First-void urine

A reservoir of HPV-related biomarkers

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First-void urine: A reservoir of HPV-related biomarkers

Eerste fractie urine: Een reservoir van HPV-gerelateerde biomerkers

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Medische Wetenschappen aan de Universiteit Antwerpen, te verdedigen door

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Antwerpen, 2024

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LIST OF ABBREVIATIONS

ACTB	β -actin
AlphaLISA	Amplified Luminescent Proximity Homogeneous Assay
AM	Amicon filtration
APC	Antigen-presenting cell
AU	Arbitrary units
BM	Basement membrane
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
CEV	Centre for the Evaluation of Vaccination
cfDNA	Cell-free DNA
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
COVID-19	Coronavirus Disease 2019
Cq	Quantification cycle
CT	*Chlamydia trachomatis*
ctDNA	Circulating tumor DNA
CVS	Cervicovaginal secretions
Cy5	Cyanine5
DAPI	4′, 6-diamidino-2′-phenylindole, dihydrochloride
DC	Dendritic cell
DELFIA	Dissociation-Enhanced Lanthanide Fluorescent Immunoassay
DKFZ	German Cancer Research Center
DNA	Deoxyribonucleic acid
DTPA	Pentetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-Linked Immunosorbent Assay

EU	European Union
FGT	Female genital tract
FVU	First-void urine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
Gluc	Gaussia luciferase
GMT	Geometric mean titer
GST	Glutathione S-transferase
HBB	β -globin
HEK	Human embryonic kidney
HI	Heat-inactivated
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR	High-risk
HSPGs	Heparin sulfate proteoglycans
HTM	High-throughput automated widefield microscopy
HTRF	Homogenous time-resolved fluorescence
IFN	Interferon
Ig	Immunoglobulin
IQR	Interquartile range
IU	International units
IUD	Intrauterine device
K	Cohen's Kappa
LCR	Locus control regions
LEEP	Loop electrosurgical excision procedure
LLOQ	Lower limit of quantification
LN	Lymph nodes
LOD	Limit of detection
LR	Low-risk
LSC	Low-speed centrifugation

MEM	Minimum Essential Medium
MFI	Mean fluorescence intensity
MG	Melon gel purification
nAbs	Neutralizing antibodies
NIBSC	National Institute for Biological Standards and Controls
NK	Natural killer
Nluc	Nanoluciferase
ori	Replication origin
PBNA	Pseudovirion-based neutralization assay
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PGMB	Protein G magnetic beads
PhHV-1	Phocine herpesvirus 1
PLL	Parallel line method
PsV	Pseudovirion
qMSP	Quantitative methylation-specific PCR
qPCR	Quantitative polymerase chain reaction
RLU	Relative light units
ROC	Receiver operating characteristic
Rpm	Rounds per minute
Rs	Spearman rank correlation coefficient
SCJ	Squamocolumnar junction
SEAP	Secreted alkaline phosphatase
SIgA	Secretory immunoglobulin A
STI	Sexually transmitted infection
THP	Tamm-Horsfall protein
TLR	Toll-like receptors
TRF	Time-resolved fluorescence
UCM	Urine conservation medium
UMOD	Uromodulin

UZA	University Hospital Antwerp
VLP	Virus-like particle
WHO	World Health Organization
2vHPV	Bivalent HPV vaccine
4vHPV	Quadrivalent HPV vaccine
9vHPV	Nonavalent HPV vaccine

CHAPTER

01

Introduction and objectives of the thesis

INTRODUCTION

1 Why first-void urine

1.1 Biomarker sampling

To effectively monitor infections, diseases, and immune responses, appropriate samples are required. Biomarkers, as defined by the World Health Organization (WHO), are any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease [1]. Examples of biomarker types are proteins, nucleic acids, metabolites, hormones, and inflammatory biomarkers [2,3]. Sampling these biomarkers enables the evaluation of the health status of an individual, which is invaluable for timely diagnosis of infection and disease and allows for further tracing of their progression or regression. Furthermore, biomarker sampling facilitates comprehensive assessment of the immune response, including pathogen-host interactions. This large amount of information helps clinicians and researchers to optimize interventions. Some examples of sample types allowing biomarker collection are blood, saliva, tissue samples, tear fluid, urine, and feces [4]. In essence, biomarker sampling is an important clinical tool for better understanding the molecular background of health and disease and corresponding immunologic responses.

1.2 Self-sampling

While certain sample types typically require the expertise of a trained individual for accurate collection, others can be self-collected and are therefore categorized as self-samples. Self-sampling covers various sample types, such as saliva, nasal swabs, hand swabs, vaginal swabs, dried blood spots, urine, and fecal samples [5–13]. While self-collected samples may be more prone to incorrect collection and lower sensitivity, the acceptability and accuracy of these self-sampling methods has been widely acknowledged. Moreover, self-sampling offers the advantage of convenience, enabling individuals to collect samples at home, thereby enhancing accessibility to diagnostic procedures and proving cost-effective by reducing the need for frequent healthcare visits. Beyond individual benefits, self-sampling supports broader public health initiatives, providing a comfortable and private

option for conducting large-scale screening programs, epidemiological studies, and vaccine trials.

1.3 Rationale for using first-void urine

Urine contains a wide variety of biomarkers [14–18]. Secretions from the entire urogenital tract are captured in urine, together with debris of whole exfoliated cells [19]. Due to substantial distinctions between the female and male urogenital tracts, the collected secretions and, consequently, the biological information present in a urine sample varies significantly [20–23]. Recent attention has been directed toward the initial stream of urine, known as first-void urine, particularly for females. In females, urogenital tract secretions accumulate between the labia minora and are washed away with the first urine flow [19,24]. This results in a sample that is rich in biological information [25].

As first-void urine serves as a non-invasive sample, capturing secretions from the female genital tract (FGT), there lies opportunity in using this sample type for monitoring urogenital tract related diseases [26–29]. Moreover, this sample type holds potential for immunological purposes, given the known exudation and transudation of antibodies from the bloodstream to the genital tract and the presence of cellular immune cells [30–33]. With the increasing utilization of body fluids for disease and vaccination monitoring, and a growing interest in liquid biopsies due to their less invasive nature compared to tissue biopsies, in-depth exploration of first-void urine has an added value [34,35]. The fact that a person urinates several times daily adds to its advantages over other minimally invasive samples, such as vaginal self-samples, serum, and plasma.

1.4 The potential and progress of first-void urine in HPV research

Over the past decade, numerous studies have assessed the utility of first-void urine in human papillomavirus (HPV) detection, the leading cause of cervical cancer in females. To screen for cervical cancer, most countries use a clinician collected cervical sample. Non-invasive, self-collected sampling at home offers the potential to reach unscreened females more effectively [35–37]. Given that FGT secretions, known to contain HPV-biomarkers, are captured in first-void urine, the presence of HPV DNA in this sample was anticipated, and ample evidence supporting this hypothesis has been gathered by research groups worldwide [38–45]. Furthermore, it has been demonstrated that the first stream of urine contains more

HPV DNA compared to the subsequent part [25]. Comprehensive studies have demonstrated non-inferiority of clinical sensitivity and specificity for HPV testing in first-void urine samples compared to clinician-collected cervical samples and vaginal self-samples [37,44,46,47].

In addition to HPV DNA detection, the same first-void urine sample has been explored as a potential sample for the categorization (triage) of females based on their likelihood of developing precancerous lesions or cervical cancer [7]. This approach offers the advantage of requiring only one sample for both HPV DNA detection and subsequent triage. As it is known that only a small percentage of females with an HPV infection develop cervical lesions or even cervical cancer, triage of these females is essential. Merging screening and triage into a self-collected, non-invasive first-void urine sample not only significantly reduces costs and manual labor but also enhances the identification of females requiring treatment using just one single sample. Consequently, researchers have examined triage markers in first-void urine samples, and as of now, methylation markers show great promise in stratifying cervical lesions according to clinical relevance [48].

Additionally, vaccine-induced humoral immune response towards HPV has been investigated in first-void urine samples [49,50]. Combining the detection of virological (DNA), diagnostic (methylation), and immunological (antibodies) markers in a single sample may ultimately lead to major progress in public health (Figure 1). This approach not only enables more timely interventions and personalized treatment strategies but also allows for the assessment of vaccination effectiveness at a population level, optimization of vaccination campaigns, and enhancement of large-scale screening programs for improved public health outcomes.

Notably, studies have demonstrated the presence of HPV-specific antibodies in first-void urine samples, establishing robust correlations between concentrations in first-void urine and serum [51]. However, the stability and potency of HPV-specific antibodies collected during urination remained unexplored. Additionally, the detection of HPV-related cellular immunological markers in first-void urine has yet to be investigated.

It is clear that the use of first-void urine as sample to detect HPV-related biomarkers has made considerable progress and that there still lies immense potential in this sample type to unravel the remaining knowledge gaps discussed in this thesis.

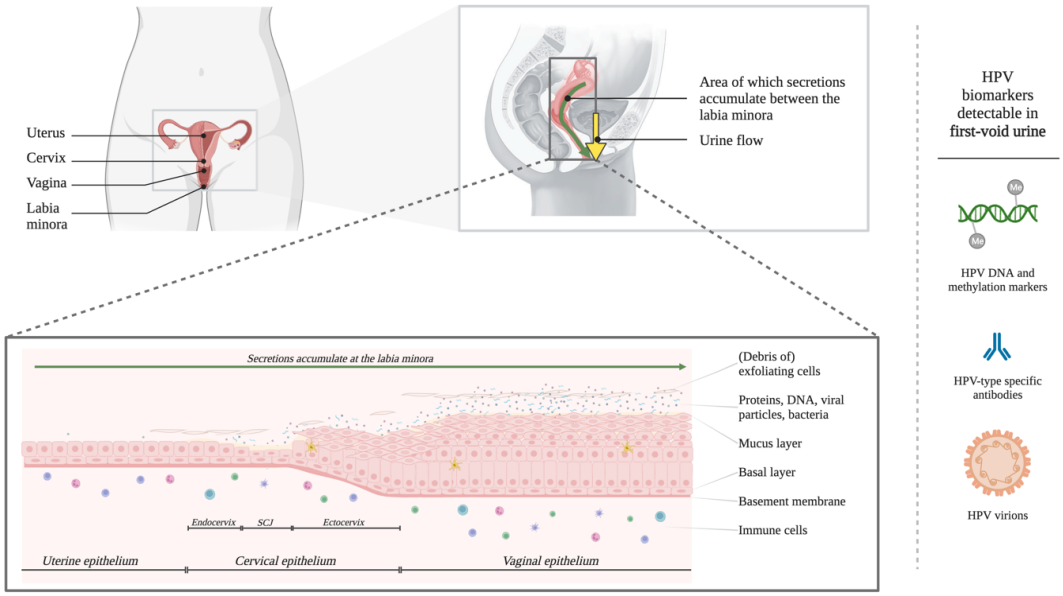


Figure 1: *Overview of female genital secretions captured in first-void urine. Secretions originating from the uterine, cervical, and vaginal epithelium migrate to the labia minora, where they accumulate. Upon urination, these accumulated secretions are collected in the initial fraction of the urine. Figure derived from Téblick et al. (2023)* [51].

1.5 First-void urine for other infections and diseases

Beyond its value for HPV-related markers, first-void urine holds significant potential as a liquid biopsy for sexually transmitted infections (STIs) and other urogenital tract diseases. Research has already demonstrated the usability of urine as a sample type for detecting infections such as *Chlamydia trachomatis* (*CT*), cytomegalovirus (CMV), human immunodeficiency virus (HIV), hepatitis A, B, and C, and gonorrhea [52–59]. Additionally, the immune responses to some of these infections could be investigated using first-void urine. For example, urogenital secretions have been assessed for antibodies and cellular immune cells in the context of *CT* [60]. As these secretions are also captured and concentrated with first-void urine, it might indeed be possible to use this sample as a source of immunological markers for *CT*. Similarly, urine samples have been explored for immunological endpoints related to HIV [61,62]. Furthermore, for diseases like kidney or bladder cancer, first-void urine may be a potential standardized biomarker sample source [18,26,28,63–66]. However, considering that cell-free tumor DNA is the primary marker for these diseases, the additional benefit of capturing the first urine void may be limited.

2 Human papillomavirus

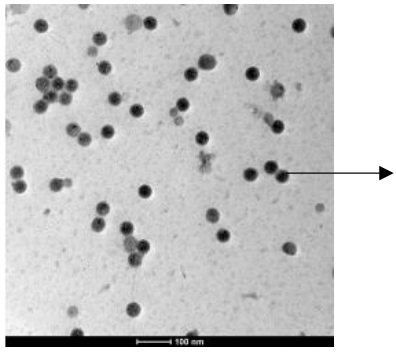
HPV is a widespread and clinically important virus with diverse implications for human health. Understanding HPV infections, the diseases associated with this virus, the modes of transmission, and prevention and control is crucial within the framework of this thesis.

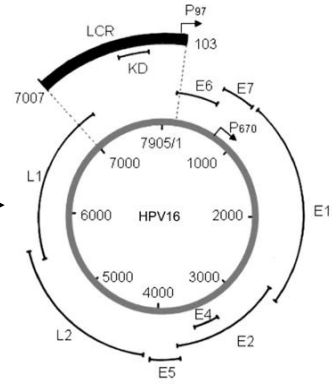
2.1 Composition and life cycle of HPV

Human papillomaviruses have a genome of approximately 8,000 bp of circular double-stranded DNA associated with histone-like proteins. The virus is part of the *Papillomaviridae* family, and the virion has a molecular weight of 5,000 kDa and a size of 50-55 nm [67–70]. The HPV genome contains three regions [69]:

I) The early (E) region containing the E genes E1, E2, E4, E5, E6, and E7
II) The late (L) region containing the late proteins L1 and L2, which are the major and minor capsid proteins, respectively
III) The non-coding region (LCR), containing the replication origin (*ori*) and other important elements for the DNA replication and transcription

An overview of the HPV16 genome organization, viral proteins, and functions is presented in Figure 2.





Viral Protein	Functions and Features
E1	It forms a heterodimer complex with E2 and controls viral replication
E2	It regulates early gene promoter and together with E1 viral DNA replication
E4	It may mediate the viral particle realease by destabilizing cytokeratin network
E5	It stimulates mitogenic signals of growth factors
E6	It inactivates many cellular proteins and is one of the major viral oncoproteins
E7	It inactivates many cellular proteins and is one of the major viral oncoproteins
L1	It is the major capsid protein and is the component of the HPV prophylactic vaccine
L2	It is the minor capsid protein

Figure 2: *Overview of the HPV16 structure and genome. Left) Electron microscopic picture of negatively stained in-house produced HPV16 pseudovirions. Particles have a size of approximately 55 nm. Right) HPV16 genome organization and overview of viral proteins and their functions. Figure derived from Tommasino et al. (2014) and reproduced with permission from Elsevier* [69].

