Forte Medical, London, UK)[26], and/or to enable the collection of specific volume, such as FirstBurst (DRW, Cambridge, UK)[25], Monovette (Sarstedt, Nümbrecht, Germany), Vacuette (BD, Franklin Lakes, New Jersey, USA)[27] and Uriswab (Copan, Brescia, Italy)[28]. However, Monovette (Sarstedt, Nümbrecht, Germany) and Vacuette (BD, Franklin Lakes, New Jersey, USA)[27] only allow volumetric collection of a sub-sample after the collection by the individual. In contrast, Colli-Pee® (Novosanis, Wijnegem, Belgium)[20] allows for standardized volumetric collection of first-void urine immediately when the patient collects the sample. The standardized volumetric collection of the Colli-Pee® device was validated previously and therefore not part of this study.

Optimized self-sampling and at home collection processes could improve participation in urine-based liquid biopsy screening programs. For example, cervical cancer, for which major progress in screening coverage has been made in the past decades via urine self-sampling. A meta-analysis showed an overall 2.14-fold increase in screening coverage due to self-samples[29]. Also, a study in France selected 5000 hard to reach women (40-65 years) who had not had a cervical cytology (PAP) smear examination over the past three years and send them a urine home-sampling kit. They received 771 urine samples by postal mail, highlighting that urinary Human Papillomavirus (HPV) testing may be useful to reach women who do not regularly have cervical smears done to find high-grade cervical lesions[30]. Studies have also shown that self-samples provided equivalent results to physician-collected samples for the detection of HPV[31]. For first-void urine, several studies already showed its value and analytical performance[32-35]. Additionally, previous studies have shown urine as the most preferred sampling

method for cervical cancer screening[34, 36-40]. These studies also showed more confidence of women for collecting a urine sample compared a cervicovaginal self-sampling[39, 40]. The World Health Organization and Human Reproduction Program have even suggested in their new guidelines that self-collected samples, such as urine, can be used when providing HPV DNA testing[41].

Limitations of the study

05

Our study had some limitations. First, the purpose of our study was to investigate the acceptability and usability of the Colli-Pee® UAS™ device and therefore the actual suitability of purpose for downstream analysis was not assessed. Secondly, the standardized volumetric collection of the Colli-Pee® UAS™ devices was previously validated by Novosanis NV (Wijnegem, Belgium) and therefore not re-evaluated as part of this study. Thirdly, since the patient recruitment was hampered during the COVID-19 pandemic (2020-2022) there is a slight bias towards healthy volunteers and pregnant women. However, the age, education level, profession and BMI distribution allows drawing parallels between healthy volunteers and cancer patients. Additionally the patient sample size was very limited as we considered this more as a feasibility assessment. The research can be considered as highly innovative, as it will ultimately explore the feasibility of biomarker detection in urine samples from breast cancer patients.

Conclusion

06

Urine is an emerging liquid biopsy with broad applicability in different analytes and cancer research. In this study evaluating Colli-Pee® UAS™ enabled first-void urine collection in both healthy and cancer participants, the device was rated positive for its usability. Participants indicated they would choose Colli-Pee® for their next urine sample, recommend using it to friends and/or family and, had the impression urine collection was performed correctly. Importantly, the self-sampling method was well received and this could offer the possibility to increase screening coverage and provides more comfort for individuals during screening and patients during their disease monitoring in a home-setting or doctor's visit.

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CHAPTER

07

URICH

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Laughter is timeless, imagination has no age, and dreams are forever.
(Walt Disney, himself)

Abstract

Evidence-based practices (EBPs) are not always straightforward to implement in a design process, as impacts can be difficult to assess in practice and appropriate methods are not yet established. User-centered design (UCD) offers opportunities to improve implementation of EBPs. UCD refers to the use of rigorous and validated engineering tools to design technology interfaces focused on user goals, such as device safety, effectiveness, efficiency, and user satisfaction. UCD has the potential to improve six core elements of patient-centered care: (i) education and shared knowledge, (ii) free flow of information, (iii) patient engagement, (iv) teamwork, (v) attention to non-medical aspects of care, and (vi) respect for patient preferences. Technological advances in medicine over the past few decades have provided patients with a variety of inventions, such as electronic health record patient portals, home-based medical devices, and smartphone apps. Effective application of UCD principles results in patient-centered inventions that are more likely to ease patients' treatment burden, improve their care experience, increase patient engagement, and enable patients to become more self-sufficient. Here, the URICH device design process is used as case-example to demonstrate the inclusion of usability testing and all users as early as possible in the product design and development process.

Introduction

01

The term medical device is used for a wide range of products used in a variety of settings for the diagnosis, prevention, monitoring or treatment of illness or disability. According to the European Medicines Agency (EMA), "medical devices are products or equipment intended for a medical purpose"[1]. In the European Union (EU) medical devices must undergo a conformity assessment to prove they meet regulatory requirements to ensure they are safe and perform as intended. Manufacturers can place a CE-mark on a medical device once it has passed the conformity assessment[1]. For this conformity assessment the European Union published in 2017 the Regulation (EU) 2017/746 (IVDR). This Regulation applies to all in vitro diagnostic medical devices that would be placed on the market or put into service in Europe. This regulation sets high standards of quality and safety for in vitro diagnostic medical devices by ensuring, that data generated in performance studies are reliable, robust and that the safety of subjects participating in performance studies is protected[2]. In addition to conformity, the International Organization for Standardization (ISO) has developed many international standards with all different focusses, such as medical device development, quality management, risk management, usability, etc.[3]. These standards are developed in any domain to assure consumers that their products are safe, reliable and of good quality[3].

In this paper, we aimed to first provide some background on the use of user-centered design and usability testing in product design and development processes. Secondly, we use the design process of the URICH device as case-example to demonstrate the inclusion of usability testing and all (end-)users as early as possible in the product design and development process.

Background

02

2.1. User-centered design

Evidence-based practices (EBPs) are not always straightforward to implement in a design process since impact in practice can be hard to assess and proper methods are not yet established. Examples of these design problems currently not supported by EPB are low ease of use, high complexity, and poor fit with the intended delivery context[4]. Looking beyond traditional implementation strategies, user-centered design (UCD) offers potential for improving the implementation of EBPs. Starting from research in human-computer interaction, user experience design, service design, and cognitive psychology, UCD experts apply concepts and strategies related to the design, evaluation, and implementation of innovations for human use and to the study of main characteristics of optimal user-interaction[4, 5]. UCD refers to the use of rigorous and validated engineering tools to design technology interfaces focused on user goals, such as safety, effectiveness, efficiency, and user satisfaction of devices[6].

UCD has the potential to improve the six core elements of patient-centered care: (i) education and shared knowledge, (ii) free flow of information, (iii) patient engagement, (iv) teamwork, (v) attention to non-medical aspects of care, and (vi) respect for patient preferences[6]. Technological advances in medicine over the past few decades have provided patients with a variety of inventions, such as patient portals for electronic health records, home-based medical devices, and smartphone apps[6]. Effective application of UCD principles results in patient-centered inventions that are more likely to ease patients' treatment burden,

improve their care experience, increase patient engagement, and enable patients to become more self-sufficient[6]. However, if the UCD principles are not applied correctly and users' needs and expectations about how the device functions are not taken into account, this can have harmful consequences[6].

2.1.1. UCD plan of approach

A first step in a UCD action plan is to make a complete overview and specification of all stakeholders and prioritize them, as the design and development team must first gain explicit view of stakeholders and then balance the needs and limitations of these stakeholders[7, 8]. Stakeholders should be identified exhaustively and in a broad context, including any user who can reasonably be expected to use the device during its lifecycle, such as users, physicians, nurses, technicians, maintenance personnel, healthcare providers, regulatory organizations, and the designing institution[6, 8, 9]. It is important to consult a wide range of specialists with different levels of experiences to address the clinical needs, human error, and patient safety[6, 9]. This is necessary because potential users range from largely untrained lay people to highly skilled professionals, and in the case of home-based devices, users may have physical or cognitive limitations or living in difficult geographic locations[10]. Another important remark to make is that users and payers (person making a purchasing decision) are often different individuals or entities in the medical context, which allows tangible benefits to end-users to be subordinated to other considerations, such as benefits to customers and other decision makers[11]. After all relevant stakeholders have been identified, their involvement is needed to identify requirements, information about users' capabilities, and methods to assess design decisions from these perspectives[8].

A second step is to compile all information to identify every stakeholder who can reasonably be expected to use the device during its lifecycle. Importantly, to design products that satisfy their target users, an understanding of relevant user characteristics is necessary. These user characteristics include cognitive aspect (e.g., technical skill, spatial reasoning, adaptability, sensitivity to stereotype, memory, and prerequisite content knowledge), personality (e.g., patience, locus of control, optimism, perfectionism, changeability, uncertainty, avoidance, self-efficacy, and exposure to marketing), demographics (e.g., age, gender, culture, income, and grown-up place), physical characteristics (e.g. strength, body dimensions, reach envelope) and use behavior (e.g., frequency of use, avoidance

of using complex products, buy decision, complaining attitude, and familiarity with devices)[7].

2.2. Usability

Usability is a broad term that has many perspectives on its definition. However, the most widely accepted definition is formulated in the ISO 9241-11 standard and is "The extent to which a system, product or service can be used by specified users to achieve specific goals with effectiveness, efficiency and satisfaction in a specified context of use."[12]. It is expected that a high level of usability leads to increased productivity and adoption of the technology for the user, increased safety, and reduced effort for operation. Furthermore, positive experiences with a product result in a stronger brand position and (re)purchase intent, while negative experiences can result in product returns, complaints or helpdesk calls[7].

To ensure that a device is the right device with the right components, meeting the un-met or poorly met needs, usability validation requires end-user involvement during the concept and pre-concept stages of device development. This is also prescribed by the regulations and found in literature[13]. However, not many manufacturers incorporate usability as early as possible, which may be because the user perspective emphasizes on the human factors engineering process at the design phase, which could interfere with the quality and risk identification factor of new products[14].

Usability is also referred to as Human Factors Engineering (HFE), Human Engineering (HE), Usability Engineering (UE), Human Computer Interaction (HCI) and Ergonomics. Much attention goes to usability requirements, heuristic evaluation and ergonomics in guidelines and standards for medical device development, therefore manufacturers have the responsibility to ensure patient safety by emphasizing on the HFE process in the design of devices[15]. HFE studies help to increase safety, reduce potential errors, decrease training, increase ease of use, improve task performance, optimize device use, enhance user satisfaction, lessen product liability risks, facilitate the regulatory approval process, and increase the chance of commercial success.

2.2.1. Regulatory base for usability testing in device development

The BS EN 62366-1:2015+A1:2020 (Medical devices – Part 1: Application of usability engineering to medical devices) is the main European harmonized standard that defines a process for a manufacturer to analyze, specify, develop, and evaluate the usability of a medical device as it relates to its safety. This last standard references the risk management BE EN ISO 14971:2019+A11:2021 (Medical devices – Application of risk management to medical devices) as the base for the identification of usability matters via a risk management process.

2.2.2. Methods for usability validation

Various methods can be used for usability testing such as heuristics, cognitive walkthroughs, focus groups, observation, explorative vs. comparative tests, etc. The design and development team must decide which methods to use or to combine in depending on several factors, such as the stage of the design of the device, the type of users involved, the expertise of the research, the type of information required, and the materials, time and money available[8, 16]. The required level of usability validation will depend on the criticality of the device, its environment of use, the primary operator, as well as the party paying for the device[16].

2.3. General product design process

Generally, the product design process (Figure 1) involves several steps or phases, starting from defining the problem and ending with commercialization. Each phase is crucial in ensuring that the product meets the requirements of its users, is functional, aesthetically pleasing, and can be produced at scale.