To date, more than 200 HPV types have been categorized into five evolutionary groups: Alpha (mucosal & cutaneous), Beta (cutaneous), Gamma (cutaneous), Mu (cutaneous), and Nu (cutaneous) papillomaviruses. Among these, the mucosal Alpha types, particularly those causing cervical cancer, are extensively studied [71]. Within the mucosotropic alpha HPV viruses, 12 types are identified as high-risk (HR) HPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, with HPV16 considered to pose the highest risk of cancer progression. HPV68 is categorized as probably carcinogenic, while HPV26, 53, 66, 67, 70, 73, 82, 30, 34, 69, 85, and 97 are deemed possibly carcinogenic to humans. HPV6 and 11 are classified as low-risk (LR) HPV types and are associated with the development of genital warts [72].

Given that cervical cancer is primarily linked to mucosal HR HPV types, an overview of their life cycle is provided in Figure 3. HPV predominantly targets basal keratinocytes, initiating the infection process through the interaction of the viral capsid with heparin sulfate proteoglycans (HSPGs) and/or laminin 5 on the cell membrane. This interaction, facilitated by the major capsid protein L1, triggers structural changes in the virion capsid, including furin cleavage of L2, facilitating transfer to basal keratinocytes [73–76]. Uptake of the HPV virus in the cells requires reorganization of the actin cytoskeleton and formation of HPV-harboring small, smooth endocytic vesicles [77]. The subsequent productive phase involves the expression of the E genes (E1, E2, E5, E6, and E7) and viral DNA replication. This is followed by the expression of the L genes (L1 and L2) and E4. L1 and L2 encapsulate the viral genomes, leading to the release of viral particles [78]. Whether a productive life cycle occurs depends on factors such as the epithelial site of infection, hormones, and cytokines. Virions undergo endosomal transport, uncoating, and sorting. The L2 protein-DNA complex facilitates the nuclear entry of viral genomes, while the L1 protein is degraded. Lesion formation requires epithelial wounding and the transformation zone, characterized by increased basal cell accessibility, being particularly susceptible to cancer progression [75].

The HPV life cycle involves infection, productive replication, and potential lesion formation. The infection occurs within the epithelium, causing no cell death and lacking systemic viremia or inflammation [69,71,78]. This absence of a 'danger' signal prevents alerting the immune system. The E6 and E7 proteins actively inhibit immune signaling pathways and downregulate toll-like receptors (TLR), strategically manipulating the immune response. This ability of HPV to evade detection and clearance is crucial for its persistence

in the host, making these immune evasion strategies a pivotal consideration in the development of HPV vaccines [79,80].

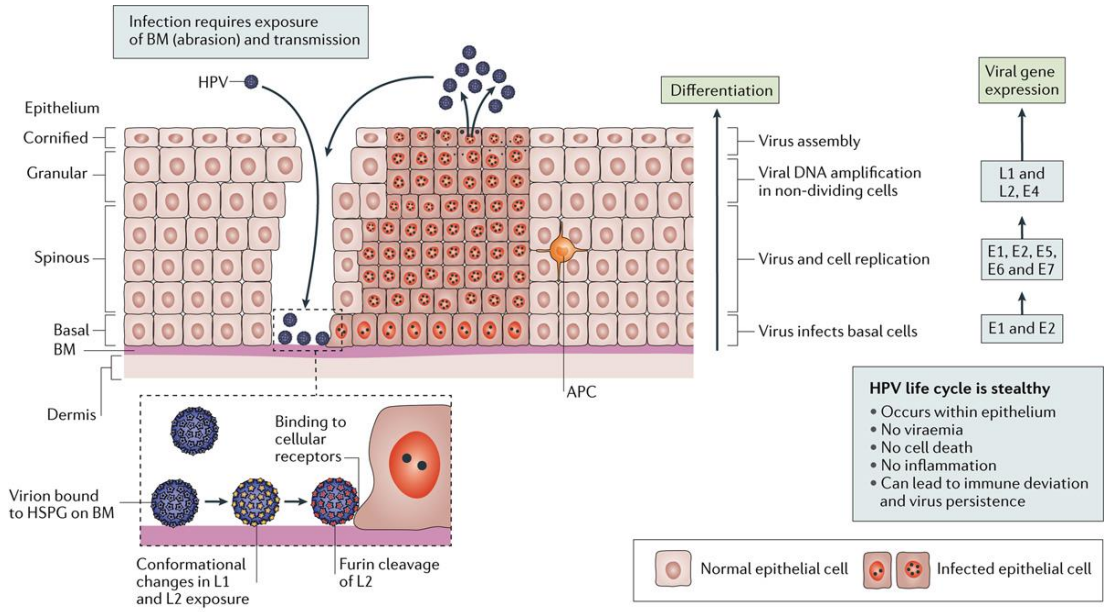


Figure 3: *The life cycle of HPV. Abrasion, causing exposure of the basement membrane (BM), allows access to basal keratinocytes by HPV. HPV binds to heparin sulfate proteoglycans (HSPGs) and/or laminin 5 on the BM through the major capsid protein L1, inducing conformational changes that expose the minor capsid protein L2. Furin cleavage of L2 leads to virus uptake, initiating viral genome replication and episome segregation. The viral life cycle, involving early proteins E6 and E7, proceeds without direct cell death, systemic viremia, or clear inflammation, and completes with the release of virions during terminal differentiation. Figure derived from Roden et al. (2018) and reproduced with permission from Springer Nature* [75].

2.2 Natural immune response to HPV infection

Upon HPV infection, both the innate and adaptive immune systems are activated (Figure 4). Although HPV cause little or no viraemia, can evade the immune system and down-regulate innate immunity, still 90% of primary infections are cleared. The immune response initiates with an innate and cell-mediated phase, followed by the activation of humoral immunity [81,82]. The innate immune system provides non-specific protection against infections by recruiting phagocytic cells, including neutrophils, natural killer (NK) cells, dendritic cells (DC), and macrophages. DCs play an important role by capturing and processing HPV particles, then migrating to regional lymph nodes, where they transform into fully functional antigen-presenting cells (APC). These APCs link the innate and

adaptive immune responses by interacting with T helper cells from the cell-mediated immune system [83].

Activated (effector) T helper cells serve various functions. Some promote the differentiation of cytotoxic T cells, capable of traveling to the infection site and eliminating HPV-infected cells where the early viral proteins E2, E6, and E7 are targeted [75]. T helper cells, together with macrophages, also induce local production of proinflammatory cytokines, chemokines, and interferons (IFNs), leading to local inflammation [81,82,84]. Another part of the T helper and cytotoxic T cells become memory T cells.

Moreover, T helper cells play a key role in activating the protective humoral immune system by generating antibodies specific to the major L1 capsid protein of HPV particles. The activation of T helper cells stimulates the differentiation of B lymphocytes into plasma cells. These plasma cells release HPV type-specific antibodies, effectively marking the pathogen for destruction by phagocytic cells. Additionally, the antibodies can neutralize the virus by binding to the L1 protein, hindering its entry into host cells.

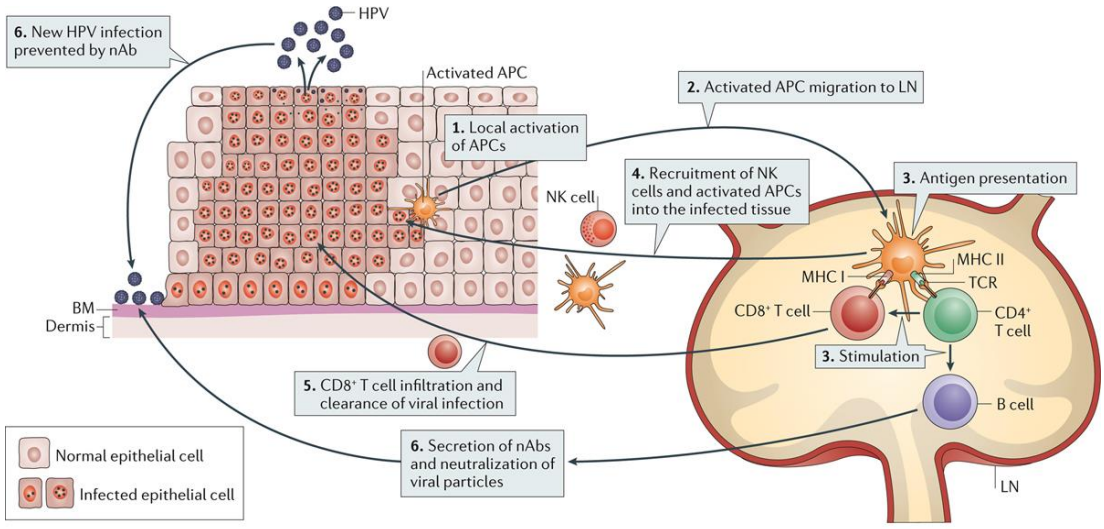


Figure 4: *Overview of the immune responses after an HPV infection. After HPV virus uptake into epithelial cells, the innate immune response is triggered, involving local APCs and pro-inflammatory cytokines. Activated APCs in the lymph nodes stimulate viral-antigen-specific helper T cells, supporting either the activation of cytotoxic T cells or the production of neutralizing antibodies (nAbs) by B cells. Figure derived from Roden et al. (2018) and reproduced with permission from Springer Nature* [75].

Some B cells specialize into long-lived plasma cells, ensuring a continuous production of antibodies for consistent protection. Another subset transforms into memory B cells, establishing humoral memory that guarantees a fast response upon re-infection by the same HPV type [83]. Although an HPV infection can lead to 50-70% seroconversion in females and 2-51% in men, and HPV type-specific IgG and secretory IgA are found locally in the female cervical mucosa, antibody levels following natural infection seem insufficient for sustained, long-term protection [82,85–87].

2.3 HPV-related diseases and transmission

HPV is the most common sexually transmitted virus globally, affecting both males and females [88]. An HPV infection is associated with cervical, penile, vulvar, vaginal, anal, and oropharyngeal cancer, representing approximately 5% of all cancers worldwide [88–90]. In this thesis, we will particularly focus on HPV-related infections and immune responses originating from the cervix, as HPV is linked to practically all cervical cancers.

Despite a significant decrease in the prevalence of cervical cancer, it remains the fourth most common cancer among females globally, with an estimated 604,000 new cases and 342,000 deaths reported in 2020 [90,91]. Cervical cancer is mainly caused by HPV16, followed by HPV18 and other HR HPV types. The oncoproteins E6 and E7 play key roles in the development of cervical cancer post-HPV infection. Both E6 and E7 are preserved after infection and are continuously transcribed to maintain malignancy and immortalize human keratinocytes [69,92]. These oncoproteins disrupt crucial cellular processes, including apoptosis, DNA repair, senescence, and differentiation, ultimately leading to the accumulation of DNA damage and the formation of cervical intraepithelial neoplasia (CIN). CIN1 represents a low-grade lesion with minimal potential for progression to cancer, while CIN2/3 are high-grade lesions with a lower likelihood of regression. If left untreated, CIN2/3 can advance to cancer [69,71]. It is essential to note that more than 90% of females experience an HPV infection in their lifetime, and only a minority (approximately 20%) fail to effectively clear the virus within 12 months [79,93–95]. In addition to its association with HPV-related cancers, HPV is linked to genital warts, with HPV6 and 11 being responsible in 96-100% of cases [88,96].

HPV is mainly transmitted through sexual contact, and significant correlations between the number of sexual partners and the presence of HPV DNA have been established decades ago [89]. HPV viruses are highly transmissible and HPV infections are generally

asymptomatic. In order for HPV to infect cells, a microlesion is required. In cases where there are multiple microlesions, the virus may infect different sites of the cervical epithelium. Furthermore, individuals can acquire multiple infections with different HPV types due to the weak and type-specific naturally induced immune response. Despite routine cleaning, HPV infections can also be transmitted through medical instruments, making it extremely important for healthcare professionals to follow a thorough decontamination protocol of their respective instruments [97].

3 HPV prevention and control

3.1 Screening for HPV infections and associated diseases

Screening for cervical cancer is required to reduce the number of cervical lesions that progress to invasive cancer. The first step within a cervical cancer screening program involves targeting specific age groups within the population [93]. This step primarily utilizes cytology, HPV testing, or a combination of both [98]. While HPV testing has higher sensitivity compared to cytology-based screening, it does come with a reduced specificity, resulting in a greater number of females diagnosed with an infection, requiring additional triage to identify clinically relevant infections. The advantage of HPV testing lies in its higher reproducibility and lower demand for operator expertise, making it more feasible for implementation in low-resource settings. This testing is typically conducted on clinician-collected cervical samples but can also be performed on self-samples [37]. A 2022 study revealed that among the 202 WHO member states and associated territories, 139 had cervical screening recommendations, with 48 recommending HPV-based screening. Out of these 48 countries, 17 (35.4%) had integrated self-sampling into their cervical screening programs. The primary motivations for this inclusion were reaching the under-screened population and establishing self-sampling as a primary screening option [5]. Self-sampling methods encompass various techniques, such as cervical swabs or brushes, lavage, tampons, labial padettes, or (first-void) urine collection [6].

Females who tested positive for HPV infection and/or have abnormal cytology are directed to undergo colposcopy and/or biopsy, aiming to identify those at an elevated risk of precancerous conditions. The primary goal in this process is to specifically identify females in need of treatment while reassuring the majority that their risk of cervical cancer is very low [93]. The identification of cervical precancer lesions or (invasive) cancer relies heavily on

various methods, including cytology (following HPV testing), HPV genotyping, cytology, histology, and host/viral methylation assays [93]. Ongoing studies are exploring the potential of self-sampling for triage purposes. Specifically, researchers are exploring methylation markers present in vaginal self-samples or first-void urine samples that are linked to the development of cervical cancer [48,99,100]. This approach holds several advantages over traditional cytology triage. It eliminates subjectivity, enables high-throughput analysis, and can be performed on the same sample used for primary screening, thereby bypassing the need for an additional cervical sample collected by a clinician, which is mandatory in reflex cytology.

Females with confirmed precancerous lesions require appropriate treatment. In cases where a CIN1 lesion is identified, it is crucial to note that the majority of females do not necessarily need treatment, as only 1% of these lesions progress to cancer [101–103]. However, despite this low progression rate, approximately 20% of CIN1 cases undergo treatment, primarily through the utilization of the loop electrosurgical excision procedure (LEEP), cryotherapy or thermal ablation. On the other hand, when a high-grade lesion such as CIN3 is detected, treatment is more frequently recommended, with over 85% of cases undergoing intervention. Treatment options for high-grade lesions include procedures like LEEP, conization, or, in more severe cases, hysterectomy [104].

3.2 HPV vaccination

The field of HPV vaccines has seen significant progress and developments over the past two decades [105]. Essential to all commercial HPV vaccines is the HPV L1 protein, playing a fundamental role in forming virus-like particles (VLPs) that trigger the production of type-specific virus-neutralizing antibodies [106]. Currently, there are six licensed prophylactic HPV vaccines, categorized into three bivalent vaccines (2vHPV targeting HPV16-18, such as; Cervarix®, GlaxoSmithKline Biologicals; Cecolin®, Innovax; and WalrinvaxV, Walvax Biotechnology), two quadrivalent vaccines (4vHPV targeting HPV6-11-16-18, such as; Gardasil®, Merck; and Cervavac®, Serum Institute of India), and one nonavalent vaccine (9vHPV targeting HPV6-11-16-18-31-33-45-52-58, namely Gardasil9®, Merck) (Figure 5) [107–109]. The most widely used vaccines are Gardasil, Cervarix, and Gardasil9, all showing excellent efficacy and safety profiles [110–112].

HPV vaccination elicits high levels of type-specific antibodies, with the effectiveness dependent on the timing and doses of the vaccine [113]. The vaccine-induced antibody levels

are at least 10-1,000 times higher than naturally-induced titers and have a higher avidity [114,115]. Although the vaccine-induced antibody levels decrease over time, they remain detectable for at least ten years after receiving one to three doses of an HPV vaccine, providing ongoing protection against infection [116–118].

Despite continuous efforts, establishing definitive correlates of protection for HPV vaccines remains challenging. To establish a defined correlate of protection, the presence of infection or disease along with the associated specific antibody response is studied. Since there is a lack of reported breakthrough precancer or cancer cases related to vaccine-targeted HPV types, it was not yet possible to define a correlate of protection for an HPV infection and associated diseases. The minimum antibody titer required for protection remains therefore an area of uncertainty.

3.3 Vaccine-induced immune response to HPV infection

The primary defense mechanism following prophylactic HPV vaccination revolves around the induction of neutralizing antibodies targeting the HPV L1 capsid proteins. All existing HPV vaccines are recombinant subunit vaccines that enable the expression of L1 from the included HPV types. These expressed L1 proteins self-assemble into type-specific HPV VLPs, which mimic the natural virus structurally but do not contain any viral DNA (Figure 5) [119].

Despite the similarity in the L1 sequence among different HPV genotypes, the elicited antibody responses are mostly type-specific but sometimes stimulate cross-protection [120,121]. This specificity originates from variances in a small number of amino acids within the exposed regions of three loops in the approximately 500-amino-acid L1 peptide chain. These loops, as part of the self-assembled conformationally intact VLP, contain the sites targeted by neutralizing antibodies [122].

The delivery of HPV VLP vaccines is intramuscular, introducing a high antigen dose in combination with a highly potent adjuvants for rapid and direct access to lymph nodes and the spleen, where adaptive immune responses initiate [123]. Similar to after natural infection, T helper cells are activated and trigger differentiation of B cells into plasma cells. These plasma cells secrete highly potent HPV type-specific antibodies, reaching titers up to four logs higher than those induced by natural infection. Some B cells transform into long-lived plasma cells, ensuring continuous protection, while others become memory B cells,

facilitating a rapid response upon re-infection. In contrast to the humoral immune response after infection, HPV vaccines lead to a seroconversion rate close to 100% [109,112,124–126].

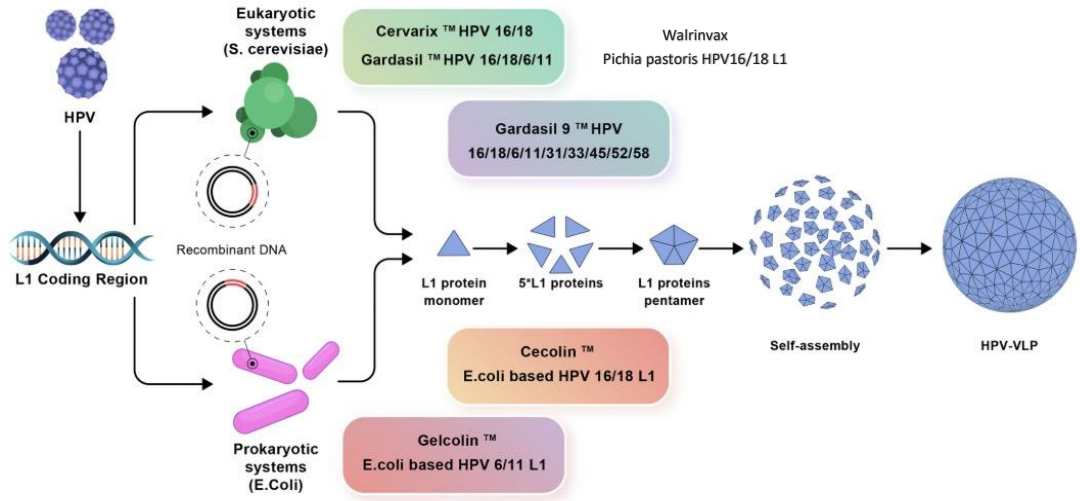


Figure 5: *Overview of the current prophylactic HPV vaccines. For all vaccine types, 72 L1 pentamers self-assemble into empty HPV-VLPs. These HPV-VLP based vaccines utilize adjuvants to enhance immune responses. Figure adapted from Yousefi et al. (2022), with permission for use by a Creative Commons Attribution License* [80].

In contrast to the L1 capsid protein, the L2 region is highly conserved between the different HPV types, making L2 an interesting target for a universal HPV vaccine [108,127]. However, the use of the L2 proteins as a vaccine antigen is challenging due to its low immunogenicity, as it lacks the ability to self-assemble into VLPs and is quickly degraded following immunization. Several approaches have been explored to enhance protective antibody responses against the L2 protein. These strategies include the conjugation of L2 peptides to a thioredoxin carrier, the fusion of L2 protein concatemers (molecules composed of repeated, identical, or similar sequences linked together) with self-adjuvanting proteins (such as flagellin), immunizations with concatemers of L2 proteins from different HPV types, and the fusion of an L2 polypeptide with a coiled-coil polypeptide to a nanoparticle derived from a thermostable thioredoxin [128–132]. The results of these studies were promising, making L2 an attractive target for next-generation HPV vaccines.

Numerous therapeutic HPV vaccines are currently in development, with encouraging results emerging. These vaccines aim to stimulate a cell-mediated immune response against persistently expressed HPV antigens in infected cells, including E1, E2, E6, and E7 [133–136]. By doing so, cytotoxic T cells are encouraged to release cytokines, marking infected and

malignant cells for elimination. Continued studies are required, but there is optimistic anticipation that therapeutic HPV vaccines will soon be introduced and provided alongside available therapies for managing HPV-associated diseases [134,137].

3.4 Impact of HPV vaccination

HPV vaccination has been introduced in over 100 countries, and while it is part of approximately 55 vaccination programs, the coverage in some of these countries is still very poor [138,139]. Most vaccination programs typically target adolescent girls and boys aged 9-13. Initially, three doses of the vaccines were recommended. However, a decade ago, studies demonstrated the non-inferiority of a two-dose schedule compared to the conventional three-dose regimen for girls under 14 years [140,141]. Consequently, the WHO adjusted their recommendation for HPV vaccination from three to two doses for both girls and boys aged 14 years and below. Recent studies have shown strong efficacy of one dose of an HPV vaccine and durable immune response ten years after receiving the vaccine [116,142]. Reducing the number of vaccine doses may further enhance vaccine uptake.

In May 2018, the WHO introduced a global cervical cancer elimination strategy, including scaling up HPV vaccination to reach 90% coverage for all adolescent girls, implementing twice-lifetime cervical screening with a target coverage of 70%, and ensuring treatment for pre-invasive lesions and invasive cancer at a rate of 90% (Figure 6) [143]. However, as of 2022, global HPV immunization coverage for at least one dose was only 21% for girls and 6% for boys [144].

Figure 6: *WHO global cervical cancer elimination strategy.*

In the past years, register-based observational studies conducted in the UK and Sweden aimed to assess the impact of HPV vaccination on cervical cancer and CIN3 [117,118,145]. The

findings revealed a significant decrease in cervical cancer and CIN3 cases among young females who received an HPV vaccine, particularly those vaccinated at the age of 12-13. While HPV vaccines specifically target two to nine HPV types, they provide sufficient cross-protection, resulting in no observed competitive advantages for other nonvaccine types and thus no type replacement [146–148]. This data suggested that the HPV immunization program holds the potential to effectively eliminate cervical cancer.

The mechanism of action for the HPV vaccine revolves around the exudation or transudation of serum antibodies to the genital tract. Local antibodies, once bound to HPV particles, play a crucial role in preventing new infections or limiting the spread of infections within the cervical mucosa (Figure 7). Additionally, this process results in a substantial reduction in viral load after shedding [83].

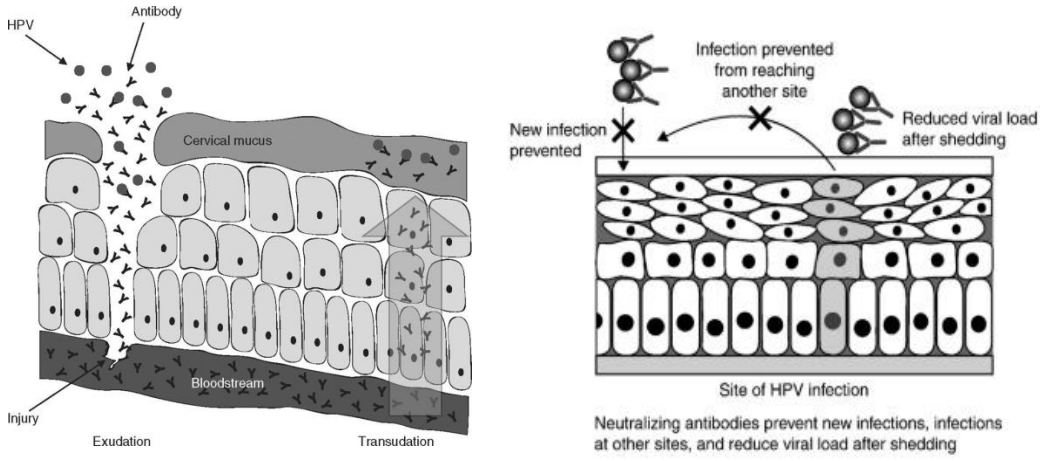


Figure 7: *Left) Transport of antibodies to the site of infection through transudation or exudation. Transudation involves the transfer of antibodies from blood vessels to the genital tract through ultrafiltration. In contrast, exudation occurs slowly from damaged blood vessels, particularly at sites of microtrauma. Figure derived and adapted from Garçon et al. 2011 and reproduced with permission from Springer Nature* [149]*. Right) Suggested mechanisms through which vaccine-induced neutralizing antibodies provide protection against HPV infection. This includes protecting against new infections, preventing infections at alternative sites, and minimizing viral load following the shedding of viral particles. Figure derived and adapted from Schwarz et al. 2008 and reproduced with permission from Elsevier* [83].

While the existing HPV vaccines are primarily preventive, there are distinct advantages to vaccination at an older age or during/after infection [150,151]. Antibody concentrations present in cervicovaginal secretions (CVS) are up to 2% of the concentrations detected in serum and good correlations have been observed between the sample types [30,31]. This suggests the presence of sufficient neutralizing antibodies at the infection site, capable of

effectively binding and neutralizing HPV particles before internalization. Vaccinating females with ongoing HPV infections has been deemed safe and has proven to generate robust immune responses [152,153]. Such vaccination induces the transudation/exudation of neutralizing antibodies to the infection site, potentially reducing autoinoculation and even transmission to sexual partners. Although these hypotheses warrant further investigation, they hold the potential to reshape how we model, evaluate, and implement HPV vaccination strategies.

3.5 (Long-term) follow-up of HPV vaccination

The follow-up of HPV vaccine trials and vaccination programs predominantly relies on serology for measuring immunological endpoints and HPV screening for virological endpoints. Assessment of vaccine effectiveness, immunogenicity, and safety is crucial [154,155]. The effectiveness of vaccines is assessed by the incidence of HPV-related diseases such as cervical cancer, genital warts, and other associated cancers. Therefore, genotyping is essential to identify prevalent HPV types and assess whether detected infections align with the HPV types included in the respective vaccine. These genotyping analyses are primarily conducted on clinician-collected cervical samples. To measure immunogenicity, serology is performed on serum samples [156]. Determining the duration of protection provided by the vaccine is also crucial for assessing the need for booster doses and maintaining sustained immunity [118]. Available serology results for HPV vaccination demonstrate enduring immunity for a minimum of ten years [116].

The current methodology requires two minimally invasive sample types (blood and cervical samples) at multiple time points, posing a labor- and time-intensive challenge for both healthcare workers and participants. Additionally, evaluating vaccine effectiveness in young adults not yet sexually active cannot be performed as collection of a cervical sample is ethically not allowed [29]. As highlighted in the initial part of this introduction, these challenges can be overcome by utilizing a non-invasive first-void urine sample that can be easily self-collected at home [157–159].

3.6 HPV-specific immunoassays

Antibody tests lack standardization, posing challenges for inter-study comparisons in HPV research. Different laboratories employ various HPV-specific immunoassays, introducing

several variables that can impact assay performance. These variables include antigen purity, integrity, conformation, sample integrity, and sample/assay stability [156].

Several HPV-specific serology tests are currently available, utilizing either Enzyme-Linked Immunosorbent Assay (ELISA) principles or pseudovirion-based neutralization assays (PBNA). PBNA, measuring neutralizing antibodies specifically, is still considered the gold standard for HPV-related serology [160]. However, PBNA protocols vary among laboratories, using different outputs such as luminescence, absorbance, or fluorescence. Additionally, setups can be singleplex or multiplex, and procedures may range from manual to high throughput [160–164]. ELISA protocols exhibit variations in both outputs and setups. These protocols moreover differ based on the antigens coated onto the plate. These antigens may include pseudovirions, VLPs, or L1 proteins fused to glutathione S-transferase (GST) [142,156,165,166].

International standards have been available to quantify HPV16 and 18 type-specific antibodies and, more recently, for other HPV types in the 4vHPV and 9vHPV vaccines (HPV6, 11, 31, 33, 45, 52, and 58) [167,168]. The use of these standards, in combination with a well-characterized control sample panel, would enhance the feasibility of accurately comparing laboratory performances.

OBJECTIVES OF THE THESIS

The aim of this thesis was to further optimize the use of first-void urine as a non-invasive sample for monitoring HPV infection and vaccine-induced HPV-specific antibodies. This includes optimizing the detection of HPV DNA (**Chapter 02-A**) and HPV type-specific antibodies (**Chapters 03-A, B, C**) in first-void urine. Additionally, we performed pilot experiments to investigate if there could be additional opportunities for HPV vaccination (**Chapters 04-A, B**).

First-void urine for HPV DNA detection

Chapter 02-A of the thesis was dedicated to the optimization of HPV DNA detection. Within this chapter, following objectives were handled:

- Evaluate the impact of different first-void urine collection volumes on the detection of biomarkers and HPV DNA.
- Investigate the influence of DNA extraction methods combined with varying collection volumes on the recovery of human DNA, HPV DNA, and other biomarkers.

Previous studies established that the initial fraction of urine contains significantly more HPV DNA than subsequent parts, however, the optimal collection volume for the highest concentration of biomarkers remained undetermined. To address this gap, we compared three collection devices, obtaining 4, 10, and 20 mL of buffered first-void urine, and investigated the influence of extraction methods combined with different sample volumes on the recovery of human DNA, HPV DNA, and other biomarkers. This chapter was the first work package within the ongoing CASUS project, which aims to maximize screening participation by identifying females with cervical cancer or precursor lesions after an HPV positive result by using a first-void urine collector and an integrated diagnostic approach for HPV detection and triage.