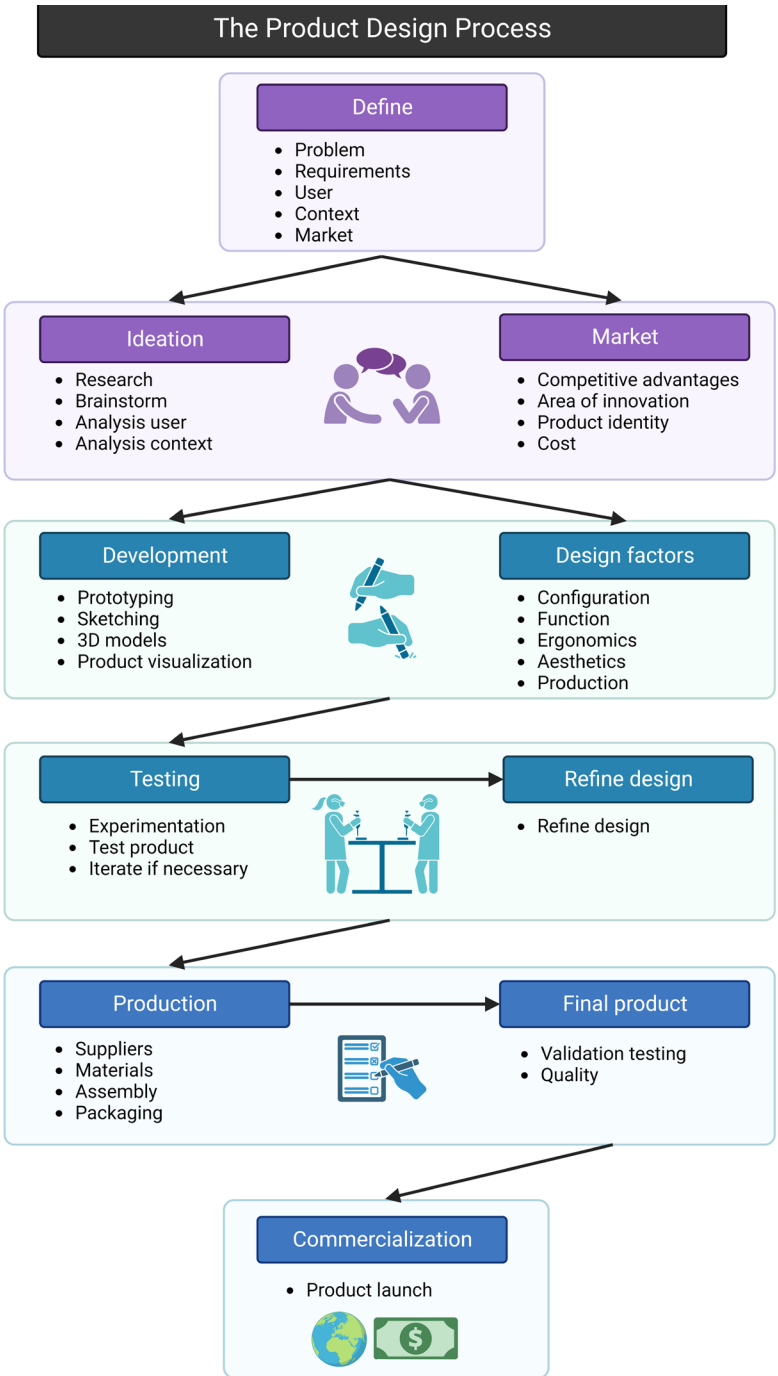

Figure 1: The Product Design Process.

The first phase is called the define phase. In this phase, the problem that the product will solve is defined, along with the requirements for the product, its target users, and the context in which it will be used. The possible market for the product is also investigated and described. The ideation phase comes next, and it involves further research, brainstorming, and analysis of the user and context of intended use. The goal of this phase is to generate new ideas for the product that ought to meet the requirements set in the first phase to solve the problem defined. The market phase is linked to the first two phases. Here, the competitive advantages, area of innovation, product identity, and costs are investigated and preliminarily determined.

Once these initial phases are complete, the development phase begins. During this phase, the product idea is transformed into sketches, prototypes, product visualizations, and 3D models. Design factors such as configuration, function, ergonomics, aesthetics, materials, and production are also investigated and determined. After the initial prototypes and 3D models are created, the testing phase begins. This phase involves experimenting with different versions of the product, eliminating options, and making improvements until a satisfactory final prototype or 3D model is created. This final prototype is then refined until it is ready for production.

In the production phase, partners needed for production are secured. The assembly and packaging of the product are also defined. The decisions made during the design factor phase, such as the product category and material types, become important in this phase. The production phase ends with the creation of the final product, validation testing, and quality assurance protocols. Finally, the product is ready for the world, and commercialization can begin. The product development team transfers the product to marketing for a product launch and further commercialization.

The URICH device, as case-example

03

The design and development process of the URICH device will be thoroughly discussed, however without mentioning intellectual property sensitive information, since possibilities for patenting are under investigation. The aim of the design and development of the URICH device is to perform an upfront enrichment of urinary analytes at the side of the user.

An important opportunity in healthcare is to optimize the process of self-sampling at home or in the clinic. It is becoming more and more clear that patient self-sampling or remote sample collection from clinical facilities offers benefits, as there is an increase in efficiency and speed of clinical trials, the allowance for additional sampling time-points, a reduction in patient burden, the opportunity for broader and more diverse participation in clinical trials and, a higher participation in screening[17]. Despite these benefits, the uptake of self-sampling in clinical trials and screening programs has been slow, mainly due to questions about the quality of self-sampling, sample stability and comparability with the gold standard methods. However, these challenges could potentially be overcome through patient training and using sample-collection devices that make sample collection easier and more convenient, which in turn could improve the data collection accuracy[18]. Urine has great potential as a non-invasive liquid biopsy for self-sampling and screening, thereby possibly optimizing healthcare. The Colli-Pee® device (Novosanis, Belgium), having different configurations to allow for volumetric and standardized collection and immediate preservation of first-void urine, which helps increasing self-sampling uptake. Preservatives such as UCM® or UAS™ guarantee the sample integrity during postal transport for up to 7 days. This makes it possible to send samples from the general practice or patient's home to the lab by postal mail or parcel services. URICH allows expanding the Colli-Pee® device portfolio to collect a urine sample that improves

meeting the requirements of current oncological research and cancer care by enabling concentrating or filtering of the urine sample.

3.1. Define phase

3.1.1. Problem statement

The three key steps in a liquid biopsy workflow include: (i) biofluid collection (including collection, storage, and handling), (ii) concentration, isolation, or extraction of biomarkers and (iii) analysis or detection of biomarkers. Each of these steps brings possible variation and thus offers opportunities for optimization and standardization[19]. For example, cancer researchers are investigating different cancer types, different analytes of interest and different sample types, all with the goals of early detection of cancer, screening for cancer, follow-up of disease progression, monitoring of treatment response and prediction of recurrence[19]. Biologically, different analytes require different preservation, storage, and handling conditions. Ideally, every analyte of potential interest in the sample is immediately properly preserved to withstand analyte degradation under transportation conditions. Furthermore, in urine samples these analytes of interest can be highly diluted. Therefore, lab technicians perform concentration or filtration steps on the sample before the sample can be used in isolation and extraction procedures to isolate the analyte of interest and prepare it for downstream analysis.

3.1.2. Identification stakeholders and user groups

Based on this application of cancer research, for the design process of URICH different stakeholders and user groups have been identified by brainstorming on all possible individuals who can reasonably be expected to use the device during its lifecycle:

- Actors in design of medical devices (University of Antwerp, Novosanis, OraSure Technologies and DNA Genotek Inc.)
- Physicians and other healthcare professionals (e.g., prescribe/recommend device, hand out/send device for screening, etc.)
- Users (patient, screening participant, person interested in own health, etc.)

- Postal service (sending samples to laboratory, etc.)
- Lab technicians (receive samples, further processing for analysis, etc.)
- Biobank personnel (receive sample, further processing for storage, etc.)
- Person making a purchasing decision (e.g., government for population screening, etc.)
- Regulatory organization (e.g., FAGG, FDA, etc.)

3.2. Ideation phase & Market research

3.2.1. Identification users and their characteristics

The URICH project will focus on sample preparation by the user and processing by the lab technician or Biobank personnel. URICH will relocate a few steps from the lab to the user to better preserve the sample during transport and storage. After collection and execution of possible additional steps, the user sends the sample to the laboratory or the Biobank. The lab technician receives the URICH-sample and performs the needed steps before using the sample for isolation or storing it for further use. On the other hand, when the Biobank personnel receive the URICH-sample they store it for clinical research.

When looking at the URICH users, being the potential patient or person interested in its health, there are three main groups that can be identified: people who would use URICH (i) for disease diagnosis, (ii) in a disease screening trajectory and, (iii) for health monitoring. These three groups will have different frameworks around their sample collection process. When a person receives URICH for disease diagnosis, there will be a large framework since the healthcare professionals will provide the device and the necessary explanation to the potential patient. For the monitoring process, there can be healthcare support when the sampling occurs in the clinic. However, monitoring usually means repeated use of the device, which allows to expect experienced users. The screening trajectory is the process in which there is almost no framework and users must rely on the instructions for use. On top of the different frameworks around the sample collection process itself, users can vary significantly when it comes to characteristics. Sadly, cancer is a disease of all people, it doesn't differentiate between cognitive aspects, personality, or demographics. People

of all ages, gender, culture, income, technical skills, adaptability, use behavior, etc. can be diagnosed with cancer and are therefore potential users of URICH. It was decided that the most critical group would be the 80+ age group, because of potential age-related ailments and strength decrease that could affect autonomous use. The aim of URICH for these 'first' users is to make the sampling process and all necessary steps as easy as possible for all potential users, while enabling self-collection in the comfort and privacy of the user's home and shipment of the URICH-sample to the laboratory.

The second and third group of URICH-users are the lab technicians and Biobank personnel, which have many similarities. One of the most important characteristics is the fact that lab technicians and Biobank personnel are trained users with developed technical skills. Furthermore, these second and third groups of users can be people between 18 and retirement age, all genders, all culture, and all use behaviors. The aim of URICH for these users is to make the laboratory processing and all necessary steps as easy, as fast and as convenient as possible.

3.3. Market research in collection, preservation, and enrichment

The scope of this project is supporting the process of collecting a urine sample, filtrate/concentrate and preserve the desired fraction and analytes, preparations in the receiving laboratory. Currently this entire workflow is not integrated into a streamlined process and is therefore performed using a chain of different products that each support 1-2 steps.

Other solutions for urine collection devices and preservatives are available. The devices are developed for the collection of urine, some enable the collection of a specific fraction and/or specific volume and/or direct preservation. While literature searches have shown that both laboratory available chemicals as well as commercially available preservatives are used for oncological research[19]. Preservatives can be developed for a specific sample type or specific analyte or as a general preservation method, however all commercially available methods have their own claims. Usually, the filtration or concentration of the urine sample is performed in the laboratory environment. Where a lab technician pipettes the urine sample into specific developed devices such as, Amicon® centrifugal filter devices and Vivaspin® centrifugal concentrators (Figure 2). Both solutions use centrifugation to provide the force to rapidly remove solvents

and small molecules through the ultrafiltration membrane. On the other hand, Per Guldberg developed a 'filtration device' for on-site collection, storage, and shipment of cells from urine[20], which is the first mention of the user performing the filtration step. Up to now the filtration device of Per Guldberg has been described in research solely and has not yet been commercialized (Figure 2). As seen in the competitive field, the process support during collection for the user and during processing for the lab technician is not yet offered, and therefore is one of URICH's biggest unique selling propositions.

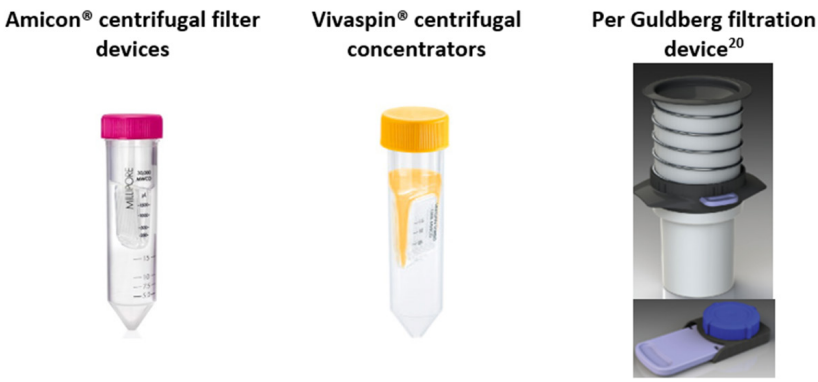

Figure 2: Overview of filtration and/or concentration devices

3.3.1. Brainstorm

Several brainstorming sessions were executed. In the first brainstorm, the full trajectory of a urine sample was mapped using scenario envisioning. Thereafter, creative sessions were performed to sketch and map out as many different ideas as possible, which resulted in a number (usually for further design processing max 5 ideas are recommended) of innovative start ideas. These innovative initial concepts were weighed against each other in a trade-off-matrix based on predetermined criteria. For URICH, these criteria were chosen with a strong emphasis on the users and their experience: readability, the number of steps, simplicity per step, lab compatibility, and feasibility. After the determination of the criteria, the innovative start ideas are scored out of 5 on each criterium, where 5 is the highest and 0 is the lowest score. The combination of these revealed the final score of that innovative start idea.

3.4. Development

The transformation of the product idea into sketches, prototypes, product visualizations, and 3D models did not involve user perspectives or engagement and will therefore not be discussed in this paper. However, the first sketches and graphical visualizations were included in Figure 3 to provide some context about the URICH device.

Figure 3: Graphical visualization of the URICH device and its packaging

3.5. Design factors

Design factors such as configuration, function, ergonomics, aesthetics, and production for URICH were investigated and determined. First, several targets of interest (TOI), also referred to as requirements, related to the users can be defined for URICH. All these different TOIs lead to their own specifications and functioning that must be considered when configuring the final product. Furthermore, the necessary ergonomics and aesthetics for the users are determined.

3.5.1 Functioning & configuration

The first step is the collection of urine, which is done using Colli-Pee®, leading to TOI 1 "a system that collects first-catch urine in a tube containing the filter module". The first TOI will not be discussed, as this is mainly the configuration of the CE-marked Colli-Pee® devices.

After the urine collection, URICH is used to perform the filtration and it should enable filtration for the user at home, translating to TOI 2 "a system that facilitates the power of the user so that the sample is filtered through the filter module". The user specifications are: (i) it must be clear which part of the package is URICH and how to assemble it, (ii) the force required must be limited to 0.8Nm or 60N torque, pushing or pulling force (e.g., user's capabilities), (iii) there should be feedback (e.g., auditive ['click'], tactile, haptic, visual feedback) to the user that the step was successful, (iv) it must be user-friendly for people of all ages and, (v) only successful execution of a step will physically enables the next step.