First-void urine for HPV-specific antibody detection

Next to the detection of HPV DNA, first-void urine can be used to detect HPV-specific antibodies. Preliminary studies have revealed promising correlations between serum and

first-void urine levels, stimulating our strong interest in exploring the potential of this sample as a source of HPV-specific immunological information. Different aspects related to HPV-specific antibody detection in first-void urine were covered in **Chapters 03-A**, **03-B** and **03-C**, including following objectives:

- Assess the stability, enrichment, and quantification of total and HPV16-specific IgG in concentrated first-void urine samples.
- Develop a time-resolved fluorescence-based assay for detecting HPV16-specific antibodies in first-void urine and compare it to results of available HPV immunoassays.
- Investigate the potential of using first-void urine as a tool for monitoring the humoral immune response after HPV vaccination.

In **Chapter 03-A**, we focused on assessing the stability, enrichment, and quantification of total and HPV16-specific IgG in concentrated first-void urine samples. The concentrated nature of a first-void urine sample enhances the levels of the biomarkers of interest. However, this concentration also introduces greater sample complexity, potentially posing challenges to the stability, extraction, and quantification of biomarkers. We aimed to evaluate the stability of IgG in first-void urine, assess its compatibility with a commonly used first-void urine DNA stabilization buffer, and compare various approaches for enriching and quantifying IgG. The findings of this chapter were crucial in establishing the suitability of this sample type for assessing HPV-type-specific immune responses.

In addition to enhancing the preprocessing of first-void urine samples, there lies potential in optimizing immunoassays to increase antibody detection. In **Chapter 03-B**, we describe the development of a time-resolved fluorescence-based assay for detecting HPV16-specific antibody concentrations in first-void urine and compare it with existing HPV immunoassays. Furthermore, recognizing the importance of normalizing inter- and intra-individual variation among first-void urine samples, we evaluated two different total human IgG assays, considering that total IgG might be a potential normalization parameter.

In **Chapter 03-C**, we set up a longitudinal cohort study, nested in a randomized controlled 9vHPV vaccine trial. This study was the first to assess if first-void urine is a suitable tool for monitoring HPV vaccination with the nonavalent HPV vaccine (9vHPV) in females, in combination with HPV DNA detection. This chapter provides essential data on the possibility of using one non-invasive, home-collected sample to monitor HPV infection and vaccination.

First-void urine to investigate the added value of HPV vaccination

While current prophylactic HPV vaccines may not have therapeutic effects, the induced neutralizing antibodies could potentially obstruct the spread of newly produced virions within the genital tract, preventing virions from spreading to other sites and transmission to the sexual partner. In the next chapters, we describe pilot experiments using first-void urine aiming to prove this hypothesis. Given that first-void urine sampling effectively captures genital secretions with intact functional antibodies, we expect the presence of intact HPV virions as well. Investigating whether vaccine-induced HPV-specific antibodies neutralize intact virions, preventing autoinoculation and transmission, could significantly influence the optimal modeling, assessment, and implementation of HPV vaccination programs. Objectives related to these chapters are:

- Investigate the neutralizing capacity of HPV-specific antibodies captured in first-void urine.
- Explore concentration strategies for diverse components in first-void urine, including HPV pseudovirions, genomic DNA (gDNA), and cell-free DNA (cfDNA).

In **Chapter 04-A**, our focus was on investigating the neutralizing capacity of HPV-specific antibodies originating from the genital tract, captured by first-void urine. With first-void urine, we collect antibodies that are present at the site of infection making it an ideal source to investigate this scientifically possible theory that vaccine-induced HPV-specific antibodies neutralize intact virions. We optimized antibody enrichment and purification from first-void urine and evaluated two different pseudovirion-based neutralization assay protocols. This chapter has important implications for understanding the mechanisms of HPV protection.

In **Chapter 04-B**, we explored concentration strategies for diverse components in first-void urine, including spiked HPV pseudovirion encapsulated DNA, spiked cfDNA, and a combination of human cfDNA and gDNA. Furthermore, the effects of naturally present and supplemented precipitating agents on the concentration protocols were evaluated. The results provide crucial insights into optimal precipitation protocols for detecting and concentrating HPV pseudovirions in first-void urine, potentially extending to naturally occurring HPV virions. Additionally, this chapter explores concentration strategies for

gDNA and cfDNA in first-void urine, broadening the sample's utility in research on STIs and as a biomarker source for cancer-related studies.

Discussion on reached goals and future perspectives

In **Chapter 05**, we present a comprehensive discussion of the research findings, offering an overview of the achieved goals and placing them within a broader context. Furthermore, this chapter provides valuable insights and suggests potential avenues for future research.

CHAPTER

02

First-void urine for HPV DNA detection

CHAPTER

02–A

Impact of collection volume and DNA extraction method on the detection of biomarkers and HPV DNA in first-void urine.

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Published in Molecules, April 2021.

Abstract

The potential of first-void (FV) urine as a non-invasive liquid biopsy for detection of human papillomavirus (HPV) DNA and other biomarkers has been increasingly recognized over the past decade. In this study, we investigated whether the volume of this initial urine stream has an impact on the analytical performance of biomarkers. In parallel, we evaluated different DNA extraction protocols and introduced an internal control in the urine preservative. Twenty-five women, diagnosed with high-risk HPV, provided three home-collected FV urine samples using three FV urine collection devices (Colli-Pee) with collector tubes that differ in volume (4, 10, 20 mL). Each collector tube was prefilled with Urine Conservation Medium spiked with phocine herpesvirus 1 (PhHV-1) DNA as internal control. Five different DNA extraction protocols were compared, followed by PCR for GAPDH and PhHV-1 (qPCR), HPV DNA, and HBB (HPV-Risk Assay), and ACTB (methylation-specific qPCR). Results showed limited effects of collection volume on human and HPV DNA endpoints. In contrast, significant variations in yield for human endpoints were observed for different DNA extraction methods ($p < 0.05$). Additionally, the potential of PhHV-1 as internal control to monitor FV urine collection, storage, and processing was demonstrated.

1 Introduction

The potential of first-void (FV) urine as a suitable, non-invasive liquid biopsy for detection of human papillomavirus (HPV) DNA and other biomarkers to detect high-grade cervical abnormality, has already been reported [169–176]. High correlates between urinary HPV DNA and cervical infections have been established and studies have confirmed the presence of HPV specific antibodies in FV urine [25,31,49,50,177–180]. Urine is considered the preferred choice of self-sampling compared to the currently available self-sampling methods used for screening; brush, lavage, spatula, swab and tampon [8,36,45,158,181–184]. Especially during the current COVID-19 (Coronavirus Disease 2019) pandemic, at-home self-sampling will provide a more accessible means for screening or follow-up than attending health care facilities. However, to use FV urine as custom liquid biopsy for cervical cancer screening, potential optimization of the sample collection and DNA extraction workflow should be considered.

The rationale behind the use of FV urine is based on the fact that the initial stream of urine – first-void – washes away secretions that accumulate between the small labia and around the urethra opening, including mucus and debris from exfoliated cells from the vagina, cervix, and uterus [19]. This explains why the first fraction of a urine void contains significantly more HPV DNA than the subsequent part [25]. However, the optimal volume of the initial urine stream to provide the highest concentration of biomarkers of interest has not been established yet. The currently used FV urine collection device (Colli-Pee®, Novosanis, Wijnegem, Belgium), prefilled with Urine Conservation Medium (UCM), collects the first fraction of urine in a total volume of 20 mL (one part UCM and two parts FV urine). The UCM buffer has already shown to positively influence detectable HPV DNA by avoiding degradation of cell-free DNA by nucleases [169]. The ideal FV urine volume would provide optimal concentration of HPV and human DNA for accurate detection of clinically relevant high risk (HR)-HPV infections. In addition, use of customized tubes, like the routinely used 10 mL tubes, could allow fully automated high-throughput testing.

An internal process control is another important factor to validate a sample for analysis. This internal control needs to be able to monitor the presence of sufficient preservative, demonstrate that the extraction was well performed, and show that no degradation of DNA/biomarker of interest took place. Earlier research in our lab showed that human DNA is not entirely sufficient as an internal process control since inappropriately stored

samples still had detectable human DNA, while the HPV DNA test became false negative [169]. In addition, the amount of human DNA is very variable between samples/individuals. As detection of cell-free viral DNA is also essential, the most suitable internal control would be DNA from a whole virus preparation.

To date, there are numerous adequate DNA extraction methods on the market, each having its own advantages and limitations [170]. However, it has not yet been investigated whether the extraction method used in combination with different FV urine volumes influences the yield of recovered human DNA, HPV DNA, and other biomarkers of interest. The evaluation of the distinct DNA extraction methods and FV urine collection volumes in combination with the introduction of an internal process control offers additional opportunities for the use of FV urine as a suitable sample for the detection of HPV.

In many European Union (EU) countries, cervical cancer screening is based on cytology. This requires a physician-taken cervical scrape and is challenged by low sensitivity (30% false-negatives) and high non-attendance (63% EU coverage) [185]. The combination of a FV urine collector and an integrated diagnostic approach for both HPV detection and triage after an HPV positive result to identify women with cervical cancer or precursor lesions could maximize screening participation.

In this study, we investigated whether the FV urine collection volume affects the detection of human and viral endpoints (i.e., glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), β-globin (HBB), HPV) by comparing three different FV urine collection volumes (4, 10, and 20 mL). In addition, we evaluated different DNA extraction protocols and introduced a universal non-human control (i.e., DNA from phocine herpesvirus 1 (PhHV-1) [186,187]) in the urine preservative to monitor sample collection, transport, storage, and DNA extraction.

2 Materials and methods

2.1 Identification of the internal control spiking concentration

To investigate the accuracy of PhHV-1 DNA as internal control, four FV urine samples were used. All four healthy female volunteers collected ± 25 mL of urine using a standard urine collection vial. These samples were divided into two falcon tubes; one tube containing 10 mL urine without UCM preservative, another one containing a total volume of 10 mL urine, and UCM in a ratio of 2:1 urine/UCM. DNA from PhHV-1 was extracted from 200 µL MEM culture medium of a PhHV-1 infected Crandell Rees Feline kidney cell line using the NucliSENS® EasyMag® (bioMérieux, off-board lysis protocol) and eluted in 100 µL. All eight samples were spiked with 25 µL of a 1:1000 dilution of this DNA eluate (stored at 4 °C). To investigate the quantity and stability of PhHV-1 DNA in the samples, 5 mL of each sample was used for DNA extractions (Figure A1). The remaining 5 mL of each sample was stored 7 days at 4 °C and an additional 5 days at room temperature after which DNA extractions using NucliSENS EasyMag on both FV urine concentrate after Amicon filtration of 4 mL (AM_EM) and 1 mL of neat FV urine (EM) were repeated for these samples. All samples were analyzed using semi-quantitative PCR (qPCR) for human DNA (GAPDH) and internal control DNA (PhHV-1) (see Section 2.5). Using this spiking volume, the initial Cq value for each UCM/FV urine combination would be 30–33, dependent on the DNA extraction method.

2.2 Study population

In total, 25 women, age 19–62 years, were included between August and November 2019 (clinicaltrials.gov ID: NCT04480866) (Figure 1). Each woman self-reported to have an HR-HPV infection, which was identified during a gynecological visit in the previous six months. Informed consent was obtained from all women before sample collection.

2.3 Sample collection and storage

Women were notified about the study by email (University of Antwerp employees) or via social media. Women interested to participate in the trial first subscribed online via the Centre for the Evaluation of Vaccination (CEV) web page. Next, the study team contacted

eligible women by phone and sent an additional e-mail to complete their registration if all inclusion criteria were met. Subsequently, a package with the information brochure, informed consent form, and collection devices with instructions for use was sent to the participants by postal mail. Each participant collected three consecutive samples at home, using prototype FV urine devices (Colli-Pee®, Novosanis) with collector tubes that differ in size, prefilled with 1/3 UCM (Novosanis) spiked with 25 µL of a 1:1000 dilution of PhHV-1 DNA extract as an internal control. This allowed us to collect an average of approximately 2.67, 6.67, and 13.33 mL urine in total volumes of 4, 10, and 20 mL respectively. Women were requested not to wash their genitals before collection, collect all samples on the same day, and not to urinate at least 2 h before each collection. All samples were collected in random order, alternating the collection volumes to eliminate potential bias associated with sampling order. Earlier research already confirmed that morning FV urine does not contain higher (HPV) DNA concentrations than FV urine collected later during the day, eliminating this potential bias [24,188]. The same day of collection (or at the latest, one day after collection), the urine samples were sent back to the University of Antwerp via postal mail where they were aliquoted and stored at – 80 °C (Biobank Antwerpen, Antwerp, Belgium; ID: BE 71030031000) prior to further analysis [189].

2.4 DNA extraction from FV urine samples

Before every extraction, buffered aliquots of urine samples were thawed (after storage for 1–2 months). For this study, five different DNA extraction methods were used (overview in Figure 1). For four out of five methods (AM_EM, EM, CF_{800}_EM, and CF_{3000}_EM), the NucliSENS® EasyMag® (bioMérieux, off-board lysis generic protocol) was used and DNA was eluted in 65 µL for further analysis. The first DNA extraction method (AM_EM) used an in-house protocol [169]. Briefly, 4 mL of the FV urine aliquot was centrifuged at 3820 *g* for 20 min at 20 °C in an Amicon Ultra-4 50 K filter device (Merck Millipore, Belgium). Next, 2 mL NucliSENS Lysis Buffer (bioMérieux Benelux, Schaarbeek, Belgium) was added to the concentrate retained on the filter. After incubation of at least 10 min at room temperature, all material was transferred to the NucliSENS Lysis buffer vial and DNA extraction was performed on the buffered concentrate. For the second method (EM), direct DNA extraction was performed on 1 mL of the FV urine sample in 2 mL lysis buffer. The third extraction method (CF_{800}_EM) started with centrifuging 4 mL of the FV urine sample at 800 *g* for 10 min at room temperature. The pellet and 1 mL of supernatant were

suspended in 2 mL lysis buffer and used for DNA extraction. The fourth extraction method (CF_{3000}_EM) used almost the same protocol as CF_{800}_EM, the only difference was centrifugation at 3000 *g* instead of 800 *g*. For the last extraction method (CF_{800}_QIA), 4 mL of the FV urine sample was centrifugated at 800 *g* for 10 min at room temperature. The pellet was resuspended in 200 µL of supernatant. DNA was extracted using the QIAamp DNA Blood Mini kit, according to the manufacturer's protocol (Qiagen GmbH, Germany) and eluted in 65 µL H_2O [169]. Different DNA extraction methods were compared for collection volumes; for 4 mL samples, extraction method EM was used; for 10 mL samples, extraction methods EM, CF_{800}_EM, and CF_{3000}_EM were used; for 20 mL samples, extraction methods AM_EM, EM, CF_{800}_EM, and CF_{800}_QIA were used (Figure 1).