Next it is important that the residue and filtrate are and remain separated throughout transport to the laboratory, which leads to TOI 3 "a system to store both samples with the correct preservatives". The following user specifications were determined: (i) the user must understand which part belongs where in which orientation, (ii) the force required must be within the user's capabilities, (iii) there should be sufficient feedback to the user that this step has been completed successfully, (iv) the sample must comply with transport requirements.

Upon arrival at the laboratory, URICH supports the lab technician in the further preparation of the sample, resulting in TOI 4a "a system that adds solvent to the residue" and TOI 4b "a system that stores the residue dissolved in the solvent during centrifugation". The user specifications were set as follows: (i) only one configuration of the parts should be possible, (ii) the mounting should be feasible by hand, (iii) the sample must be compatible with standard centrifuges, (iv) the product should withstand centrifugal forces up to 6000g, (v) the product should be balanced to avoid vibration during centrifugation and, (vi) the product can be disassembled by a lab technician after centrifugation.

Finally, the lab technician needs to be able to pipette the filtrate from the URICH components into the isolation components, leading to TOI 5 "a system that

enables pipetting". Therefore, only one user specification is determined, being that the URICH configuration after centrifugation requires an angled bottom for easy pipetting.

3.5.2. Ergonomics & Aesthetics

URICH should be intuitive, easy, and user-friendly for all user groups. To achieve these ergonomic specifications and the above described TOIs, some ergonomic and aesthetic choices will be made. The number of steps to be performed by the user for collecting and filtrating the urine sample and shipping the preserved sample and the complexity of these steps will be limited, to keep URICH as intuitive and easy as possible. The decisive for ease of use will be the time and power required by the user to perform the filtration step. Additionally, the decipherability of the various parts of the URICH components will be improved by smart color coding of the various parts. By coloring the components that will replace each other in the same color, the process becomes more intuitive and clearer.

3.6. Testing

During the testing phase, different simulations, experiments, and studies can be performed to examine the functioning of product components, critical aspects, functioning of the product, usability, and readability of URICH, instructions for use (IFU), and packaging. All these different tests can be executed with prototypes, 3D prints or with the actual molded product. In this paper, the critical user-related tests are described.

3.6.1. Pressure test

This experiment was designed to investigate the pressure required for the filtration steps in the URICH variants. For this experiment, the final design has been approached as closely as possible by working with syringe filters and collecting first-morning first-catch urine and first-catch urine (corresponding to the most critical scenarios possible). The set-up for this experiment consisted of three formats wherein raw urine is aspirated with the syringe and then filtered using (i) a 5.0-micron syringe filter, (ii) the combination of a 5.0- and 0.8-micron syringe filter or, (iii) the filtrate from the 5.0-micron syringe filter is

refiltered with a 0.8-micron membrane. This specific set-up of formats enables to find the resistance of each membrane separately and together. Each test is repeated three times at a different speed. The measurements of these tests allow to assess the flux of each membrane, which is linearly related to the pressure. This estimation of pressure determines the necessary torque from which the diameter of the plunger head to accommodate the user's strength can be derived. Further analysis of the results showed that the components are calculated for a maximum axial force of 100N at 10s. And that the maximum measured and calculated force for URICH at a filtration time of 10s is 88N.

3.6.2. Usability of URICH – related to readability of IFU and packaging.

The usability and readability of URICH in combination with the IFU and designed packaging was examined with low-fidelity prototypes of URICH in the packaging, without the actual urine collection and filtration, by asking users what steps they would take and why. Because the 3D prints could not be made transparent and were not printed in the right colors, the test-persons were shown a picture after each step of how the device/sample would look at that point. For this test, at least seven naïve people were included. Each of their responses and actions were recorded until the entire process from collection to shipment to the lab was completed. Based on the observations of this experiment some changes were made to the packaging and IFU to decrease the potential human errors and increase the intuitiveness of URICH.

Future perspectives

04

The first steps in the URICH product design process have been taken, but there are still some phases to be completed such as the development phase, researching the design factors, testing and finally refining the final design. After completing the design, the Design and Development control phases can be started, with research into the production information to determine the supply chain and the verification, validation and quality assurance of the final product[21]. When the final product is fully validated, and the quality assurance protocols are in place the commercialization phase can begin in which the product is launched to the market.

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05

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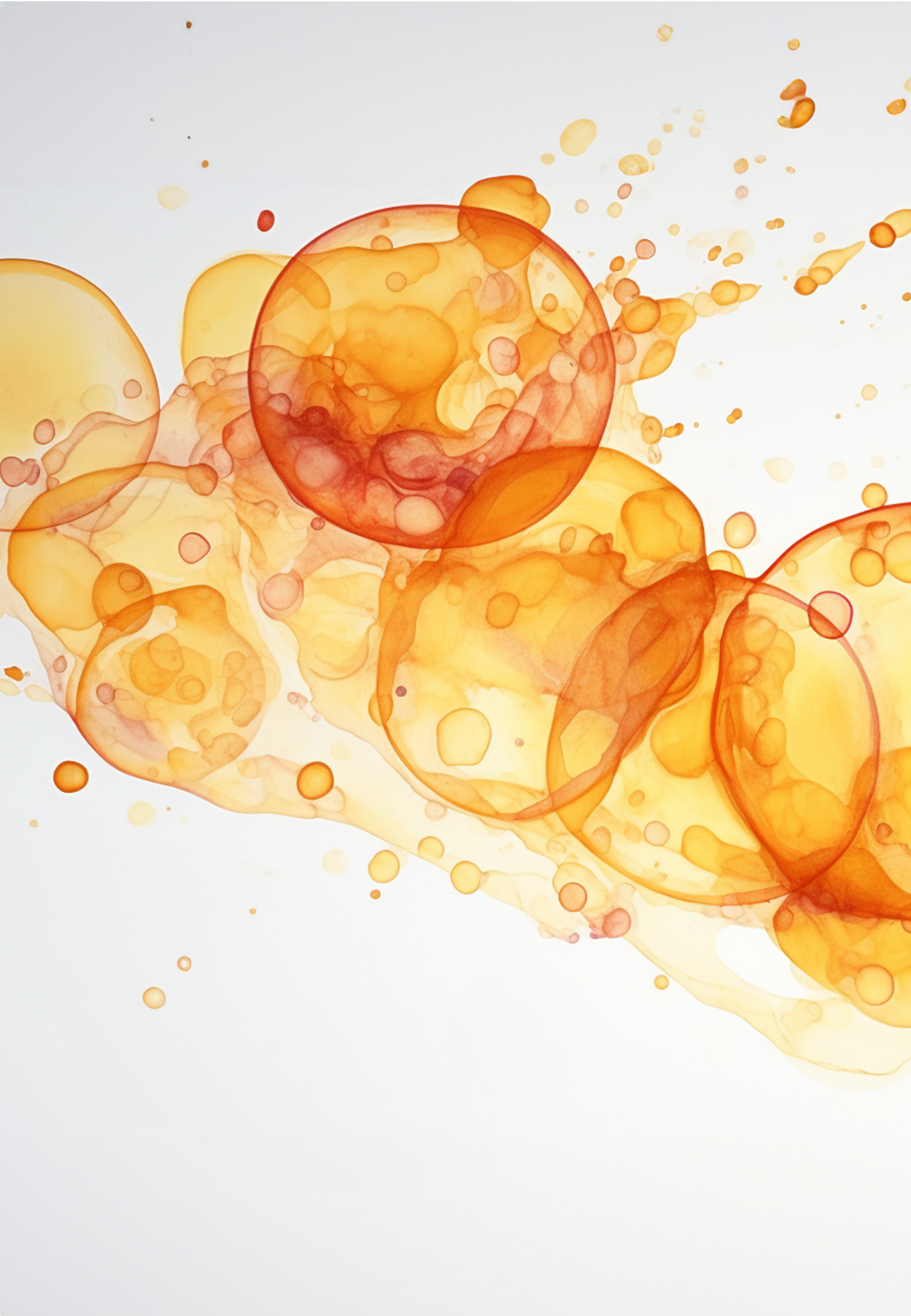

PART III

Concluding remarks

CHAPTER

08

Discussion and Future perspectives

If you focus on what you left behind, you will never see what lies ahead.
(Gusteau, Ratatouille)

Discussion

01

Cancer has a large and increasing burden from a clinical, economic and social point of view[1]. Globally, it has been estimated that 19.3 million new diagnoses would be made in 2020, and the estimated number of deaths was 9.96 million. Additionally, the global incidence is expected to increase to 30.2 million and the mortality to 16.3 million by 2040[2]. Furthermore, the clinical, economic, and social burden associated with cancer can be divided into direct and indirect costs. These direct costs are linked to services patients receive, such as physician visits, hospitalizations, medication (anti hormone therapy, chemotherapy, and immunotherapy), radiation therapy and surgery. Cancer-related medical costs vary significantly by cancer site and by cancer stage[3]. The indirect costs are the monetary losses associated with time spent receiving medical care, such as time lost to work or perform other activities and lost productivity due to premature death. These costs are paid by patients as well as their caregivers and families, since these losses are not reflected in monetary transactions their value can only be estimated[3]. Around the world, different organizations and policymakers have implemented various preventive interventions, such as lowering risk factors, to reduce the health and economic burden of cancer[4]. One of these policymakers, the European Institute of Innovation and Technology (EIT), set up a knowledge and innovation community (KIC) in 2015, called EIT Health. The idea behind EIT KICs is that innovation thrives best when the right people are brought together and share their expertise. They are based on the knowledge triangle, which states that when experts from industry, research and education work together as one, an optimal climate for innovation is created[5]. Similarly, the Flemish Government Agency for Innovation and Entrepreneurship (VLAIO) also aims to bridge the gap between the academia and industry, through Baekeland Mandates from which this doctoral thesis was funded. Another policy-maker, the European Commission, has launched their

Europe's Beating Cancer Plan in February 2021 around: (i) prevention through actions addressing key risk factors, (ii) early detection of cancer by improving access, quality and diagnostics, (iii) diagnosis and treatment through actions to ensure better integrated and comprehensive cancer care and addressing unequal access to quality care and medicines and, (iv) improve quality of life of cancer patients and survivors[6]. In 2023, EIT Health launched three major flagships: (i) new models to deliver healthcare, (ii) facilitating the uptake of digital medical devices in Europe and, (iii) harnessing the full potential of health data for innovation[5].

The work executed in this dissertation can be linked to four clear trends seen in the healthcare: (i) transition from an invasive or minimally invasive sample to a non-invasive sample, (ii) standardization of liquid biopsy workflows, (iii) transition from therapeutic care to preventive care and, (iv) rise of so-called self-care where individuals take their health into their own hands. A key factor to support all these trends to optimize healthcare is the process of non-invasive self-sampling at home or in the clinic. Patient self-sampling or remote sample collection from clinical facilities offers benefits, as there is an increase in efficiency and speed of clinical trials, the allowance for additional sampling time-points, a reduction in patient burden, the opportunity for broader and more diverse participation in clinical trials and, a higher participation in screening. The next part of this discussion will discuss these four trends and link them to the first two flagships as identified by EIT driving innovation in healthcare as well as the Europe's Beating Cancer Plan.

1.1. Transition from an invasive to non-invasive sample

A first clear trend seen in cancer research is the transition from an invasive sample like tissue biopsies to a minimally invasive sample like blood or even non-invasive sample like urine. The use of minimally invasive procedures such as liquid biopsies is gaining interest[7]. Body fluids used as liquid biopsies have several advantages: they allow repeated sampling, they can better reflect the tumor heterogeneity, they are also associated with significantly less morbidity and can prevent complications associated with traditional tissue biopsies[7]. The most commonly used minimally invasive liquid biopsy is blood. It requires a brief time and sampling is considered low cost[8]. However, the key consideration is that taking blood is not always easy and problems may arise depending on the age and condition of the patients[9].

This led us to explore urine as a sample type, which is fully non-invasive, easily accessible, enables cost-efficient rapid and serial sampling. Furthermore, urine as source of biomarkers for oncology applications has gained increasing interest over the past three decades and is expected to continue into the current decade. When looking into PubMed data, the number of publications for urine and liquid biopsy has 7-folded the number of registered publications in the last decade compared to the previous decade, 268 publications vs a mere 37 publications. When looking more specifically at urine and oncology, the number of publications in the last decade has almost doubled compared to two decades ago, with close to 9000 publications. The increasing global interest in urine is also reflected in the increasing number of clinical trials conducted in North America and Europe as leading continents. Our systematic review of the literature showed that urine has already been used as a sample type for several cancers, both urological and non-urological cancers, and has potential for a broad variety of cancers. Furthermore, we demonstrated that urine can be used as a true multi-omics sample type, including DNA, RNA, proteins, metabolites, and even essential metals (**Chapter 3**). Urine has also been proposed as an alternative biofluid for detecting and monitoring treatment of cancers. Additionally, urine is applicable for home collection, allowing for patient monitoring[10, 11]. However, exploitation of the potential of urine as a liquid biopsy in cancer research and its potential for screening and patient monitoring is needed.

1.2. Standardization of liquid biopsy workflows

A second trend in healthcare highly linked to this dissertation is the standardization of workflows. This clearly wins in importance making researchers and policymakers investigate and develop guidelines and standards operating procedures. A liquid biopsy workflow typically exists of three main steps: (i) biofluid collection (including collection, storage, and handling), (ii) isolation or extraction of biomarkers and (iii) analysis or detection of biomarkers. Each of these steps brings possible variation and thus offers opportunities for optimization and standardization[12]. When further elaborating on the clinical applicability of urine as liquid biopsy, it is of utmost importance to use appropriate pre-analytical conditions, such as optimized collection and preservation methods and, storage and transport temperatures and sample handling, to achieve accurate detection of urinary biomarkers[13-16]. Pre-analytical parameters for urine processing may lead to water evaporation, host cellular

lysis, nuclease activity, bacterial growth and/or contamination, and potentially changes in urine composition[17, 18]. Preventing bacterial growth is important as it can cause the urine sample to reach a higher turbidity, making the extraction and detection of specific analytes more difficult[19]. Furthermore, urine contains potassium, calcium, magnesium, sodium, and zinc, and has a pH between 5.0 and 7.0, supplying a suitable environment for nuclease activity. DNase I, one of these DNA-hydrolyzing enzymes, has a more than 100-fold higher activity in urine compared to its activity in blood. DNase II is also present in urine although its activity is 30-times lower than that of DNase I. On the other hand, RNA-hydrolyzing enzymes; including RNase I and RNase II; and phosphodiesterase I are also present in urine. All these nucleic acid-hydrolyzing enzymes jeopardize the preservation of DNA and RNA fragments[20]. Addition of preserving agents, like ethylenediaminetetraacetic acid (EDTA), has demonstrated to improve the stability of urinary analytes upon storage[13, 16, 18, 21-26]. EDTA is a well-established chelating agent that binds ions required for DNase activity, thereby inhibiting nucleic acid-hydrolyzing enzymatic activity, and reducing DNA degradation[27]. EDTA is a frequently used preservative, however it can only protect DNA[13, 16, 24, 26]. Other preservatives have been frequently used for other analytes; e.g., guanidine thiocyanate preservatives for RNA[26] and, boric acid or thymol preservatives for metabolites[18]. Currently, it appears that there is no one-size-fits-all and Teach analyte might require a different preservative. However, from a collection and processing point of view the need for multiple reagents is not convenient for the continuously evolving multi-omics scene, emphasizing the need to validate an efficient preservative in urine that can preserve multiple analytes. Concerns have been raised on quality and stability of the collected samples, as well as comparability of results to standard sampling methods[28].

Therefore, a novel preservative, UAS™, has been designed to prevent chemical and enzymatic degradation of nucleic acid and cellular lysis, as well as microbial growth. The UAS™ preservative can be included in the Colli-Pee® devices, which are developed for self-sampling of first-void urine. Other devices have been developed or are in development to collect first-void urine, such as FirstBurst (DRW)[29] and Peezy First Stream (Forte Medical)[30], and/or to enable the collection of a specific volume, such as FirstBurst (DRW)[29], Monovette (Sarstedt), Vacuette (BD)[31] and Uriswab (Copan)[32]. However, Monovette (Sarstedt) and Vacuette (BD)[31] only allow volumetric collection of a sub-sample after the collection by the individual. In contrast, Colli-Pee® (Novosanis)[33] allows for standardized volumetric collection of first-void urine as well as immediate mixing with

preservative upon collection of the sample by the patient. We demonstrated the performance of UAS™ for various sample handling conditions, as well as its potential in a clinical setting. Also, we showed that the performance of Colli-Pee® UAS™ was independent of the standardized and volumetric collected volume of urine, since all three variants: Colli-Pee® UAS™ FV-5010, Colli-Pee® UAS™ FV-5020 and Colli-Pee® UAS™ FV-5040 performed comparable.

Colli-Pee® UAS™ devices are developed for self-sampling of first-void urine and allows the storage of urine prior to analysis, whether due to home collection, sample shipment or biobanking. Home-sampling allows individuals to use the comfort and privacy of their home environment to collect a sample, without a need for visiting a health care institution, this in turn increases donor compliance and allows for easier recruitment of larger cohorts. Furthermore, home-sampling reduces the pressure on healthcare workers and protective materials required for clinic-based testing[34]. However, the implementation of home-sampling has been slow in clinical trials and in routine clinical diagnostics, mainly due to questions about the quality of self-sampling, sample stability and comparability with the gold standard methods. To maximize the potential of self-sampling and home-sampling, the entire process calls for optimization. However, despite the need for further optimization, the acceptance of urine self-sampling is high among study participants, due to its non-invasive nature and confidence that a urine sample has been correctly collected[35-37]. Furthermore, a recent study by Aquarius Population Health (London, UK) even showed that self-sampling, first-void urine collection using Colli-Pee® (Novosanis NV, Wijnegem, Belgium) or vaginal self-sampling using FLOQSwab (COPAN Diagnostics, Brescia, Italy), could provide a cheaper alternative to clinician-collected sampling for routine HPV primary screening and offer opportunities to extend the reach of cervical screening to under-screened women[38].

All together, this demonstrated the added value of Colli-Pee® UAS™ medical devices in addressing the critical issue of standardization of pre-analytical workflows by allowing user-friendly, volumetric urine collection combined with optimized preservation, storage and transportation conditions thereby improving downstream processes in oncology applications (**Chapter 4-6**).

Importantly, the optimization of cfDNA isolation, including sample preparation steps, concentration steps and the isolation step, towards methods capable of efficiently capturing short, diluted DNA fragments is critical to maximize the

clinical sensitivity and reproducibility[39]. For cfDNA derived from blood, several studies have investigated the optimization of yield and stability of cfDNA by comparing a range of commercial cfDNA isolation/extraction kits[12, 40-48]. While, for urine-derived cfDNA only a few studies have compared several cfDNA isolation/extraction methods[39, 49, 50].

In this dissertation, we compared the isolation efficiency of four cfDNA isolation methods for urine and concluded that they all performed comparably. However, it can be considered that the nRichDX Revolution® system offers the best capabilities for high-throughput and clinical setting of the liquid biopsy workflow (**Chapter 5**). Furthermore, many interesting analytes, such as: circulating DNA forms, circulating RNA forms, extracellular vesicles, proteins, are present in urine, albeit in low concentration. There are already some solutions to filter or concentrate these analytes of interest in the lab, such as: syringe filters, Amicon® centrifugal filter devices and Vivaspin® centrifugal concentrators. Importantly, if this filtration or concentration could take place earlier in the process, these analytes could be immediately well-preserved. This led us to the design and development pathway of a potential new Colli-Pee® variant for the enrichment of urinary analytes of interest. Here, the early integration of users, user needs and usability in the design and development process is highlighted. We show that it is possible to include all (end-)users from the beginning of the design and development process, which could increase device acceptability and user-friendliness (**Chapter 7**).

Despite the great potential of urine as liquid biopsy, no standardized protocols for pre-analytical parameters are available[51], which was surprising as it has been shown that most of the test errors are due to problems during the pre-analytical phase[52, 53]. Currently, mainly general guidelines such as the Biospecimen Reporting for Improved Study Quality (BRISQ) and European Guidelines for Urinalysis have been established for urine biobanking and studies[54, 55]. Moreover, during the past years, for EV research some guidelines, such as the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018)[56], and knowledgebases, such as the EV-TRACK[57], have been developed. To enhance the reporting of all pre-analytical variables, the evaluation of these could be used in the future to establish best practice protocols. Additionally, in 2021 the Urine Taskforce of the International Society for Extracellular Vesicles (ISEV) published their position paper, in which they present the state of the art and identify challenges and gaps in current urinary EV analysis as well as recommend on reporting of different

pre-analytical parameters for urinary EV research to improve reproducibility and interoperability in urinary EV research[58]. Aligned with their recommendations, we proposed a standard parameter checklist or template to be used when reporting on research using urine as liquid biopsy (**Chapter 3**).

1.3. Transition from therapeutic care to preventive care

A third trend in healthcare is the shift from therapeutic to preventive healthcare. Currently healthcare and healthcare budgets are mostly reactive, with patients being treated linked to disease and associated symptoms, pain, and discomfort. Preventive care, meaning people considered to be healthy are being monitored through screening programs to detect possible cancers (and other diseases) at an early stage. Unfortunately, cancers are still often diagnosed at a later stage, making treatments more difficult for the patient, challenging for the healthcare professionals and more expensive for the healthcare system. The challenge for healthcare professionals is to identify cancers at an early stage so preventive actions can still be taken. To allow for faster and earlier diagnosis, organizations and policymakers are striving to implement and improve screening programs for different cancers. However, the main challenge remains for a screening program to be acceptable, accessible, economically efficient, equitable, sustainable, next to the health system being equipped to manage the increased number of cases in terms of treatment, support, and follow-up[59]. The current tissue samples can be considered less attractive for screening because of their invasive nature, while blood as liquid biopsy is minimally invasive. The serum PSA level is used to determine the risk of developing prostate cancer, however, PSA is not sensitive and specific enough as a person's PSA level can rise due to other benign conditions such as prostatitis and benign prostatic hyperplasia[60, 61]. To meet the screening challenges, it is important to discover and validate diagnostic methods including biomarkers to enable and improve screening programs. Interestingly, several companies have developed or are developing urine-based biomarker tests for detection of prostate cancer, including MDxHealth[62], ExosomeDx[63], and LynxDx[64]. Here, urine sampling forms an interesting alternative as it is non-invasive.

The previous three trends clearly link to the EIT Health's first flagship '*new models to deliver healthcare*', which looks at how the community can define new models of healthcare delivery and the concept of value-based healthcare, where

measures of success are based on patient outcomes and the shift from treatment to prevention[5].

1.4. The rise of so-called self-care

Linked to the trend seen in uptake of digital health, there is also a rise of so-called self-care, in which individuals take their health into their own hands. This emergence has already led to the presence of medical devices including wearable devices such as smartwatches and fitness trackers in our daily life. Linked to the scope of the dissertation and thus looking at cancer in particular, at-home cancer test kits are starting to be introduced. These currently contain instructions and materials for a person to collect and return a sample for testing. This sample can be blood, saliva, stool, vaginal swab, or a urine sample. These tests screen for cancer by looking for specific biomarkers in the sample that may indicate a higher chance of developing a particular type of cancer. Companies are starting to capitalize on this rise in self-care by offering private health check test kits (e.g., 23andme[65], Check4cancer[66] and, Galleri[67]). Also, the sexually transmitted infections (STI) market already has many of these direct-to-consumer test kits. The fact that people buy and perform these tests to learn more about their own health opens up prospects for bringing home tests to the oncology market as well. However, this rise of self-care requires more efficient and reliable communication channels to be developed and used, to provide psychological support to a patient, especially when envisioning virtual patient-doctor meetings or when a test result needs to be communicated[34]. Additionally, healthcare professionals and policymakers will most likely have to change their habits and way of thinking about healthcare in order to further develop and benefit from this digital healthcare.

Currently, companies, such as MDxHealth, ExosomeDX and LynxDx, have incorporated self-sampling devices for urine collection (Colli-Pee®) in their diagnostic kits. However, these kits are still aimed for collection at the clinic or general practitioner's office. While other companies, such as 23andMe, Check4Cancer and Galleri, have self-sampling devices for urine collection in their kits that are sent to the individual's home. Home-sampling allows individuals to collect their samples in the comfort and privacy of their home environment. This could even mean that individuals can conduct diagnostic tests directly at home, without the need for visiting a health care facility. Furthermore, another benefit is that individuals will experience less anxiety and stress to pee at the

clinic. However, to enable self-sampling at home, there is a clear need for proper preservation of the urinary analytes and the self-sampling devices must be user-friendly and convenient. Additionally, the acceptability of at-home self-sampling devices is important for their adoption in the further development of the digital health era. Here, the Colli-Pee® UAS™ devices offer a potential solution for the user-friendly and convenient standardized and volumetric collection of first-void urine combined with immediate preservation of analytes of interest. We clearly demonstrated that Colli-Pee® UAS™ devices perform satisfactorily for the preservation of urinary analytes and are considered very easy and therefore user-friendly. Furthermore, home-sampling with Colli-Pee® UAS™ devices was very well received by the participants of our studies (**Chapter 4-6**). Furthermore, Novosanis offers solutions for home-sampling, where the Colli-Pee® devices can be included directly in a postal package. The postal kits are designed to utilize postal services for the distribution of the device to the individual and/or the return of the collected sample. Therefore, the combination of our results and Novosanis' postal solutions can already provide a digital medical device solution for self-sampling of urine at home.

And this fourth trend obviously links to the EIT Health's second flagship '*facilitating uptake of digital medical devices*', which aims to support the digital health transformation in Europe and focusses on the development of and access to digital health medical devices. It will also explore how the use of digital medical devices and diagnostics can be transformed, harmonized and enhanced to look at the prescription and reimbursement in EU countries for already certified medical devices and the harmonization of clinical trial protocol design to ensure the replicability and consistency of medical outcomes between member states for faster reimbursement[5]. The policymakers, governments, healthcare professionals and individuals play a key role in the uptake of self-sampling in research and diagnostics, if they don't accept the potential and process of self-sampling and support its use, there will be no further progress. It will become important that they open up to self-sampling and that prescriptions and reimbursement strategies are evaluated with a view to self-sampling.