2.5 Semi-quantitative PCR for GAPDH and PhHV-1

All DNA extracts obtained from FV urine samples were analyzed using semi-quantitative PCR (qPCR) for human DNA (GAPDH) and internal control DNA (PhHV-1). This qPCR technique is based on TaqMan technology and performed with the LightCycler 480 Real-Time PCR instrument (Roche Diagnostics, Machelen, Belgium) as described by Vorsters et al. (2014) [169]. Briefly, a 20-µL portion of the GAPDH PCR mixture containing 1× LightCycler® 480 Probes Master (Roche Diagnostics, Machelen, Belgium), 0.5 µM concentrations of each primer, 0.1 µM concentrations of probe, and 5 µL of DNA solution was loaded into the LightCycler. Primers and probes for GAPDH are defined by Payan et al. (2007) [190]. For PhHV-1 qPCR was performed on 20 µL of the following mixture; 1 × LightCycler® 480 Probes Master (Roche Applied Science, Belgium), 0.05 µM forward primer, 0.2 µM reverse primer, 0.1 µM probe, and 5 µL of DNA solution. PhHV-1 primers and probes are described by Van Doornum et al. (2002) [186]. The thermal cycles were as follows: an initial 10 min at 95 °C for FastStart Taq DNA Polymerase activation, followed by 45 cycles of 10 s of denaturing at 95 °C, 15 s of annealing at 60 °C. Both positive and negative controls were used on each run to confirm reproducibility.

2.6 HPV-risk assay

High-risk HPV testing was done using the HPV-Risk assay (Self-screen B.V., Amsterdam), which is a multiplex, real-time PCR assay targeting the E7 region of 15 (probably) HR-HPV types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -67, and -

68), and providing additional genotype information for HPV16 and HPV18 [191]. The assay includes amplification of the reference gene HBB to assess DNA quality and quantity. The assay was performed using 5 µL of the DNA extract according to the manufacturer's instructions, on a Rotor-Gene Q MDx 5plex HRM instrument (Qiagen GMBH, Hilden, Germany). The Cq value cut-offs of the assay for calling a sample HPV-positive were not used. Samples were scored HPV positive when there was a Cq value for any of the HPV targets.

2.7 Semi-quantitative PCR on bisulfite converted DNA

For testing the suitability of the DNA for quantitative Methylation-Specific PCR (qMSP), DNA extracted from FV urine was treated with bisulfite and amplified using primers and probes in the ACTB gene area containing at least three cytidines, as described previously [99,192–194]. Bisulfite-conversion was performed with the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's specifications. Standard DNA input for bisulfite-conversion was 250 ng. Elution was done with 12.5 µL M-elution buffer yielding 20 ng/µL bisulfite-converted DNA. For samples with insufficient DNA yield to accomplish an input of 250 ng (around 50% of the samples), a standard amount of 45 µL was used.

2.8 Statistical analysis

For the statistical analysis, we used R statistical software version 3.6.0. The data were first checked for their normality using Shapiro–Wilk test. If the data were normally distributed, significant differences between different collection volumes and DNA extraction methods were examined using paired *t*-test, otherwise non-parametric Wilcoxon signed-rank testing was used. Statistical significance was defined as p-adjusted < 0.05 (using Holm–Bonferroni method for p-value adjustment). To analyze possible correlations between housekeeping genes (GAPDH, ACTB, HBB), Pearson correlation coefficients were calculated. To investigate HPV positivity agreement between different FV urine collection volumes and DNA extraction methods, Cohen's Kappa (K) when maximum three conditions were compared and Fleiss Kappa values when more than three conditions were compared, were calculated. For the calculation of cut-off within the internal control data, the mean Cq plus two standard deviations were used [186]. To check whether there was an

effect of time between collection and freezing on DNA yield for all endpoints, including internal control, we used a linear regression model on all EM data.

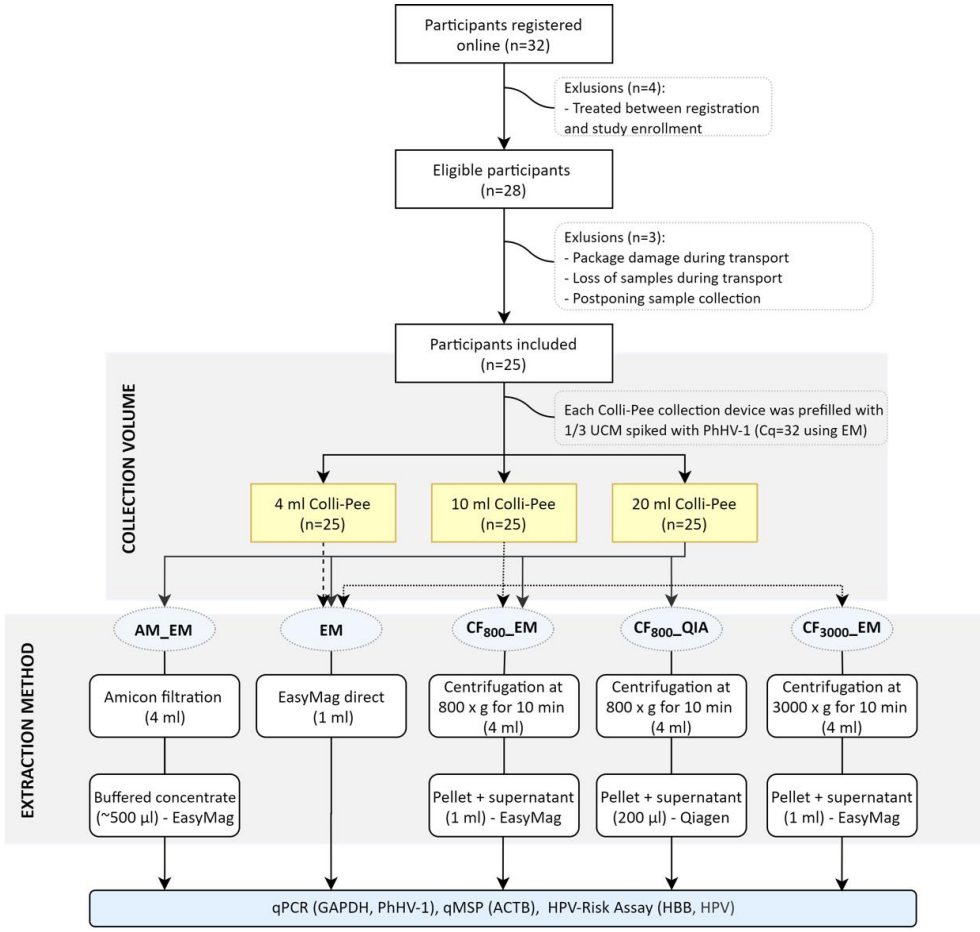

Figure 1: Flow diagram of the study*. 32 women subscribed online, whereof 28 eligible women were included. Each woman received a study package at home as described. From those 28 women, three women were excluded (reasons for exclusion listed in the figure). All 25 included women collected three consecutive samples using three different Colli-Pee® urine devices with collector tubes that differ in size. On the same day of collection (or at the latest, one day after collection), the urine samples were sent back to the lab via postal mail where they were aliquoted and stored at – 80 °C prior to further analysis. On each sample, DNA was extracted using the EM. On the samples collected with Colli-Pee® 20 mL and 10 mL, three and two other DNA extraction methods were performed, respectively. All DNA extracts were analyzed for GAPDH and phocine herpesvirus 1 (PhHV-1) using an in-house qPCR. In addition, samples were analyzed using the human papillomavirus (HPV)-Risk Assay and methylation-specific qPCR at Self-Screen B.V. n; number, EM; extraction method, SN; supernatant.*

3 Results

3.1 Sample collection

Three samples, all different volumes at random order, were received from 25 women aged between 19 and 62 years old (overview in Figure 1). The median age in the cohort was 31 (interquartile range (IQR): 27–35). Median (IQR) estimated collection volumes were 4.1 (3.8–4.4) mL, 9.8 (9.4–10.3) mL, and 20.3 (19.8–20.8) mL with collector tubes estimated to collect 4 mL, 10 mL, and 20 mL, respectively. The three samples were collected within a median (IQR) time interval of 3h 2min (2:30–4:26). Two women collected one sample on another day than the other two samples. Data for GAPDH, PhHV-1, ACTB, HBB, and HPV DNA was generated from 3 × 25 FV urine samples.

3.2 Evaluation of PhHV-1 as internal process control

Internal control quantification cycle (Cq) value results of the 25 women included in the study were generated by qPCR (Figure 2). Since spiked PhHV-1 concentrations in the different FV urine collection volumes were identical and no significant differences in Cq values were observed between the different collection volumes when using the same extraction method ($p \geq 0.36$), cut-offs were calculated for each DNA extraction method separately, but on all volumes combined (cut-offs; AM_EM: 36.26, EM: 35.83, CF_{800}_EM: 36.40, CF_{800}_QIA: 40.00, CF_{3000}_EM: 35.81). Comparing three different collection volumes using EM and four extraction methods on 20 mL, three IDs (1, 8, and 19) exceeded the calculated cut-offs. Comparing two collection volumes using CF_{800}_EM and three extraction methods on 10 mL, also three samples (8, 15, and 19) exceeded the calculated cut-offs. Since the sample size is too low to validate the exploratory defined cut-off, all samples were included in further analyses. The distribution of Cq values varied between different extraction methods. A linear model was used to investigate the effect of time (days) between sample collection and freezing of aliquoted samples on the DNA yield of PhHV-1, GAPDH, HBB, and ACTB using extraction method EM (Figure A1). For none of the human endpoints, a significant effect was found (all $p \geq 0.05$). However, there was a significant effect of time between collection and freezing on the PhHV-1 Cq values ($p = 0.017$). For each extra day between FV urine collection and storage at – 80 °C for samples extracted with EM, the median Cq value increased by 0.19 (Figure A2).

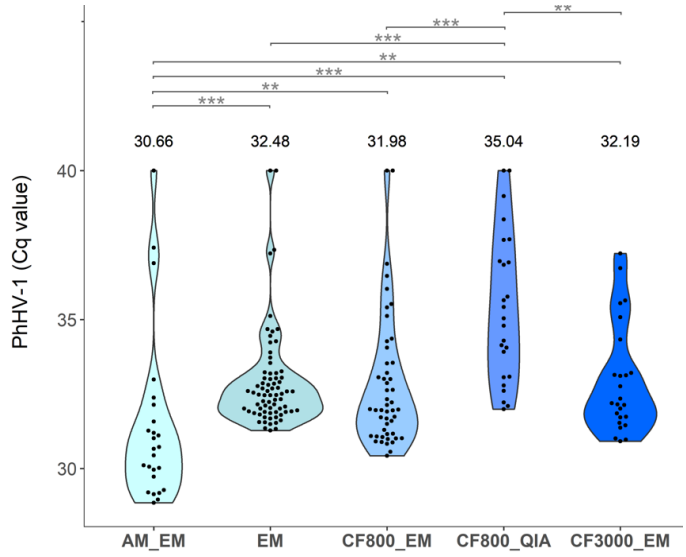

Figure 2: *Violin plot of qPCR Cq values for PhHV-1 for all 25 included samples. EM was used for all extraction volumes, $CF_{80}0$_EM for both 10 mL and 20 mL, AM_EM, CF_{800}_QIA only for 20 mL, and $CF_{300}0$_EM only for 10 mL. Calculated cut-offs for each extraction method are; AM_EM: 36.26, EM: 35.83, CF_{800}_EM: 36.40, CF_{800}_QIA: 40.00, CF_{3000}_EM: 3 5.81. Significance levels are represented in the figure by an asterisk (** $p < 0.01$; *** $p < 0.001$).*

3.3 Comparison of DNA and biomarker yields between FV urine collection volumes

3.3.1 Human endpoints

PCR Cq value results, inversely proportional to concentrations, for GAPDH, HBB, and ACTB were generated for all three FV urine collection volumes using extraction method EM and on 10 mL, and 20 mL samples using CF_{800}_EM. Results are presented as boxplots (Figure 3). Using EM, we observed no significant differences between the three FV urine collection volumes for all human endpoints (GAPDH: $p \geq 0.07$, ACTB: $p \geq 0.40$, HBB: $p \geq 0.28$) (Figure 3). In addition, similar and all highly significant ($p \leq 3.9 \times 10^{-4}$) Pearson correlations were found between all three housekeeping genes; GAPDH, HBB, and ACTB, for all collection volumes (Figure A3). The timing of the three FV urine collections did also not have a significant effect on the DNA Cq value for all three human endpoints (data not shown). For CF_{800}_EM, there was no significant Cq difference for HBB ($p = 0.85$) and ACTB ($p = 0.083$) between 10 and 20 mL collections. We did observe significant differences in Cq for GAPDH ($p = 0.033$). Here median Cq value for 20 mL (28.3) was higher than for 10 mL (27.6). However, the same Pearson correlation trend

between all housekeeping genes was seen for CF_{800}_EM ($p \leq 1.36 \times 10^{-12}$) compared to EM (Figure A4).

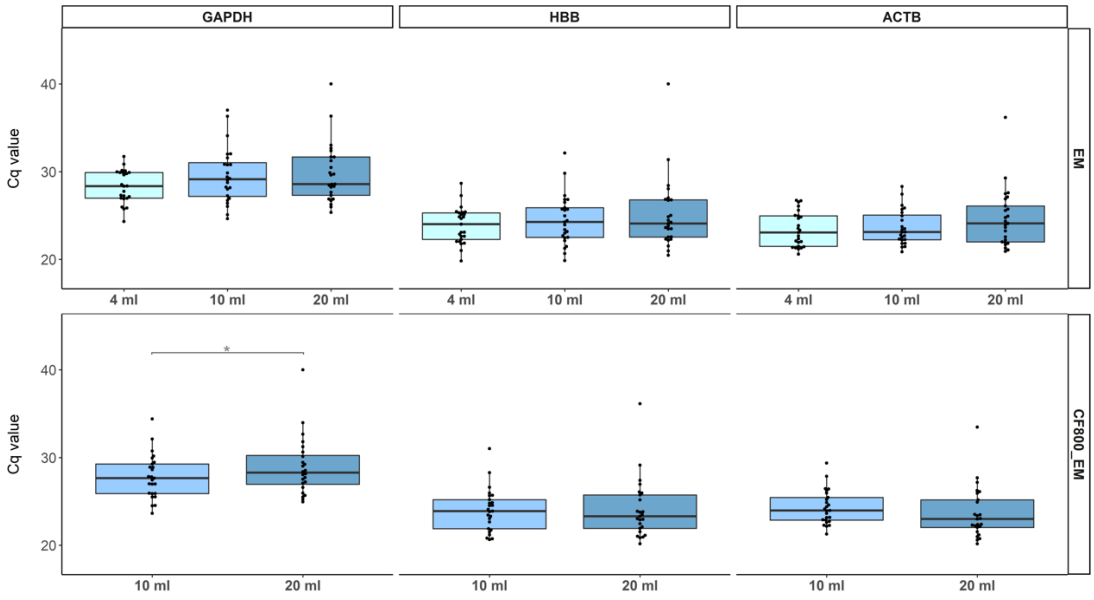

Figure 3: *Boxplots representing the Cq values of GAPDH, HBB, and ACTB for each evaluated collection volume using EM (upper figures) and CF_{800}_EM (lower figures). Individual Cq values are represented as dots. All data were analyzed using the non-parametric Wilcoxon signed-rank testing. Significant differences are represented in the figure by an asterisk ($p < 0.05$).*

3.3.2 Viral endpoints

HPV DNA results for different volumes were compared using the DNA extraction method EM (Table 1). Samples were classified as HR-HPV positive when HR-HPV DNA was present. Here, no samples showed presence of HPV-16 or -18 DNA. However, in total 37% (28/75) of the DNA samples extracted with EM and 44% (23/50) with CF_{800}_EM contained HPV DNA of an HR-HPV type included in the HPV-Risk assay. Using EM, FV urine samples collected with the 4 mL device showed 32% (8/25) HR-HPV positivity, the 10 mL device 44% (11/25), and the 20 mL 36% (9/25). No significant differences were found ($p \geq 0.14$). Concordance of the HR-HPV positivity results over the different FV urine collection volumes are $K_{4/10} = 0.58$ and $K_{4/20} = 0.91$, $K_{10/20} = 0.67$. For CF_{800}_EM, FV urine samples collected with the 10 mL device showed 52% (13/25) HR-HPV positivity and the 20 mL 40% (10/25). Here, Cohen's Kappa value is 0.60. While not all volumes gave HR-HPV positive results, the Cq values were high (34.71; 28.89–38.78) in the sample where HR-HPV DNA was detected.