Future perspectives

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2.1. Future perspectives

Breast cancer is the leading cause of cancer-associated death among women and the most prevalent cancer around the world[2]. Breast cancer is a very heterogenous disease that can be classified into distinct molecular subtypes based on the expression of estrogenic hormone receptors (ER), progesterone receptors (PR), human epidermal growth factor 2 (HER2), and Ki-67 proliferative index. These distinct molecular subtypes are luminal A (ER and/or PR+, Her2−, Ki-67 low), luminal B (ER and/or PR+, Her2−, Ki-67 high) or (ER and/ or PR+ Her2+), HER2-enriched (ER and PR− Her2+), and triple-negative (ER− PR− Her2−) breast cancer[68, 69]. The most aggressive subtype is the triple-negative breast cancer, of which the majority are of high grade and show a high proliferation rate[68]. And one of the most significant oncogenic pathways in breast cancer is PI3K, where PIK3CA mutations are the most common. Several studies have already shown the prognostic value and correlation to treatment response of these PIK3CA mutations[70-72]. A study has already shown that the detection of early-stage breast cancer using cfDNA is challenging due to the low concentrations compared to studies examining advanced-stage cancers. They investigated the presence of cfDNA in both plasma and urine and found a strong correlation (correlation coefficient >0.9) between both[73]. Furthermore, they confirmed previous results[72], demonstrating that PIK3CA profiling had a high clinical specificity and sensitivity when using plasma and urine compared to tumor tissues. These results highlight the potential of supplementing current testing methodologies and confirming key driver mutations for certain groups of patients[73]. Additionally, for patients with breast cancer disease relapse is a

critical concern, and these patients will benefit from more frequent testing, where urine can offer an easy-access sample for serial sampling. Therefore, we have an ongoing prospective collection of urine samples from breast cancer patients with HER2 amplification or PIK3CA hotspot mutations to perform a proof-of-concept study on the use of urinary cfDNA for the detection, diagnosis, follow-up, monitoring of recurrence or screening of breast cancer.

Additionally, further research is ongoing to elaborate on the potential of Colli-Pee® UAS™ for standardized and volumetric collection as well as preservation of first-void urine. First of all, the potential of UAS™ has already been explored for cfDNA but is now also under investigation for other urinary analytes such as extracellular vesicles, RNA, and proteins. Furthermore, research is ongoing to investigate improvements of the ratio between urine volume and preservative volume, including (i) reducing the amount of liquid preservative in the collector tube, and (ii) exploring possibilities for the creation of a powder/solid version of the preservative using technologies like lyophilization and spray drying, among others.

Other interesting studies to be conducted to further develop the potential of urine as liquid biopsy for oncology applications and Colli-Pee® UAS™ as at-home self-sampling device include: (i) studies comparing different commercially available preservatives to determine the potential of a one-size-fits-all preservative suitable for home-sampling, (ii) studies exploring possible optimizations of the different steps in the liquid biopsy workflow to standardize this workflow, (iii) investigating optimization of urine self-sampling devices to enable compatibility with high-throughput systems, and (iv) research into biodegradable materials to reduce the environmental impact of single-use plastic self-sampling devices.

2.2. Long-term future

Urine is an ideal non-invasive sample for a multitude of applications in cancer research and clinical practice in the future, including screening, patient stratification, early diagnosis, prediction of treatment response, drug or treatment selection, longitudinal monitoring during treatment, and detection of recurrence[74, 75]. All these applications support the era of precision medicine, in which personalized treatment-related decision-making is an important pillar[75].

However, before urine can be used to its full potential, there are some opportunities for improvement and challenges to overcome. First, there is a need for standardization of the pre-analytical workflow, including sample collection, preservation, storage, and isolation of analytes of interest. Colli-Pee® UAS™ devices can help achieve this standardization of the pre-analytical workflow, by providing standardized volumetric collection of first-void urine and immediate preservation of urinary analytes. Thereby, it could enable individuals to collect a urine sample in the comfort of their own home and send the sample to the laboratory, all in a more standardized manner. However, there is a need to develop and improve preservatives and techniques to isolate analytes of interest. Second, the development, optimization and standardization of detection methods would further increase the potential of urine in cancer research and care. These developments and improvements are necessary to increase the sensitivity and specificity of tests for them to be safe and suitable for several clinical applications, including early detection, prognosis, and monitoring[74, 76]. Additionally, improving computational and mathematical methods based on machine learning could further elaborate the applicability of liquid biopsy for routine setting[76]. Thanks to the promising results for urinary multi-omics biomarkers and the continuous development of isolation and analysis platforms, diagnostics based on body fluids has entered the next stage of development[74, 77].

Furthermore, EIT Health's third flagship '*harnessing the full potential of health data for innovation*', may help to elaborate further on the potential of urine as liquid biopsy. Because it supports the implementation of the European Health Data Space, so that the full potential of health data for innovation can be unleashed through the secondary use of data for the development of technologies or solutions that deliver outcomes that matter to patients. It also looks at how to educate and develop patients, citizens and healthcare professionals to understand the importance and relevance of data sharing in inform and improve the continuum of care pathways[5].

There are currently only screening programs for breast, colon and cervical cancer in Flanders (Belgium)[78], which have a good uptake and participation. However, urine could potentially even help to increase these the participation rate for cervical and breast cancer. Despite the usefulness of cancer screening, there are no screening programs in place for other types of cancer, mostly likely because these cancers are rare, current screening methods still yield many false-

positive or false-negative results, or a screening program is not cost-effective. Optimized urine self-sampling and home-sampling could be the ideal tool for screening programs, because (i) it could reduce the costs associated with screening[38], (ii) there is promising research on sensitive and specific urinary biomarkers that could lower the false-positive and false-negative rates and, (iii) it is highly accepted by study participants due to its non-invasive nature and confident in self-collection. The positive impact of urine self-sampling is already clearly proven in cervical cancer screening. Self-sampling collection of urine substantially increased cervical cancer screening participation rates with a major impact on morbidity and mortality[36, 79-84].

Additionally, previous studies have shown that urine is the most preferred sampling method for cervical cancer screening[36, 37, 85-88]. The World Health Organization and Human Reproduction Program have even suggested in their new guidelines that self-collected samples, such as urine, can be used when providing HPV DNA testing[89]. However, not only for cervical cancer can urine collection be a great solution for cancer screening, research has shown promising results for other cancers as well, such as breast cancer[74], colorectal cancer[90-92], hepatocellular carcinoma[93], non-small-cell lung cancer[14], and pancreatic cancer[94]. Importantly, social, economic, religious, and cultural barriers have a major impact on screening programs and participation rates. However, not only for other types of cancer and in Belgium urine can be a promising tool for screening programs, but it will also be a great method for cancer screening in low- and middle-income countries, remote locations (e.g., not close to hospitals or healthcare professionals) and, in certain cultures. In future, screening programs using urine could clearly facilitate the shift from therapeutic healthcare to preventive healthcare for diverse types of cancer, countries, cultures, religions, social and economic populations.

Here, we take the screening, follow-up and treatment of prostate cancer as an example to discuss the future possibilities on how urine can be implemented as a sample in this setting. Currently, the ESMO guidelines state that first PSA levels are evaluated, followed by multi-parametric magnetic resonance imaging (mpMRI), if PSA levels are elevated, and/or other risk factor calculators before a decision is made to perform a tumor biopsy[95]. And for monitoring, there are guidelines around active surveillance where elevated PSA, imaging and repeat biopsies are the key players (Figure 1)[95]. Interestingly, using a urine sample provides options to simplify screening and monitoring procedures and lower

the burden for the patients or individuals taking part in screening programs (Figure 1). A first way to lower the burden for patients and individuals would be to investigate PSA in urine instead of serum. Currently, urine biomarker based tests, such as the already commercially available SelectMDx, Mi-ProstateScore (MIPS), Progensa Prostate Cancer Antigen 3 (PCA3) and ExoDx Prostate Intelliscore (EPI) tests, are performed after PSA measurements as part of the risk factor calculators. Another way to simplify or lower burden is to replace PSA measurements by urinary biomarker based tests. Although this replacement will require changes to current reimbursement systems and guidelines. These commercially available urine biomarker tests target combinations of different biomarkers: SelectMDx from MDxHealth measures HOXC6, DLX1 and KLK3; University of Michigan's MIPS measures TMPRSS2:ERG and PCA3 in urine and serum PSA; PCA3 from Progensa only measures PCA3; and EPI from Exosome Diagnostics measures ERG, PCA3 and SPDEF. An important difference between these test is that SelectMDx, MIPS and PCA3 require a digital rectal examination (DRE) before collecting the urine sample, while EPI does not. A recent systematic review and network meta-analysis has already shown that SelectMDx and MIPS are the tests with the most suitable urine markers for prostate cancer screening and diagnosis[96]. Furthermore, they ranked the different markers compared to PSA based on the cumulative ranking area under the curve for the specificity: SelectMDx [MD=0.49, 95% CI: (-0.00,0.99), 85.2%] > MIPS [MD=0.27, 95%CI: (-0.22,0.75), 56.5%] > PCA3 [MD=0.22, 95%CI: (-0.01,0.45), 51.5%] > EPI [MD=0.19, 95%CI: (-0.57,0.95), 45.0%] > PSA (11.8%), which clearly indicated that all urinary biomarker based tests where more specific than PSA although the differences were not significant. Their overall conclusion was that all five urinary biomarker based tests were superior to PSA in terms of sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy[96]. And maybe one day urine can even replace mpMRI. A recent study already showed that the mpMRI strategy avoided 49% of unnecessary biopsies and that the SelectMDX test avoided 38% of unnecessary biopsies. More interesting for now, however, is that a conditional approach combining a positive SelectMDx test with a suspicious mpMRI already avoided 60% of unnecessary biopsies[97]. These recent studies clearly indicate the high potential of urine for screening, diagnosis and disease and treatment monitoring by simplifying and lowering the burden to patients and individuals. Since urine is a completely non-invasive sample type and can be taken by the patients themselves and without the need of a healthcare practitioner.

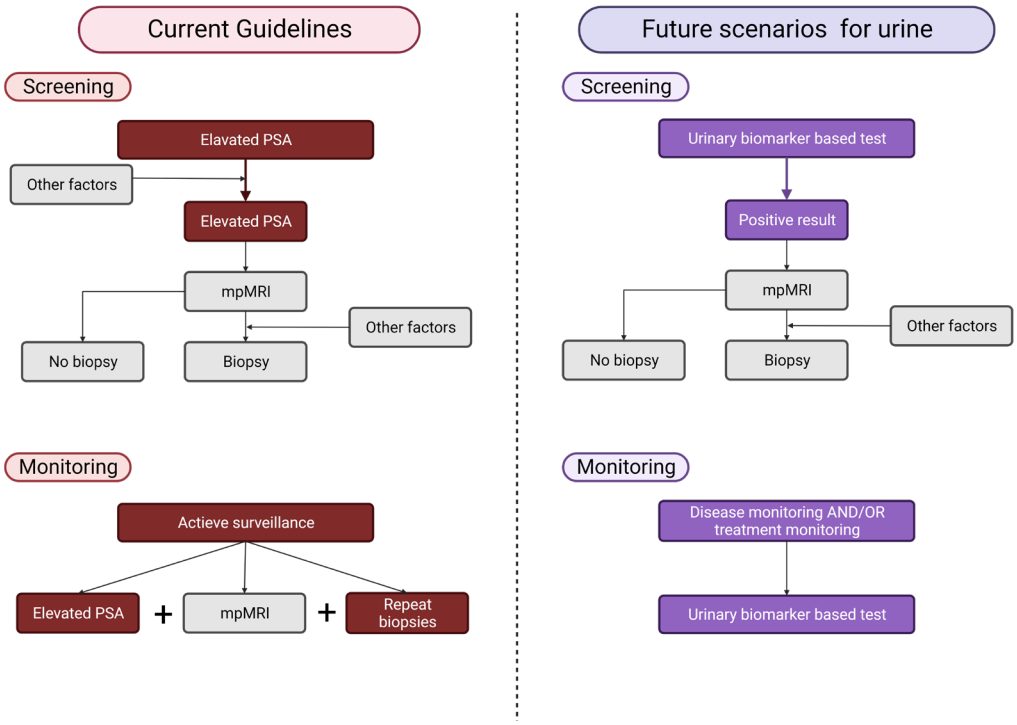

Figure 1: Overview of current guidelines vs future scenarios urine for prostate cancer screening and monitoring. Figure based on ESMO guidelines 2020[95].

Urine, as liquid biopsy, is a promising non-invasive and easy-to-repeat sampling approach for a multitude of clinical applications in cancer research and care. Thanks to the efforts of the scientific community, large clinical trials can be set up to perform clinical validation. Which will ultimately lead to the acceptability of urine as sample type for cancer research and care, and the recommendation of urine as liquid biopsy by major guidelines from governmental organizations.

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CHAPTER

09

Summary

Giving up is for rookies.
(Philoctetes, Hercules)

Summary

01

Cancer is a major public health challenge worldwide, with more than 19.3 million new cases in 2020 and an estimated 30.2 million new cases in 2040. In the era of precision oncology, the main goal is to provide the most accurate and effective treatment strategy based on the molecular profile of cancer and the individual patient. To perform large-scale cancer triage or screening in healthy individuals in the future, a more general cost-effective approach is needed. A new promising and emerging area in this regard is the field of liquid biopsy. The term liquid biopsy refers to the examination of analytes from various biological fluids, including blood, saliva, stool, air and urine. Compared to tissue biopsies, liquid biopsies have many advantages. They are more feasible, less invasive and have the potential to better evaluate inter- and intratumor heterogeneity. Urine has many advantages that make it a useful candidate for non-invasive liquid biopsy that enables serial sampling and multi-omics analysis for several types of cancers.

The dissertation started off with a comprehensive overview of the current literature-based data on urine sampling as liquid biopsy for non-invasive cancer research (**Chapter 3**). The two main health sciences databases, PubMed and Web of Sciences, were searched and information was extracted from all eligible publications (2010-2022) regarding: (i) study population characteristics, (ii) cancer type, (iii) urine pre-analytics, (iv) analyte class, (v) isolation method, (vi) detection method, (vii) comparator used, (viii) biomarker type, (ix) conclusion, and (x) sensitivity and specificity. Interestingly, we found 924 publications investigating urine for many types of cancers, different analytes and various applications. Our analysis demonstrated that most publications (73.8%) reported no information on a pre-analytical parameter of their samples, and almost 25.0% did not report any pre-analytical parameter, although several other studies have

emphasized the importance of standardization of sample handling. Interestingly, it was noted that urine is used for many cancer types and not just cancers originating from the urogenital tract, and urine can be used as a multi-omics sample type, covering DNA, RNA, proteins, metabolites, and essential metals. In this chapter, we describe the details of analytes of interest and pre-analytical parameters of urothelial cancer, prostate cancer, cervical cancer, ovarian cancer, endometrial cancer, colorectal cancer, hepatocellular carcinoma, pancreatic cancer, gastric cancer, cholangiocarcinoma, lung cancer, breast cancer, renal cell carcinoma, head and neck carcinoma and thyroid cancer. Furthermore, we provide future considerations regarding the heterogeneous nature of urine, the need for standardized practice protocols and the road towards the clinic. Since biomarker discovery and validation rely on proper sample handling and processing, we propose a standard parameter checklist or template for use in reporting research using urine as liquid biopsy. Because standardization will ultimately help elaborate the clinical utility of urine in cancer research, early detection, screening, and disease and treatment monitoring. Despite the lack of reporting of pre-analytical parameters, urine is an emerging liquid biopsy with broad applicability and potential in different analytes and several cancer types.

Next, we investigated the potential of urine for different cancer types. Two cancer types were addressed: prostate cancer and breast cancer. These types were chosen because they are the most common cancers in men and women. To further elaborate on the clinical applicability of urine as liquid biopsy, there are some challenges to overcome, including but not limited to: the degradation of interesting urinary analytes, the collection of the correct fraction of urine using appropriate and user-friendly devices, and the low concentration of urinary analytes of interest.

To allow for accurate and clinically relevant analysis of urinary analytes, it is critical to prevent analyte degradation during pre-analytical sample storage and handling. Therefore, we investigated the performance of a recently developed preservative, UAS™, for the preservation of urinary analytes (**Chapter 4**). First, the need for urine preservation was examined using urine samples from healthy volunteers. Secondly, the performance of UAS™ was assessed for different urinary analytes during storage at room temperature (RT) and after freeze-thaw cycling. Finally, UAS™ was used in a clinical setting on samples from breast and prostate cancer patients. Furthermore, this preservative was included in the Colli-Pee® device for the studies performed in Chapter 5. Results clearly demonstrated that

in the absence of a preservative, urinary cell-free DNA (cfDNA) was degraded, and bacterial overgrowth occurred at RT. No microbial growth was observed in urine samples stored in UAS™ and cfDNA and cellular integrity were maintained for up to 14 days at RT. The UAS™ preservative demonstrated satisfactory performance in preventing bacterial growth, preserving host cell integrity and cfDNA. After freeze-thaw cycling, the preservation of host cell integrity and cfDNA showed significant improvements when using UAS™ compared to unpreserved urine samples. Additionally, the agnostic nature of the UAS™ preservative was demonstrated by its compatibility with commercially available isolation methods based on different mechanisms using clinical samples. Furthermore, this study demonstrated the applicability of the first-void at-home urine collection device Colli-Pee® UAS™ by Novosanis as a standardized, volumetric device to collect urine and immediately preserve urinary analytes for oncology applications.

Another pre-analytical parameter in the liquid biopsy workflow is the isolation of cfDNA. Therefore, the isolation efficiency of four cfDNA isolation methods was compared (**Chapter 5**). For this optimization study, samples from pregnant women, breast and prostate cancer patients and healthy female and male volunteers were included. In particular, the presence of trans-renal urinary biomarkers was investigated by detecting male genes, such as SRY and DYS14, in women pregnant with a boy. First, the DNA concentration was shown to be higher in female samples compared to male samples, irrespective of the isolation method. Secondly, all isolation methods were shown to perform similarly with respect to the cfDNA fragmentation profiles. The nRichDX cfDNA isolation method that allowed the highest starting volumes of urine yielded the highest concentrations of DNA in the samples without the need to pool different isolation aliquots. Furthermore, three different Colli-Pee® UAS™ device variants (FV-5010, collecting approx. 10 mL; FV-5020, collecting approx. 20 mL; FV-5040, collecting approx. 40 mL) were compared for the standardized and volumetric collection of urine with immediate preservation of analytes. All three variants performed similarly, indicating their potential in cancer research.

Since standardization of urine collection and preservation could assist in elaborating the clinical utility of urine as source of biomarkers, it is important that devices developed for immediate preservation and collection of the correct fractions are easy, hygienic and user-friendly. Therefore, we examined the experiences and preferences of the study participants (**Chapter 5**) regarding the first-void urine collection and preservation using the Colli-Pee® UAS™ devices,

both for collection during the doctor's visit and as home-sampling (**Chapter 6**). The experiences and preferences were examined through the analysis of the questionnaires from 98 participants (females [n=59], males [n=39]) who participated in the URODETECT studies between June 2020 – April 2023. The vast majority was very positive about the urine collecting process and goals for which it could be used. Participants even indicated they would choose Colli-Pee® for their next urine sample and recommend using it to friends and/or family. Interestingly, the at home-sampling method was well received and could allow for higher screening coverages and allow for more comfort for patients during their disease monitoring.

Many interesting analytes are present in urine, albeit in a low concentration. In **Chapter 7**, we describe user-centered design and usability testing in design and development processes. Furthermore, we use the design process of a potential new Colli-Pee® variant for the enrichment of urinary analytes of interest, the URICH device, as case-example to demonstrate the inclusion of users and usability testing as early as possible in the design and development pathway.
In conclusion, this dissertation identified the gap in reporting and standardization of pre-analytical workflows for urine as liquid biopsy and provided some initial solutions to this gap. Furthermore, the potential of Colli-Pee® UAS™, as a device for the volumetric collection of first-void urine and immediate preservation of urinary analytes for oncology applications, was clearly demonstrated as well as its user-friendliness and further innovative potential.

CHAPTER

10

Samenvatting

All our dreams can come true, if we have the courage to pursue them.
(Walt Disney, himself)

Samenvatting

01

Kanker is wereldwijd een grote uitdaging voor de volksgezondheid, met meer dan 19,3 miljoen nieuwe gevallen in 2020 en naar schatting 30,2 miljoen nieuwe gevallen in 2040. In het tijdperk van precisie-oncologie is het belangrijkste doel om de meest nauwkeurige en effectieve behandelstrategie te bieden op basis van het moleculaire profiel van de kanker en de individuele patiënt. Om in de toekomst op grote schaal kankertriage of -screening bij gezonde individuen uit te voeren, is een meer algemene kosteneffectieve aanpak nodig. Een nieuw veelbelovend en opkomend onderzoeksgebied in dit opzicht is het onderzoeksgebied van vloeibare biopsies. De term vloeibare biopsie verwijst naar het onderzoek van analieten uit verschillende biologische vloeistoffen, waaronder bloed, ontlasting, speeksel, lucht en urine. In vergelijking met weefselbiopten hebben vloeibare biopsieën veel voordelen. Ze zijn haalbaarder, minder invasief en hebben het potentieel om inter- en intratumor heterogeniteit beter te evalueren. Urine heeft veel voordelen die het tot een bruikbare kandidaat maken voor niet-invasieve vloeibare biopsie die seriële staalafname en multi-omische analyse voor verschillende soorten kanker mogelijk maakt.

Deze doctoraatsthesis begon met een uitgebreid overzicht van de huidige op de literatuur-gebaseerde gegevens over urinestalen als vloeibare biopsie voor niet-invasief kankeronderzoek (**Hoofdstuk 3**). De twee belangrijkste gezondheidswetenschappelijke databases, PubMed en Web of Sciences, werden doorzocht en uit alle in aanmerking komende publicaties (2010-2022) werd informatie geëxtraheerd met betrekking tot: (i) kenmerken van de onderzoekspopulatie, (ii) kankertype, (iii) pre-analytische parameters van urine, (iv) analietklasse, (v) isolatiemethode, (vi) detectiemethode, (vii) gebruikte comparator, (viii) biomarkertype, (ix) conclusie en (x) gevoeligheid en specificiteit. Interessant is dat we 924 publicaties vonden die urine

onderzoeken voor vele soorten kanker, verschillende analieten en verschillende toepassingen. Onze analyse toonde aan dat de meeste publicaties (73,8%) geen informatie rapporteerden over een pre-analytische behandeling van hun stalen, en bijna 25,0% rapporteerde geen enkele pre-analytische parameter, hoewel verschillende andere studies het belang van standaardisatie van alle staalbehandelingen hebben aangetoond. Interessante bevinding is dat urine wordt gebruikt voor veel soorten kanker en niet alleen voor kankers die afkomstig zijn van het urogenitale kanaal, en dat urine kan worden gebruikt als een multi-omisch monstertype dat DNA, RNA, eiwitten, metabolieten en essentiële metalen omvat. In dit hoofdstuk, beschrijven we de details van interessante analieten en pre-analytische parameters van urotheelkanker, prostaatkanker, baarmoederhalskanker, eierstokkanker, endometriumkanker, colorectale kanker, hepatocellulair carcinoom, pancreaskanker, maagkanker, cholangiocarcinoom, longkanker, borstkanker, nier-cel-carcinoom, hoofd-hals-carcinoom en schildklierkanker. Verder hebben we enkele overwegingen toegelicht met betrekking tot de heterogene aard van urine, de behoefte aan gestandaardiseerde praktijkprotocollen en de weg naar de kliniek. Aangezien de ontdekking en validatie van biomerkers afhangt van de juiste behandeling en verwerking van stalen, hebben we een standaardparameterchecklist of -sjabloon voorgesteld voor gebruik bij het rapporteren van onderzoek waarbij urine als vloeibare biopsie wordt gebruikt. Omdat standaardisatie uiteindelijk zal helpen om de klinische bruikbaarheid van urine bij kankeronderzoek, vroege opsporing, screening en ziekte- en behandelingsmonitoring uit te werken. Ondanks het gebrek aan rapportage van pre-analytische parameters, is urine een opkomende vloeibare biopsie met brede toepasbaarheid en potentieel in verschillende analieten en verschillende soorten kanker.

Vervolgens onderzochten we het potentieel van urine voor verschillende soorten kanker. Twee soorten kanker werden behandeld: prostaatkanker en borstkanker. Deze typen zijn gekozen omdat ze de meest voorkomende vormen van kanker zijn bij mannen en vrouwen. Om de klinische toepasbaarheid van urine als vloeibare biopsie verder uit te werken, zijn er enkele uitdagingen die moeten worden overwonnen, waaronder maar niet beperkt tot: de afbraak van interessante urinaire analieten, het verzamelen van de juiste fractie urine met behulp van geschikte en gebruiksvriendelijke apparaten, en de lage concentratie van urinaire analieten van belang.