3.4 Comparison of DNA and biomarker yields between DNA extraction methods

3.4.1 Human endpoints

DNA yield results for GAPDH, HBB, and ACTB on the different DNA extraction methods were generated and presented as boxplots (Figure 4). Comparing four DNA extraction methods (AM_EM, EM, CF_{800}_EM, and CF_{800}_QIA) used on 20 mL FV urine samples, GAPDH Cq values were significantly higher for EM in comparison to all other methods ($p \leq 0.12 \times 10^{-3}$). In addition, AM_EM had a lower Cq value for GAPDH than CF_{800}_EM ($p = 0.04$). The HBB Cq values for EM were significantly higher than for AM_EM ($p = 0.21 \times 10^{-2}$), CF_{800}_EM ($p = 0.26 \times 10^{-3}$), and CF_{800}_QIA ($p = 0.79 \times 10^{-2}$)). The quantitative Methylation-Specific PCR (qMSP) results for ACTB showed significantly lower yield for EM ($p = 0.16 \times 10^{-3}$) and CF_{800}_QIA ($p = 0.52 \times 10^{-2}$) compared to results for AM_EM. Moreover, CF_{800}_EM had significantly lower Cq-values than EM ($p = 7.40 \times 10^{-5}$) and CF_{800}_QIA ($p = 0.01$), but no significant difference was observed between CF_{800}_EM and AM_EM ($p = 1.00$). There was a significant Pearson correlation between all human endpoint combinations ($p \leq 1.80 \times 10^{-10}$) for all extraction methods (Figure A5). Since extraction method CF_{800}_EM showed promising results and a smaller collection volume has transport and storage advantages, the remaining volume of the 10 mL FV urine sample was used to compare two centrifugation speeds (CF_{800}_EM and CF_{3000}_EM). In total, three different DNA extraction methods (EM, CF_{800}_EM, and CF_{3000}_EM) were compared using 10 mL FV urine samples. Here, GAPDH Cq values were significantly lower, using CF_{800}_EM or CF_{3000}_EM compared to EM ($p \leq 0.35 \times 10^{-2}$). The HBB Cq values showed no significant differences for all methods ($p \leq 0.08$). Results for ACTB showed significantly higher yield for EM compared to CF_{3000}_EM ($p = 0.016$). Other observed variations were not significant (Figure 4). For all three extraction methods, there was a significant Pearson correlation between all human endpoint combinations ($p \leq 3.91 \times 10^{-4}$) (Figure A6). For the 4 mL FV urine samples, only extraction method EM was used since there was not enough volume left to perform additional extractions.

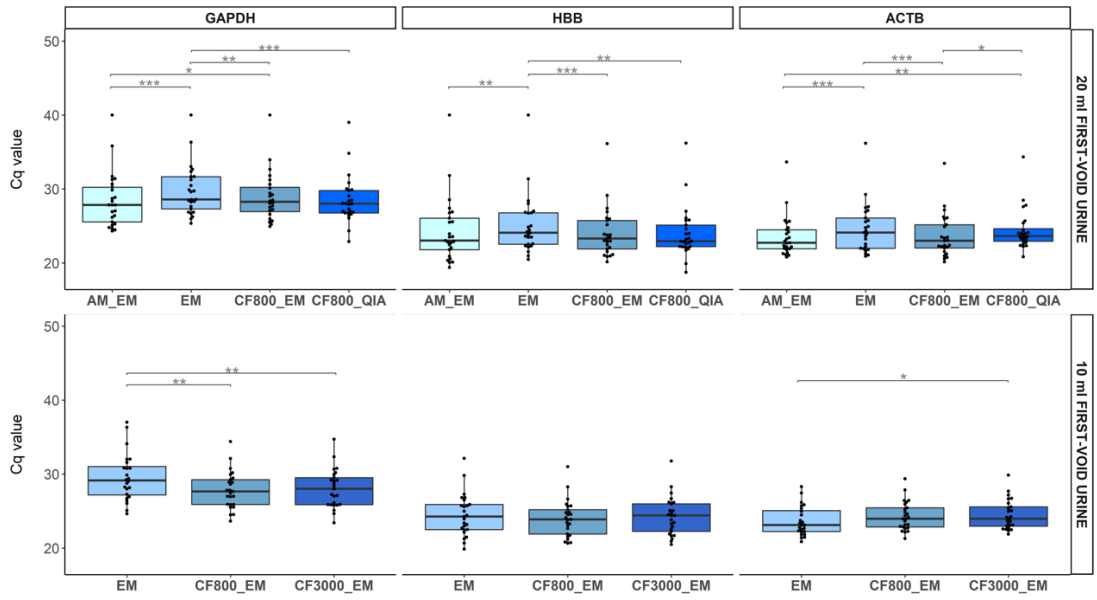

Figure 4: *Boxplots representing the Cq value of GAPDH, HBB, and ACTB for each DNA extraction method on both 20 mL samples (upper figures) and 10 mL sample volume (lower figures). Individual Cq values are represented as dots. All data were analyzed using the non-parametric Wilcoxon signed-rank testing. Significance levels are represented in the figure by an asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

3.4.2 Viral endpoints

Results for different extraction methods were evaluated on 20 mL and 10 mL samples (Table 1). No samples showed the presence of HPV16 or -18 DNA. HR-HPV infections were found in 39% (39/100) of the 20 mL FV urine DNA extracts. DNA extracted with AM_EM showed 44% (11/25) HR-HPV positivity, with EM 36% (9/25), with CF_{800}_EM 40% (10/25), and with CF_{800}_QIA 36% (9/25). No significant differences were found ($p \geq 0.09$). Here, the Fleiss Kappa value is 0.87. In total 48% (36/75) of the 10 mL FV urine DNA extracts contained HPV DNA of an HR-HPV type included in the HPV-Risk assay. DNA extracted with EM showed 44% (11/25) HR-HPV positivity, CF_{800}_EM 52% (13/25), and CF_{3000}_EM 48% (12/25). Concordance of the HR-HPV positivity results over the different DNA extraction methods on 10 mL samples was 0.78. Whenever not all methods gave HR-HPV positive results, the Cq values were high (36.39; 30.83–39.24) in the extracts where HR-HPV DNA was detected.

Table 1: *HPV DNA Cq values (either HPV-31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -67, or -68) for all different first-void (FV) urine collection volumes and DNA extraction method combinations. Percentage of positive samples/extract is noted underneath. There were no significant differences between groups ($p > 0.05$). EM; EasyMag, CF; Centrifugation.*

	HPV Cq values							
	AM_EM	*EM*			*CF_{800}_EM*		*CF_{800}_QIA*	*CF_{3000}_EM*
	20 mL	4 mL	10 mL	20 mL	10 mL	20 mL	20 mL	10 mL
ID1	-	-	-	-	38.78	-	-	-
ID2	22.08	17.90	19.17	23.17	17.28	22.75	24.75	21.65
ID3	-	-	-	-	-	-	-	-
ID4	-	-	35.46	-	32.26	-	-	32.57
ID5	-	-	-	-	-	-	-	-
ID6	36.71	34.32	-	29.11	-	28.89	25.97	33.53
ID7	29.64	23.58	24.65	26.25	23.62	22.81	24.89	25.70
ID8	-	-	-	-	-	-	-	-
ID9	-	-	-	-	30.83	-	-	37.46
ID10	-	-	-	-	-	-	-	-
ID12	22.16	25.09	24.04	23.73	20.94	21.66	22.80	21.12
ID13	19.82	21.97	25.61	20.89	24.15	19.40	18.99	24.57
ID14	-	-	-	-	-	-	-	-
ID15	39.24	-	37.87	-	-	-	-	-
ID16	-	-	-	-	-	-	-	-
ID17	34.92	30.34	33.52	34.55	35.94	38.59	33.96	35.50
ID18	36.19	-	37.00	-	31.25	36.39	38.16	31.07
ID19	24.63	22.92	24.57	26.17	22.58	24.43	25.26	23.38
ID21	-	-	-	-	-	-	-	-
ID22	-	-	-	-	-	-	-	-
ID23	31.59	-	35.11	35.73	32.98	38.98	-	36.29
ID24	-	-	-	-	-	-	-	-
ID25	29.90	38.90	27.93	30.65	30.12	30.67	33.10	28.37
ID27	-	-	-	-	-	-	-	-
ID28	-	-	-	-	-	-	-	-
% positive	44.00	32.00	44.00	36.00	52.00	40.00	36.00	48.00

4 Discussion

The use of FV urine as a self-sample for HPV DNA testing has already proven promising and high correlates between urinary HPV DNA and cervical infections have been established [171]. Combining this non-invasive and user-friendly sample with a tailored molecular assay for the detection of pre-cancer biomarkers in urine [176,195–197] could provide an alternative for the current non-attendees of screening programs and ultimately the entire screening population. We studied several sample collection and extraction workflow (i.e., internal control, collection volume, DNA extraction method) to investigate if we could improve the performance of FV urine as a liquid biopsy.

Internal process control

As part of the optimization process, we introduced phocine herpesvirus type 1 (PhHV-1) as internal process control in the UCM and observed degradation in PhHV-1 DNA when samples were stored for 12 days at ambient temperature (Figure A1) whereas this was not seen for GAPDH when samples were stored with UCM. This difference can be due to the fact that GAPDH concentrations are subsidiary to interpersonal variation and PhHV-1 DNA is spiked in a constant concentration. PhHV-1 Cq values furthermore linearly increased with an average of 0.19 Cq per additional day passed between sample collection and storage at – 80 °C in this specific sample set of 75 FV urine extracts (extraction method EM on all three collection volumes), confirming its potential as an internal control for sample storage. The selection of a cell-free internal control is based upon previous studies where it was shown that non-cell associated DNA in FV urine samples is most vulnerable to degradation [169,170]. Since the buffer is spiked with PhHV-1, not detecting this control will indicate inappropriate sample collection. Due to PhHV-1 DNA being cell-free, and thus not able to pellet, it is impossible to combine this control with an extraction method that only uses the pellet for qPCR (CF_{800}_QIA). Small fluctuations between PhHV-1 Cq values within the same DNA extraction method can be due to different sample composition and storage conditions [24,170]. The internal control cut-off calculated in this pilot sample set will be evaluated in a larger external validation set (CASUS, clinicaltrials.gov: NCT04530201). Combining the UCM [169] with an internal process control will allow laboratories to set up a strict sample control quality system, confirming the presence of the preservative and absence of substantial nucleic acid degradation.

FV urine collection volume

The most widely used method for urine collection to screen for infectious agents or other biomarkers is the use of a urine collection cup. However, these cups are not designed to collect the first urine fraction, making it more difficult to confidently collect the initial urine stream with high concentrations of certain biomarkers [24,25]. Colli-Pee® is a commercially available device that collects a standardized, pre-defined volume of FV urine without the need to interrupt the urine current. With a view toward volume optimization, different FV urine collection devices collecting a fixed volume of the initial urine stream were evaluated. Results of the experimental arms compared in this study show limited differences between the FV urine collection volumes for all human endpoints using EM.

The human endpoints evaluated within this study represent the sample quality for both HPV DNA testing and biomarker testing and are therefore important factors to evaluate. Since HPV DNA detection using the 20 mL collection device already provided promising analytical and clinical accuracy results [24,42,158,169,198]. These additional results also support the potential of the 4- and 10- mL collection volume. For CF_{800}_EM, there was a significant difference between 10- and 20-mL samples for GAPDH. When comparing HPV DNA Cq values, more HR-HPV infections were detected using Colli-Pee® for the collection of 10 mL compared to 4- and 20-mL. Based on these results, we can conclude that collecting only 10 mL could provide an increased sample quality for the detection of HPV DNA. In addition, we hypothesize that 4 mL of FV urine (approximately 2.33 mL when taking the buffer into account), is not sufficient to wash away all secretions that have accumulated between the urethra and small labia. Since there is a clear biological link between HPV infection and cervical pre-cancer and cancer, the highest HPV DNA concentration will also be a good representation for the highest amount of biomarkers, including methylation markers [93,199–201]. Besides, the smaller collector tube of the 10 mL in contrast to the 20 mL device has advantages in transport (by, e.g., postal mail), storage, and high throughput testing if a fully integrated nucleic acid extraction and testing platform is used.

DNA extraction method

To date, there has not been a comparison between DNA extraction methods in combination with different FV urine collection volumes. Since the DNA extraction can most likely influence the concentration of a certain biomarker, we compared five different extraction methods overall, including a direct comparison of four methods on 20 mL FV urine samples. Almost all differences in Cq value can directly be linked to the difference in implemented volumes. Variation in centrifugal forces did not cause different DNA yields (CF_{800}_EM vs. CF_{3000}_EM). For both human and viral endpoints using only the pellet for extraction (CF_{800}_QIA) gave similar DNA yields in comparison to other methods. However, this extraction method is not compatible with the selected internal control given that cell-free viral DNA is not concentrated by the pellet. Considering all factors, including costs and amount of laboratory work, which are higher for AM_EM and CF_{800}_QIA and lower yields of human and viral endpoints for EM; preference is given to CF_{800}_EM.

Limitations of the study

A number of limitations regarding this study need to be acknowledged. First, the relatively small sample size making it necessary to be cautious while interpreting the results. Second, samples exceeding the internal control cut-offs were not excluded from the data since calculations were only performed on this training set and need further evaluation in an independent validation set. Third, since all women self-reported to have an HR-HPV infection in the previous six months, no documentation concerning their infection was provided to the study team and could therefore not be verified. We also did not receive information on whether the HPV infection was associated with a cervical intraepithelial lesion (CIN). Only 13 out of 25 women still had a detectable HR-HPV infection in at least one of the three collected samples. However, the objective of this study was not to compare urinary and cervical HR-HPV infections but to develop a reproducible optimal urine sampling and DNA extraction protocol. As this study is the first study to optimize the combination of FV urine collection volume, DNA extraction methods, and introduction of an internal control we are confident that these limitations will not have a major effect on the study outcomes.

Conclusions

In conclusion, we have evaluated a potential internal control of which the exact cut-off concentrations for sample validity will need to be determined in a validation study (NCT04530201). Good agreement between the three different FV urine collection volumes was found for all human endpoints. Because of the advantages of a lower collection volume in high-throughput testing and the highest percentage of HPV DNA-positive DNA extracts, the 10 mL collection volume is preferred. Overall, the preferred collection and processing combination within this study would be a 10 mL collection device, prefilled with UCM containing PhHV-1 as internal control, together with DNA extraction using 4 mL of the collected FV urine for centrifugation at 800 *g* whereof the pellet and 1 mL of supernatant will be used for NucliSENS EasyMag extraction (CF_{800}_EM).