Om nauwkeurige en klinisch relevante analyse van urinaire analieten mogelijk te maken, is het van cruciaal belang om analiet-degradatie te voorkomen tijdens pre-analytische opslag en hantering van stalen. Daarom hebben we de prestaties onderzocht van een recent ontwikkeld bewaarmiddel, UAS™, voor het bewaren van urine-analieten (**Hoofdstuk 4**). Eerst werd de noodzaak van urinebewaring onderzocht met behulp van urinestalen van gezonde vrijwilligers. Ten tweede werden de prestaties van UAS™ beoordeeld voor verschillende urinaire analieten tijdens opslag bij kamertemperatuur (KT) en na vries-ontdooicycli. Ten slotte werd UAS™ in een klinische setting gebruikt bij stalen van patiënten met borst- en prostaatkanker. Bovendien werd dit bewaarmiddel toegevoegd aan het Colli-Pee®-apparaat voor de studies uitgevoerd in **Hoofdstuk 5**. De resultaten toonden duidelijk aan dat bij afwezigheid van een bewaarmiddel cel-vrij DNA (cfDNA) in de urine werd afgebroken en bacteriële overgroei optrad bij kamertemperatuur. Er werd geen microbiële groei waargenomen in urinestalen die bewaard waren met UAS™ en het cfDNA en de cellulaire integriteit werden tot 14 dagen bij kamertemperatuur behouden. Het UAS™-bewaarmiddel vertoonde bevredigende prestaties bij het voorkomen van bacteriële groei, het behoud van de integriteit van de gastheercel en cfDNA. Na cycli van bevriezen en ontdooien vertoonde het behoud van de integriteit van de gastheercel en cfDNA significante verbeteringen bij het gebruik van UAS™ in vergelijking met niet-geconserveerde urinestalen. Bovendien werd de agnostische aard van het UAS™-bewaarmiddel aangetoond door zijn compatibiliteit met in de handel verkrijgbare isolatiemethoden op basis van verschillende mechanismen met behulp van klinische stalen. Bovendien toonde deze studie de toepasbaarheid aan van het eerste-fractie urine collectie-apparaat Colli-Pee® UAS™ van Novosanis als een gestandaardiseerd, volumetrisch apparaat om urine op te vangen en urine-analieten onmiddellijk te bewaren voor oncologische toepassingen.

Een andere pre-analytische parameter in de workflow voor vloeibare biopsie is de isolatie van cfDNA. Daarom werd de isolatie-efficiëntie van vier cfDNA-isolatiemethoden vergeleken (**Hoofdstuk 5**). Stalen van zwangere vrouwen, borst- en prostaatkankerpatiënten en gezonde vrouwelijke en mannelijke vrijwilligers werden geïncludeerd in deze optimalisatiestudie. In het bijzonder werd de aanwezigheid van trans-renale biomarkers in de urine onderzocht door mannelijke genen, zoals SRY en DYS14, te detecteren bij vrouwen die zwanger waren van een jongen. Ten eerste bleek de DNA-concentratie hoger te zijn in vrouwelijke stalen in vergelijking met mannelijke stalen, ongeacht de isolatiemethode. Ten tweede bleken alle isolatiemethoden vergelijkbaar te

presteren met betrekking tot de cfDNA-fragmentatieprofielen. De nRichDX cfDNA-isolatiemethode die de hoogste startvolumes urine mogelijk maakte, leverde de hoogste concentraties DNA op in de stalen zonder de noodzaak om verschillende isolatie-aliquots samen te voegen. Bovendien werden drie verschillende Colli-Pee® UAS™-apparaat varianten (FV-5010, collecteert ca. 10 ml; FV-5020, collecteert ca. 20 ml; FV-5040, collecteert ca. 40 ml) vergeleken voor de gestandaardiseerde en volumetrische collectie van urine met onmiddellijke bewaring van analieten. Alle drie de varianten presteerden vergelijkbaar, wat wijst op hun potentieel in kankeronderzoek.

Aangezien standaardisatie van urinecollectie en -opslag zou kunnen helpen om de klinische bruikbaarheid van urine als bron van biomerkers te ontwikkelen, is het belangrijk dat apparaten die zijn ontworpen voor collectie en onmiddellijke opslag van de juiste fracties eenvoudig, hygiënisch en gebruiksvriendelijk zijn. Daarom onderzochten we de ervaringen en voorkeuren van de studiedeelnemers (**Hoofdstuk 5**) met betrekking tot het collecteren en bewaren van eerste-fractie urine met behulp van de Colli-Pee® UAS™-apparaten, zowel voor het collecteren tijdens het doktersbezoek als voor thuiscollectie (**Hoofdstuk 6**). De ervaringen en voorkeuren werden onderzocht door de analyse van de vragenlijsten van 98 deelnemers (vrouwen [n=59], mannen [n=39]) die tussen juni 2020 – april 2023 deelnamen aan de URODETECT-studies. De overgrote meerderheid was zeer positief over het urine collectieproces en de doelen waarvoor het zou kunnen worden gebruikt. Deelnemers gaven zelfs aan dat ze Colli-Pee® zouden kiezen voor hun volgende urinestaal en het aanbevelen aan vrienden en/of familie. Interessant is dat thuisafname goed werd ontvangen en een hogere screeningdekking mogelijk zou maken en patiënten meer comfort zou bieden tijdens hun ziektemonitoring.

Veel interessante analieten zijn aanwezig in urine, zij het in een lage concentratie. In **Hoofdstuk 7** beschrijven we user-centered design en usability testing in ontwerp- en ontwikkelprocessen. Verder gebruiken we het ontwerpproces van een mogelijke nieuwe Colli-Pee®-variant voor de verrijking van urine-analieten, het URICH-apparaat, als voorbeeld om de inclusie van gebruikers en bruikbaarheidstesten zo vroeg mogelijk in het ontwerp en ontwikkelingstraject aan te tonen.

Concluderend identificeerde deze doctoraatsthesis de tekortkoming in rapportage en standaardisatie van pre-analytische workflows voor urine als vloeibare biopsie en bood het enkele eerste oplossingen voor deze tekortkoming. Bovendien werd het potentieel van Colli-Pee® UAS™, als een apparaat voor de volumetrische opvang van eerste-fractie urine en onmiddellijk bewaring van urinaire analieten voor oncologische toepassingen, duidelijk aangetoond, evenals de gebruiksvriendelijkheid en het verdere innovatieve potentieel.

CHAPTER

11

Curriculum Vitae

I give myself very good advice, but I very seldom follow it.
(Alice, Alice in Wonderland)

Education

- **University of Antwerp, Faculty GGW, PhD**

  2020- current

- **University of Antwerp, Faculty FBD, Biochemistry – Biotechnology**

  2016-2019

  Shortened bachelor program for academic bachelor biochemistry and biotechnology

  Master biochemistry and biotechnology – specialization cell biology and physiology – minor research

  Research project (6 weeks): "PD-1 sensitive natural killer cell model assay as a tool to study PD-1 interactions in dendritic cell – natural killer cell crosstalk"

  Master thesis (ERASMUS in Hull; UK): "Identification and characterization of novel antigens in patients with adult B-cell acute lymphoblastic leukaemia"

- **University of Ghent, Department of Science, Biochemistry – Biotechnology**

  2015-2016

  Shortened bachelor program which I started at the University of Ghent and finished at University of Antwerp.

- **Karel de Grote hogeschool, IWT Hoboken, Chemistry – Biochemistry**

  2012-2015

  Professional bachelor Chemistry specialization biochemistry

  Bachelor thesis: "Vasculaire calcificatie bij chronische nierfalen"

- **Mater Salvatoris Instituut Kapellen – Science – Mathematics**

  2006-2012

Career overview

- **University of Antwerp – PhD student (Baekeland Mandate)**

  January 2020 - current

- **Novosanis NV – Biomedical scientist / PhD Student**

  Augustus 2019 - current

Publications

International peer-reviewed publications

During PhD research:

Jordaens S., Ríos Cortés A., Tjalma W., Pauwels P., Vankerckhoven V., Beyers K. Acceptability and usability of Colli-Pee® UAS™ for first-catch urine self-sampling at home. Cancers (MDPI) (ISSN 2045-2322). (2023). Submitted

Jordaens S., Ríos Cortés A., Polis R., Neyrinck J., Verwulgen S., Vankerckhoven V., Beyers K. Human factors engineering and usability as key drivers for design of device suited for urine filtration and enrichment. Current Trends in Biomedical Engineering and Biosciences (ISSN 2572-1151). Volume 21, Issue 5 (2023).

Jordaens S., Arora A., MacDonald K., Wood C., Hendrickx J., Zwaenepoel K., Deben C., Tjalma W., Pauwels P., Beyers K., Vankerckhoven V. UAS™ - a urine preservative for oncology applications. Cancers (ISSN 2072-6694). Volume 15, Issue 12 (2023).

Jordaens S., Oeyen E., Willems H., Ameye F., De Wachter S., Pauwels P., Mertens I. Protein biomarker discovery studies on urinary sEV fractions separated with UF-SEC for the first diagnosis and detection of recurrence in bladder cancer patients. Biomolecules (ISSN 2218-273X). Volume 13, Issue 6 (2023).

Jordaens S., Zwaenepoel K., Tjalma W., Deben C., Beyers, K., Vankerckhoven V., Pauwels P., Vorsters A. Urine biomarkers in cancer detection: a systematic review of pre-analytical parameters and applied methods. International Journal of Cancer (ISSN 1097-0215). Volume 10, Issue 152 (2023)

Augustus E., Zwaenepoel K., Siozopoulou V., Raskin J., **Jordaens S.**, Baggerman G., Sorber L., Roeyen G., Peeters M., Pauwels P. Prognostic and predictive biomarkers in non-small cell lung cancer patients on immunotherapy - the role of liquid biopsy in unraveling the puzzle. Cancers (ISSN 2072-6694). Volume 13, Issue 7 (2021)

Before PhD research:

Jordaens S., Cooksey L., Bonney S., Orchard L., Coutinho M., Van Tendeloo V., Mills K.I., Orchard K., Guinn B.A. Serum profiling identifies ibrutinib as a treatment option for young adults with B-cell acute lymphoblastic leukaemia. Britisch Journal of haematology (ISSN 0007-1048). Volume 189, Issue 3 (2020) p. 500-512

Jordaens S., Cooksey L., Boullosa L.F., Van Tendeloo V., Smits E., Mills K.I., Orchard K.H., Guinn B.A. New targets for therapy: antigen identification in adults with B-cell acute lymphoblastic leukaemia. Cancer immunology and immunotherapy (ISSN 0340-7004), 69 (2020) p. 867-877

National peer-reviewed publications

Jordaens S., Boullosa L.F., Cooksey L., Van Tendeloo V., Smits E., Orchard K.H., Guinn B.A. Identificatie van antigenen als therapiedoelwitten voor volwassenen met acute lymfoblastische B-celleukemie. Onco-Hemato (ISSN 2030-2738). Volume 14, Issue 3 (2020) p. 6-10

Oral (invited) presentations

- Urine biomarkers in cancer detection: a systematic review of pre-analytical parameters and applied methods – EUROGIN (February, 2023, Bilbao, Spain).
- Potential of urine as liquid biopsy for oncology applications – Webinar EACR – Novosanis (Virtual).
- Moving urine as a liquid biopsy into clinical practice: bridging the gap – Circulating Biomarkers, Exosomes & Liquid Biopsy Europe 2022 (March 2022, Rotterdam, The Netherlands).
- Urine as the golden sample for cancer biomarker detection – Liquid biopsy congress (Virtual).
- When tissue is an issue – urine as a liquid biopsy in oncology screening – Webinar Frontline Genomics (Virtual).
- Urine as the golden sample for cancer biomarker detection – International Liquid Biopsy Society (ISLB) congress (Virtual).
- Urine as an emerging liquid biopsy – 5th Advanced Circulating Tumor Congress (ACTC) (September 2021, Kalamata, Greece).
- Urine as promising biomarker source for cancer detection – International Liquid Biopsy Society (ISLB) congress (Virtual).

Abstracts accepted for oral presentation

2022

Beyers K., Ríos Cortés A., **Jordaens S.**, Meers N., Hendrickx J., Pasmans D., Tijsen J., Vankerckhoven V. Explorative Study on Device Architecture for defined Ranges of Firs-Void Urine Collection. EUROGIN, Düsseldorf, Germany, April 11, 2022. (oral presentation by K. Beyers)

Hendrickx J., **Jordaens S.**, Meers N., Ríos Cortés A., Pasmans D., Beyers K., Vankerckhoven V. First-void urine: a reliable approach for cervical cancer screening. EUROGIN, Düsseldorf, April 11, 2022. (oral presentation by J. O. Hendrickx)

2020

Laeremans M., Van Avondt Q., Pasmans D., Meers N., Ríos Cortés A., **Jordaens S.**, Baks C., Beyers K., Vankerckhoven V.. First-void urine as an alternative to physician-taken samples for HPV screening. IPVC 2020, Barcelona, Spain, July 20-24, 2020. (oral presentation by M. Laeremans)

Abstracts accepted for poster presentation

2023

Meers N., Macdonald K., Wood C., Ríos Cortés A., Richer M., **Jordaens S.**, Hendrickx J., Crawford Parks T., Beyers K., Vankerckhoven V. Validation of Colli-Pee UCM FV-5004: A user-friendly device for volumetric first-void urine collection and HPV DNA preservation compatible with high-throughput instruments. EUROGIN, Bilbao, Spain, February 8-11, 2023.

Hendrickx J., **Jordaens S.**, Meers N., Ríos Cortés A., Beyers K., Vankerckhoven V. Home-based self-sampling of first-void urine for hr-HPV testing in the casus study: attitudes and preferences from a Belgian colposcopy referral population. EUROGIN, Bilbao, Spain, February 8-11, 2023.

Beyers K., Ríos Cortés A., Meers N., MacDonald K., Wood C., **Jordaens S.**, Hendrickx J., Vankerckhoven V. Colli-Pee device architectures for defined volumes of first-void urine collection, compatible with high-throughput carriers. IPVC, Washington DC, United States of America, April 17-21, 2023.

Jordaens S., Zwaenepoel K., Tjalma W., Deben C., Beyers K., Vankerckhoven V., Pauwels P., Vorsters A. Urine biomarkers in cancer detection: a systematic review of pre-analytical parameters and applied methods with focus on breast cancer. SGBCC 2023, Vienna, Austria, March 15-18, 2023.

2022

Hendrickx J., **Jordaens S.**, Meers N., Pasmans D., Beyers K., Vankerckhoven V. First-Void Urine and Vaginal Self-Sampling in Cervical Cancer Screening: Feedback from a Colposcopy Referral Population. EUROGIN, Düsseldorf, Germany, April 9-12, 2022.

Hendrickx J., **Jordaens S.**, Meers N., Ríos Cortés A., Beyers K., Vankerckhoven V. Home-Based Self-Sampling of First-Void Urine for hrHPV testing in Cervical Cancer Screening: Usability feedback from a Belgian Colposcopy Referral Population. EUROGIN, Düsseldorf, Germany, April 9-12, 2022.

Arora A., MacDonald K., Wood C., Wong J., Narula G., Crawford Parks T., Doukhanine E., Morse G., **Jordaens S.**, Tijsen J., Hendricks J., Beyers K., Vankerckhoven V., Iwasiow R. Stabilization and characterisation of urinary cell-free DNA to facilitate home based sampling. EACR Liquid Biopsies, Bergamo, Italy, May 24-26, 2022.

Jordaens S., Arora A., Zwaenepoel K., Vorsters A., Tijsen C.J., Beyers, K., Deben, C., Tjalma, W., De Wachter S., Van Dam P., Pauwels P., Vankerckhoven V., Jafari N. Colli-Pee® UAS™ Combined with nRichDX Revolution System™ , a Promising Urinary Cell-free DNA Collection, Preservation and Extraction Workflow. AMP Annual Meeting & Expo, Phoenix Arizona, November 1-5, 2022.

2021

Meers N., **Jordaens S.**, Van den Bossche R., Beyers K., Vankerckhoven V. Home-based self-sampling of first-void urine as a valuable and accessible HPV screening method during and after the COVID-19 pandemic. IPVC, virtual congress, November 15-19, 2021.

Scalco Vasconcelos D., **Jordaens S.**, Hendrickx J., Van den Bossche R., Meers N., Van Keer S., Téblick L., Beyers K., Vorsters A., Vankerckhoven V. Usability evaluation of Colli-Pee Small Volumes for HPV-based cervical cancer screening (CASUS). IPVC, Virtual congress, November 15-19, 2021.

Hendrickx J., **Jordaens S.**, Meers N., Beyers K., Vankerckhoven V. First-void urine self-sampling for hrHPV testing in cervical cancer screening: sample preference from a Belgian triage population. ISLB, October 22, 2021.

Jordaens S., Zwaenepoel K., Vorsters A., Beyers K., Deben C., Tjalma W., De Wachter S., Van Dam P., Vankerckhoven V., Pauwels P. Comparison of three commercially available kits for cfDNA isolation from first-void urine collected with Colli-Pee containing UAS. ISLB, October 22, 2021.

Jordaens S., Zwaenepoel K., Vorsters A., Deben C., Tjalma W., De Wachter S., Van Dam P., Vankerckhoven V., Pauwels P. cfDNA isolation from first-void urine using the Colli-Pee device containing UAS. ACTC, September 22-25, 2021.

2020

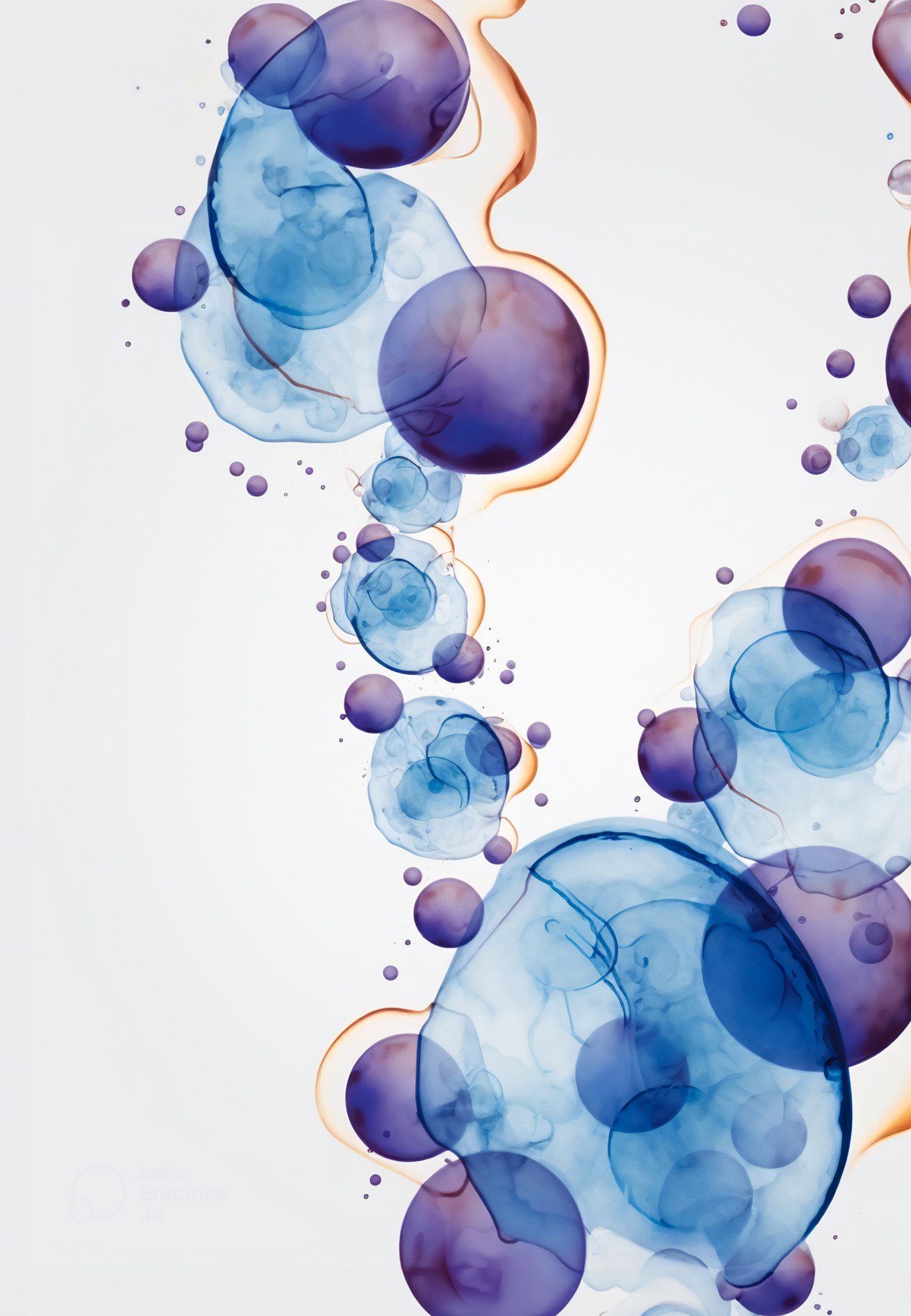
Jordaens S., Laeremans M., Mehta A., Pasmans D., Bruyninckx S., Beyers K., Vankerckhoven V. Colli-Pee, the urinomics solution. Knowledge for Growth, September 9-10, 2020.

Baks C., Beyers K., Rios Cortes A., Meers N., Laeremans M., **Jordaens S.**, Bastiaens J., Vankerckhoven V. Design optimization of the Colli-Pee first-void urine sample device to enable HPV screening using Computational Fluid Dynamics. IPVC 2020, Barcelona, Spain, July 20-24, 2020.

Laeremans M., Meers N., Rios Cortes A., **Jordaens S.**, Van den Bossche R., Baks C., Beyers K., Vankerckhoven V. Development of a cervical cancer screening approach based on first-void urine: verification of volumetric and standardized collection. IPVC 2020, Barcelona, Spain, July 20-24, 2020.

(Co)-Promotor Bachelor and Master students

- Industrial Promotor Master Thesis Rani Moens Industrial Sciences – Biochemistry entitled 'Exploration of microbial DNA in different urine fractions using two different preservation chemistries (2021-2022)
- Industrial Promotor EMOTION Master thesis Daniele Scalco Vasconcelos entitled 'Implications of the European Medical Device Regulation for Clinical Requirements in the conformity assessment of medical devices (2021)
- Industrial Promotor Master thesis Lucas Scheper Biomedical Sciences entitled 'Colli-Pee® for applications in infectious diseases for LMIC and rapid diagnostic methods for detection of HPV in first-void urine' (2020-2021)
- Industrial Promotor Master thesis Dorien Scholiers Industrial Sciences – Biochemistry entitled 'Evaluation of HPV DNA and RNA stability in urine using various preservatives (2020-2021)
- Industrial Promotor Bachelor thesis Mauranne Bauwens and Cato Vangenechten Biomedical Sciences entitled 'Detectie van prostaatkanker biomerkers in eerste fractie urine' (2020-2021)



CHAPTER

12

Acknowledgement

Ohana means family, family means nobody gets left behind.
(Stitch, Lilo & Stitch)

Acknowledgements

01

En dan zijn we nu aangekomen bij het hoofdstuk dat door velen als eerste gelezen zal worden ☺, daarom wil ik hier dan ook uitgebreid iedereen bedanken die mij de afgelopen vier jaar gesteund en geholpen heeft om dit doctoraatsonderzoek tot een goed einde te brengen. Maar ook om mij de kansen en ruimte te geven om mezelf te ontwikkelen tot de persoon die hier vandaag staat.
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overtuigen dat er wel degelijk zaken zijn waar je zelf geen fout aan hebt of geen invloed op hebt, zoals de COVID-19 pandemie. Ik apprecieerde ten zeerste dat je eind 2021 langer in de vergaderzaal van pathologie bleef om te babbelen over 'waarom ik zo teleurgesteld was dat er zaken moesten wijzingen met oog op de tijd'. Je begreep oprecht waarom ik het er lastig mee had, maar je verklaarde ook heel duidelijk dat wat er ook op papier stond in het begin, dat het zelden is wat er op het einde afgeleverd wordt in het leven. Je opende mijn ogen dat ik misschien iets te streng was voor mezelf en echt iets meer voldoening mocht halen uit wat er uiteindelijk wel terecht zou komen van de experimenten. Daarnaast benadruk ik graag hoe hard ik onze wekelijkse meeting sinds September 2022 in onze agenda apprecieer, want tijdens deze meetings was je altijd zeer betrokken. En dit niet alleen over de lopende gang van zaken omtrent de patiënten inclusies en de experimenten maar ook hoe het mogelijks met mij ging. U was ook altijd bereidt manuscripten, abstracts en posters na te lezen zo snel als het drukke doktersleven u toeliet en als die gekke computerprogramma's met vreemde beveiligingen eindelijk wilden meewerken. Waarom instituties alles IT-gewijs zo moeilijk maken dat blijft voor ons beide toch de vraag ☺. Maar bij deze dus Professor Hartelijk bedankt voor de persoon die u bent en alle ondersteuning die u geboden heeft!

Prof. Vorsters, ik wil jou graag bedanken voor alle ondersteuning en advies die je me gegeven hebt tijdens dit doctoraat. Ik vond het enorm leerzaam om samen met jou de Systematic Review te schrijven, de eerste echte publicatie van dit doctoraat. Daarnaast wil ik je ook bedanken voor mij deel te maken van uw bi-weekly team meeting en mij hiermee de mogelijkheid te bieden om kennis te maken met uw fantastisch team dat verschillende kanten van de HPV problematiek bekijkt maar vooral ook werkt met urine en zelfs Colli-Pee®. Maar natuurlijk wil ik u vooral bedanken voor het delen van uw expertise en uw ervaring omtrent cfDNA en het trans-renale onderzoek. Bovendien was u ook altijd zeer snel en efficiënt in het geven van feedback op abstracts, posters en manuscripten. We konden dan ook altijd op een efficiënte manier tot een beslissing komen en daarmee zaken afronden. Maar ook het nalezen van de nog niet ingediende manuscripten en hoofdstukken van dit doctoraat deed u zorgvuldig en zo snel als mogelijk was. Beste Alex, hartelijk bedankt voor alles!

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proberen ontwikkelen en optimaliseren voor urine. Als ik een vraag had over wat dan ook, dan kon ik altijd op uw deur kloppen en was je bereidt hier samen over na te denken en een antwoord te gaan zoeken. En ook tijdens COVID kon ik u bereiken, dan wel via mail, maar we ontdekten al snel dat mailen over dergelijke zaken vooral leidde tot misverstanden. Dan maar overschakelen op Teams meetings om samen te bespreken wat er mogelijk was en wat er mogelijk misliep. Daarnaast wil ik jou bedanken voor je altijd snelle feedback en reviews op abstracts, posters & publicaties. Als je iets doorgestuurd kreeg voor review dan had ik van jou altijd op 1-3 dagen de feedback ontvangen! Dat is natuurlijk super-efficiënt, waar jij ook voor staat! En waar ik ontzettend van hou ☺. Karen, bedankt voor alles!!!

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University of Antwerp

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