5 Supplementary material

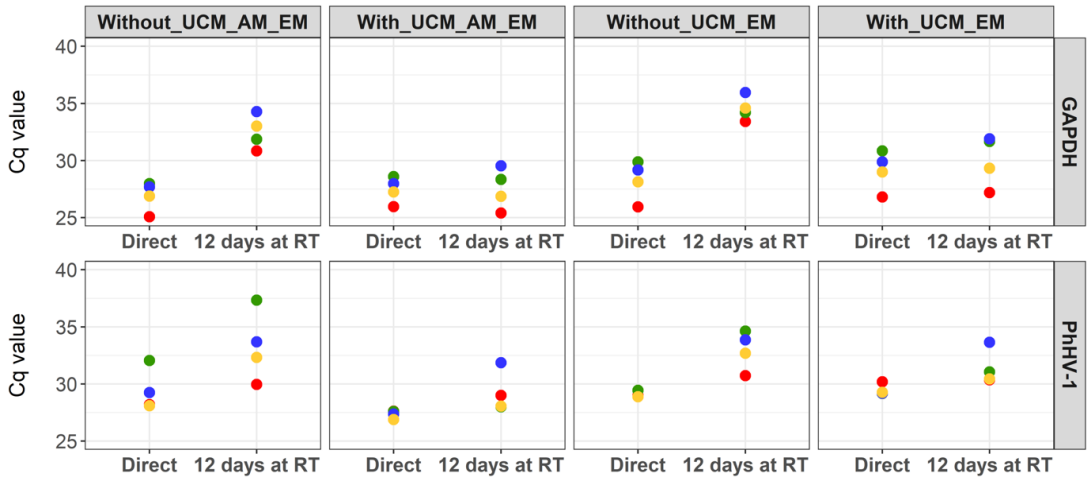

Figure A1: *Results for GAPDH (upper figures) and PhHV-1 (lower figures) on 4 FV urine samples. Each ID has a unique color mating its Cq values. Samples were processed directly (Direct) and after 12 days of storage at room temperature. Presence of UCM in the samples is referred to as either "With_UCM" or "Without_UCM". The use DNA Extraction method is referred to as well; AM_EM or EM. No PhHV-1 result was obtained for ID3 at time point 0 for category With_UCM_AM_EM due to pipetting error. UCM; Urine Conservation Medium, EM; EasyMag, AM; Amicon filtration.*

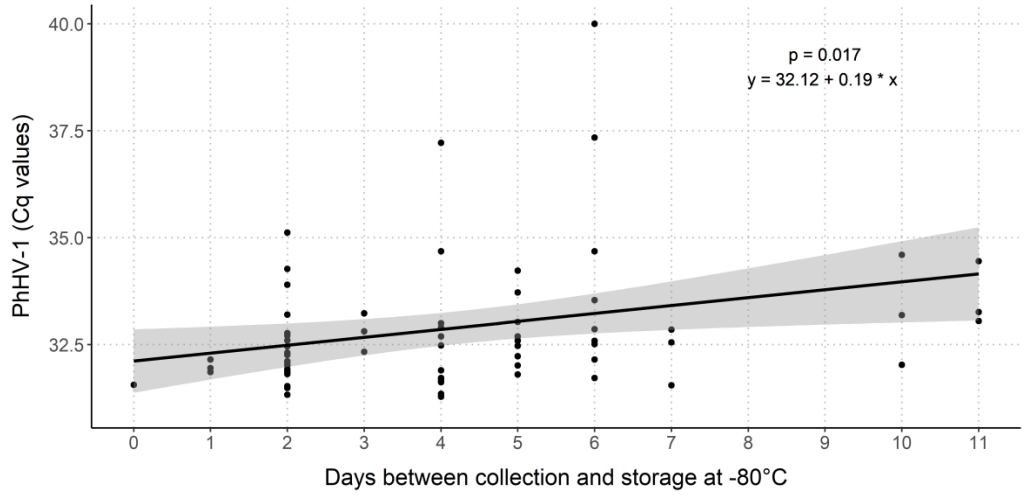

Figure A2: *Spreading and correlation curve of PhHV-1 Cq values of DNA extracted with EM over timespan between collection of the urine sample and storage at −80 °C. A linear model was used to evaluate this effect. For each extra day between FV urine collection and storage at −80 °C, the Cq value increased with 0.19. This effect was significant. No such significant effect was seen for all other human endpoints.*

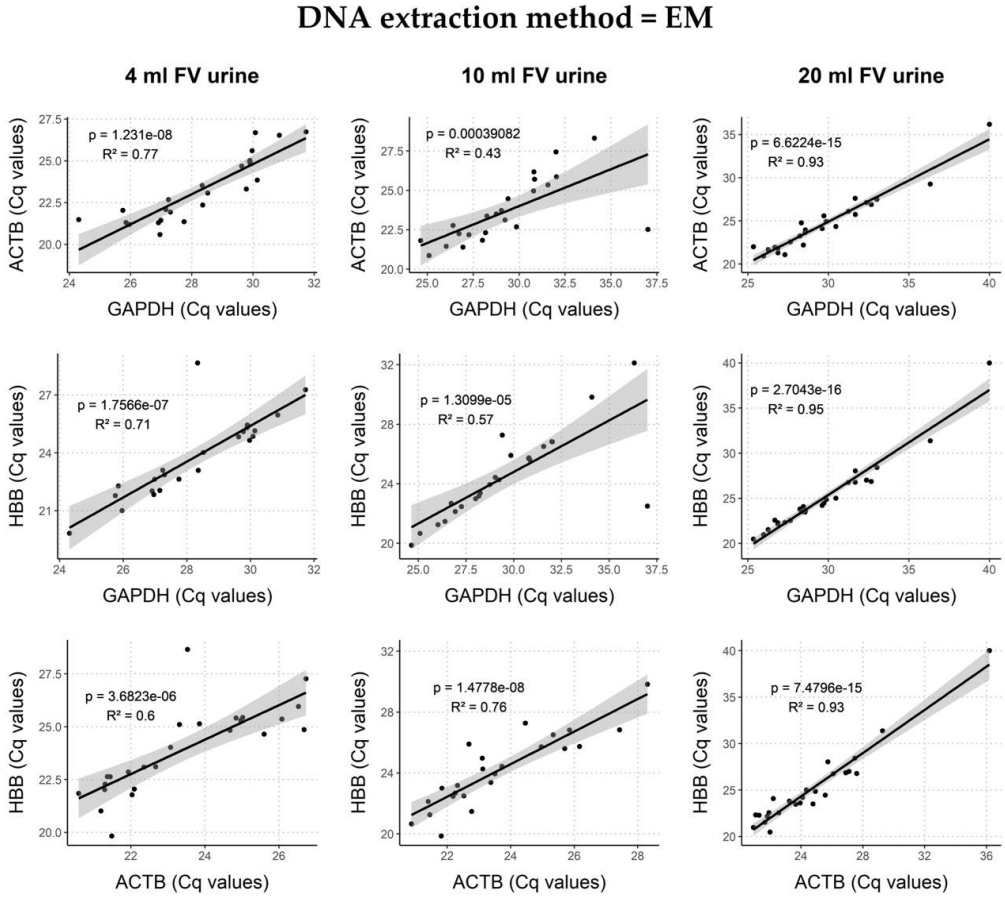

Figure A3: *Correlation curves for all human endpoint combinations on DNA extracted using EM for 4, 10, and 20 mL FV urine collection volumes. High risk (HR)-HPV positive samples are marked grey. All correlations are highly significant. R2 values are highest for 20 mL and lowest for 10 mL samples.*

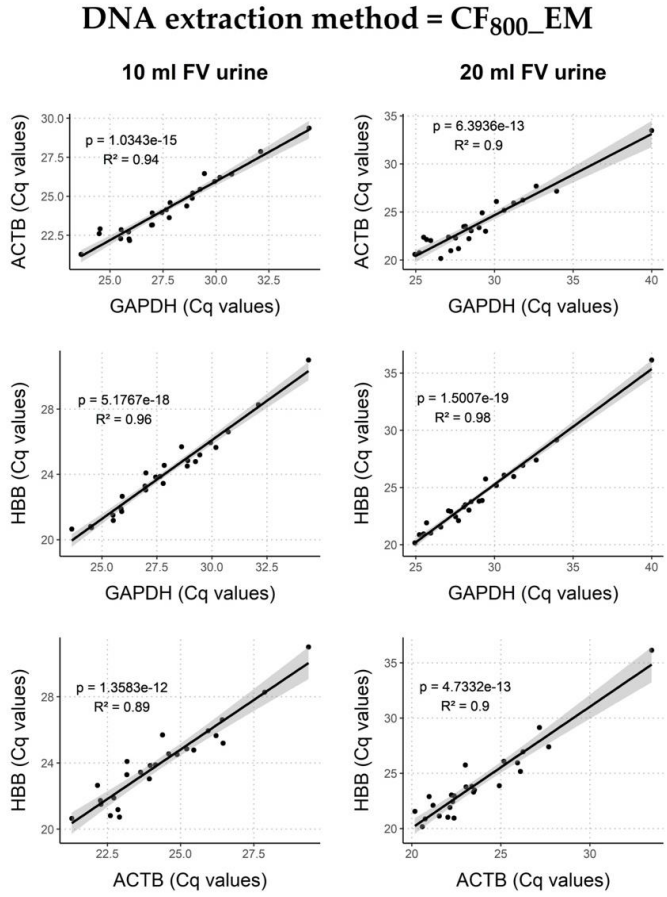

Figure A4: *Correlation curves for all human endpoint combinations on DNA extracted using CF_{800}_EM for 10 and 20 mL FV urine collection volumes. HR-HPV positive samples are marked grey. All correlations are highly significant, and R2 values are similar for both volumes.*

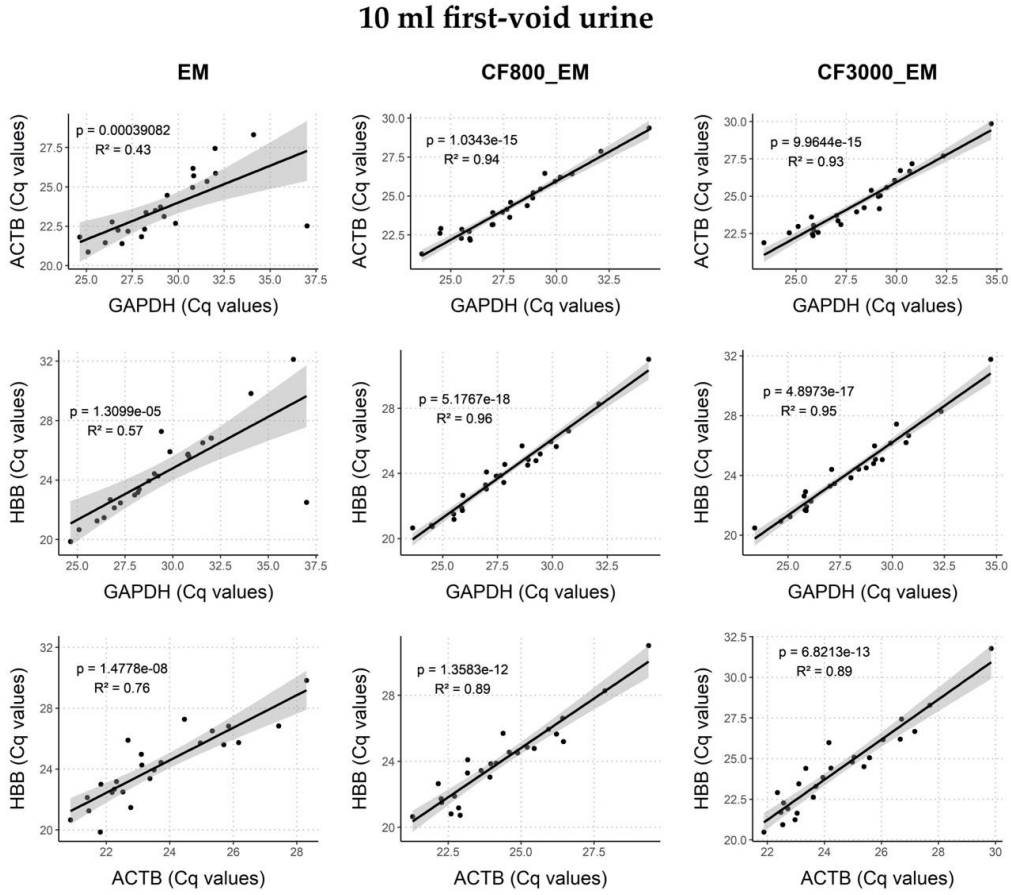

Figure A5: *Correlation curves for all human endpoint combinations on 20 mL FV urine samples extracted with AM_EM, EM, CF_{800}_EM, and CF_{800}_QIA. HR-HPV positive samples are marked grey. All correlations are highly significant.*

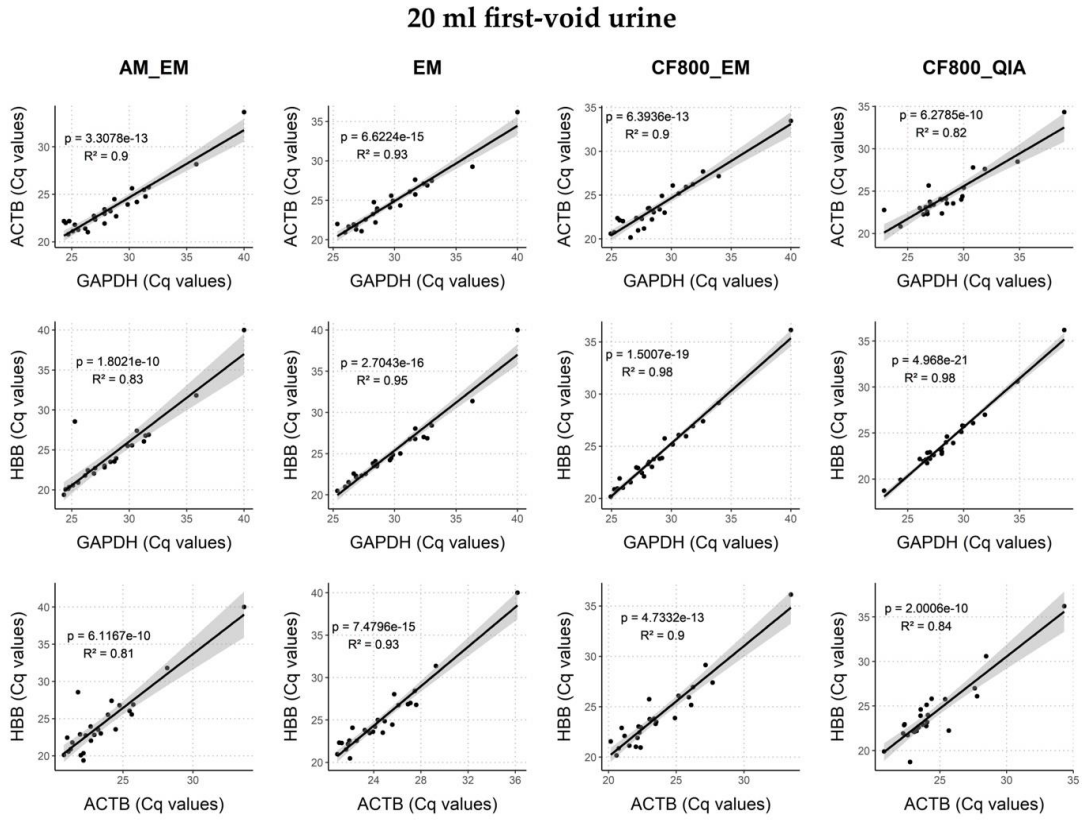

Figure A6: *Correlation curves for all human endpoint combination on 10 mL FV urine samples extracted with EM, CF_{800}_EM, and CF_{3000}_EM. HR-HPV positive samples are marked grey. All correlations are highly significant. Using the method EM, R2 values are lower than CF_{800}_EM, and CF_{3000}_EM.*

CHAPTER

03

First-void urine for HPV-specific antibody detection

CHAPTER

03–A

Stability, enrichment, and quantification of total and HPV16-specific IgG present in first-void urine.

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Submitted to Scientific Reports.

Abstract

First-void urine (FVU) samples, containing human papillomavirus (HPV)-specific IgG from female genital tract secretions, provide a non-invasive option for disease monitoring and vaccine impact assessment. This study explores the utility of FVU for IgG quantification, exploring stability and compatibility with DNA preservation methods, alongside various IgG enrichment methods. Healthy female volunteers provided FVU and serum samples. FVU was collected with or without urine conservation medium (UCM) and stored under different conditions before freezing at – 80 °C. Four IgG enrichment methods were tested on FVU samples. All samples were analyzed using three total human IgG quantification assays and an in-house HPV16-specific IgG assay. Samples stored with UCM buffer had higher total and HPV16-specific IgG concentrations ($p \leq 0.01$) and IgG remained stable for at least 14 days at room temperature. Among IgG enrichment methods, Amicon filtration (AM) and AM combined with Melon Gel purification (AM-MG) provided similar HPV16-IgG concentrations, correlating strongly with serum levels. Protein G magnetic beads methods were incompatible with time-resolved fluorescence-based assays. This study highlights FVU as a reliable and convenient sample for IgG quantification, demonstrating stability for at least 14 days at room temperature and compatibility with UCM DNA preservation. It emphasizes the need to select appropriate IgG enrichment methods and confirms the suitability of both AM and AM-MG methods, with a slightly better performance for AM-MG.

1 Introduction

First-void urine (FVU) samples have emerged as a valuable source for non-invasively detecting biomarkers related to human papillomavirus (HPV), including HPV DNA and HPV-specific immunoglobulins (Ig) in women [14,19,25,26,49,50,169,171,172]. Collecting these secretions through FVU sampling offers distinct advantages over invasive methods such as blood collection and cervical smears, as it allows for convenient self-collection at home [35,38].

FVU is characterized by its rich concentration of DNA, proteins, bacteria, viruses, and other female genital tract (FGT) secretions, exceeding those found in mid-stream urine [25]. This results in elevated levels of biomarkers of interest although it does introduce greater complexity, which can potentially challenge the stability, isolation/extraction, and quantification of these biomarkers [169,202]. To address one of these issues, the use of a DNA stabilization buffer has become a standard practice to preserve the integrity of nucleic acids in FVU samples [46,169,178]. However, the impact of such buffers on the stability and integrity of IgG in FVU, has not been explored. Furthermore, while studies have demonstrated urinary IgG stability under various conditions for specific viral pathogens [15,203], a knowledge gap exists regarding the stability of (HPV-specific) IgG in FVU.

Earlier studies showed that FVU contains limited quantities of IgG, approximately 0.5% of total IgG and 0.07% of HPV-specific IgG compared to serum levels [51]. While these studies have demonstrated good correlations between serum and FVU HPV-specific antibodies, FVU samples still exhibit lower antibody positivity than serum [49–51]. Apart from assay optimization, there lies potential in enhancing FVU sample preprocessing to increase antibody concentration, thus enabling the detection of even lower antibody titers in FVU. Additionally, inter- and intra-individual variation has been reported in FGT secretions [204], underlining the importance of normalizing HPV-specific antibody concentrations, where total human IgG could play a crucial role. To address these aspects, this study explores various IgG enrichment and quantification methods.

This study aims to evaluate the stability of IgG in FVU, assess its compatibility with a commonly used FVU DNA stabilization buffer, and compare diverse approaches for enriching and quantifying IgG. The outcomes of this study serve as fundamental steps in demonstrating the suitability of this sample type not only for disease monitoring but also

for assessing vaccine impact, ultimately having broad implications for clinical diagnostics and public health interventions [205].

2 Materials and methods

2.1 Study population

Women aged between 20 and 50 years old, employed at the University of Antwerp were recruited by addressing them personally. Next, the study team briefly informed the participants and provided them with the information brochure, including an informed consent form. They were asked to read these documents thoroughly before participating in the study. Informed consent was obtained from all volunteers, and data and samples were coded to ensure privacy of the participants. Key exclusion criteria included individuals who had previously received treatment for cervical cancer or precancerous lesions. This study was approved by the Institutional Review Board of UZA/University of Antwerp (B300201734129).

2.2 Sample collection and storage

For each analysis, women were asked to provide FVU samples. Before collecting a FVU sample, the participants were instructed not to wash their genitals thoroughly, not to use a tampon, and to avoid urinating for at least 2 h before collection. FVU samples were collected using the Colli-Pee® 20 ml device (Novosanis, Wijnegem, Belgium).

To assess IgG stability and compatibility of IgG storage with urine conservation medium (UCM) (Novosanis, Wijnegem, Belgium), 11 female volunteers collected one FVU sample without UCM (Figure1). Ten ml FVU was transferred to a Falcon tube and 5 ml UCM was added to obtain a UCM:FVU ratio of 1:2. All samples were vortexed and aliquoted in 4 ml aliquots for UCM buffered samples and in 2.66 ml aliquots for samples without UCM. A UCM buffered and unbuffered aliquot was I) directly stored at – 80 °C, II) stored at room temperature (RT) for seven days before storage at – 80 °C, III) stored at RT for 14 days before storage at – 80 °C. The remaining aliquots were stored immediately at – 80 °C for future use.

To compare different FVU enrichment methods gain 11 volunteers – partially overlapping with those from the stability analysis, collected two FVU samples using collection devices prefilled with 1/3 UCM with a time interval of at least two hours between two collections (Figure 1). The two samples were pooled in a 50 ml Falcon tube to reach a final volume of approximately 26.7 ml FVU and 13.3 ml UCM. Pooled samples were vortexed and aliquoted in 4 ml aliquots. All aliquots were stored immediately at – 80 °C before processing. We measured the pH of all samples and determined the presence of erythrocytes using Hemastix® reagent strips (Siemens Healthcare Diagnostics Inc., Dilbeek, Belgium). Paired blood samples were collected using 10 ml BD Vacutainer® Serum Tubes without anticoagulant (BD Benelux N.V., Erembodegem, Belgium). The blood samples were allowed to clot for 30–60 min, whereafter they were centrifuged for 10 min at 1000 × *g* and 20 °C. The serum was divided into aliquots before storage at – 80 °C. All aliquots were registered in the Antwerp Biobank (Biobank Antwerpen, Antwerp, Belgium; ID: BE71030031000) before further analysis.

2.3 Sample processing

To compare different enrichment methods, FVU samples were subjected to four different protocols. The first method included centrifugation of a 4 ml FVU aliquot at 4000 × *g* for 20 min at 20 °C in an Amicon Ultra-4 50K filter device (Merck Millipore, Belgium), this method is further referred to as AM. Centrifugation continued until the volume of FVU remaining on the filter was less than 50 µl. Next, the sample was diluted with dPBS until a total volume of 500 µl was reached. The second method included AM filtration and an extra purification step using the Melon™ Gel IgG Spin Purification Kit (Thermo Scientific, Belgium), this method is further referred to as AM-MG. Here, a 4 ml FVU aliquot was centrifugated using the Amicon Ultra-4 50 K filter device as mentioned above. The sample was diluted with 450 µl purification buffer to obtain a final 1:10 dilution of the sample and further purified according to the manufacturer's instructions. All centrifugation steps were performed at 4000 × *g*.

For the next two methods, Pierce™ Protein G magnetic beads (PGMB) were used (Thermo Scientific, Belgium). For each 4 ml FVU aliquot, 0.5 mg PGMB were placed into two 2 ml Protein Lo-Binding microcentrifuge tubes (1 mg PGMB per sample in total). According to the manufacturer's instructions, the beads were washed twice using binding-wash buffer (0.05 M Tris, 0.15 M NaCl, 0.05% Tween 20) and a DynaMa™-2 Magnet

(Invitrogen™, Belgium). Afterwards, the 4 ml FVU aliquots were added to the beads by adding 2 ml in each prepared tube. Tween detergent was added to a final concentration of 0.05%. The samples were incubated for approximately 1 h at RT with end-over-end mixing. Magnetic beads were collected with the magnet, and the supernatant was discarded. 500 µl binding-wash buffer was added to the first tube, the beads were dissolved, and the dissolved beads were added to the same sample's second tube. The beads were washed three times whereafter the total IgG was eluted in 100 µl using two different elution buffers: 0.1M glycine at pH 2 (further referred to as PGMB-G), and 0.1M citric acid (further referred to as PGMB-CA) at pH 2.5. Four additional elution buffers were tested: I) 0.1 M glycine elution at pH 3.5, II) 0.1 M citric acid elution at pH 3.5, III) elution using the Pierc™ Gentle Ag/Ab Elution Buffer (Thermo Fisher Scientific, Dilbeek, Belgium), and IV) elution using the Pierc™ IgG Elution Buffer (Thermo Fisher Scientific, Dilbeek, Belgium). After elution, the samples were neutralized using 1 M Tris at pH 8.5. All samples were brought to a pH of 7–7.5 using NaOH or HCl.

For the storage and stability analysis, all aliquots were processed using the Amicon Ultra-4 50K filter device as mentioned above. The concentrate was diluted in dPBS until a final volume of 500 µl was reached. All purified samples were stored up to 14 days at – 20 °C before further analysis. No purification step was performed for serum samples.

2.4 Total human IgG quantification

2.4.1 Bio-Rad BioPlex Luminex assay

Total human IgG concentrations were assessed for both processed FVU samples and for serum samples using the BioPlex Pro™ Human Isotyping Assay for total human IgG (Bio-Rad, USA) following the manufacturer's instructions. For FVU samples, dilutions of 1:128 and 1:512 were used, while serum samples were diluted at 1:10,000 and 1:100,000. UCM, diluted similarly to the samples was used as control. The LX200 platform (Luminex, Austin, Texas, USA) was used for measurement, and concentrations were derived from median fluorescence intensity (MFI) values using a five-parameter logistic regression. The average of two dilution-corrected concentrations was reported as the total human IgG concentrations. The dynamic range of this assay is 3.00 – 30,270 ng/ml.

2.4.2 HTRF homogeneous assay

The second total IgG quantification method used was the homogeneous time-resolved fluorescence (HTRF) Cisbio Human IgG kit, designed for the fast quantification of human IgG (Cisbio, Belgium). After optimization experiments, FVU dilution 1:300 and serum dilution 1:500,000 were selected for total human IgG quantification to fit within the assay's dynamic range. Other dilutions tested were 1:100, 1:1000, and 1:10000 for FVU and 1:1000, 1:10000, 1:100000, and 1:1000000 for serum (data not shown). UCM, diluted similarly to the samples, was used as control. Total IgG concentrations were determined according to the manufacturer's instructions, and signals were measured for 665 nm and 620 nm using the Victor Nivo multimode plate reader (Perkin Elmer, Belgium). The measurement ratio (665nm signal/ 620 nm signal) $\times 10^4$ was calculated for each measurement and the average background well signal was subtracted from all values. Total human IgG concentrations were calculated using the sigmoidal dose-response curve with variable slope. All calculations were performed using GraphPad Prism version 9.5.1. The dynamic range of this assay is 0.91–2,000 ng/ml.

2.4.3 AlphaLISA assay

The third total human IgG immunoassay used was the homogeneous highly sensitive IgG (human) Amplified Luminescent Proximity Homogeneous Assay (AlphaLISA) Detection Kit (Perkin Elmer, Belgium) for total IgG quantification to fit within the assay's dynamic range. Similar to the previous assays, optimized dilutions of 1:300 for FVU and 1:500,000 for serum samples were applied. UCM, diluted similarly to the samples, was used as control. The kit was used according to the manufacturer's instructions. A sharp peak of light emission at 615 nm was measured using the Alpha technology on the Victor Nivo multimode plate reader (Perkin Elmer, Belgium). The average signal of four background wells was subtracted from all sample values before analysis. Concentrations were calculated using nonlinear regression with the 4-parameter logistic equation. A sigmoidal dose-response curve with variable slope was constructed using $1/Y^2$ data weighting. All calculations were performed using GraphPad Prism version 9.5.1 The dynamic range of this assay is 0.24–1,000 ng/ml.

2.5 HPV16-IgG quantification (DELFIA)

HPV16-specific IgG were measured for all processed FVU samples and serum samples using an in-house Dissociation-Enhanced Lanthanide Fluorescent immunoassay (DELFIA). Sample testing was performed using the HPV DELFIA protocol as described previously [206]. For FVU samples, the testing began undiluted, while serum samples, standards, and controls were started at a dilution of 1:400. Serial dilutions at a 1:2 ratio were made for each sample, and four dilutions were tested. Plates were read using either the EnVision® multimode plate reader (Perkin Elmer, Belgium) or the Victor Nivo® multimode plate reader (Perkin Elmer, Belgium). Background counts were subtracted from the sample measurements. To determine IgG concentrations, the parallel line method (PLL) described in the World Health Organization (WHO) HPV Labnet Manual 2009 was employed [207]. Samples were assigned titers if they exhibited linear titration and fulfilled all PLL conditions. The results are reported as HPV16 IgG titers in International Units (IU/ml). The HPV16 (05–134) standard (10–140) used in the assay was obtained from the National Institute for Biological Standards and Controls [NIBSC] (Potter's Bar, UK). The results of the HPV16-DELFIA assay were in-house compared to the concentrations derived with the M9ELISA [50,164], and excellent Spearman rank correlations were established (≥ 0.9) (data not shown).

2.6 Statistical analysis

R statistical software version 4.2.2 was used for statistical analysis. Firstly, normality of the data was assessed using the Shapiro-Wilk test, assuming that the data on one variable were monotonically related to the other variable. If the data followed a normal distribution, significant differences between different storage conditions and IgG enrichment methods were analyzed using paired *t*-tests. However, if the data did not meet the normality assumption, non-parametric Wilcoxon signed-rank testing was applied. Statistical significance was determined as p-adjusted < 0.05, and the Holm-Bonferroni method was used for adjusting p-values to counteract the problem of multiple comparisons. To explore potential correlations between human IgG quantification kits and between paired FVU and serum, Spearman's rank correlations were calculated. Additionally, we generated Bland Altman plots to assess concordance between the total IgG quantification kits [208]. To evaluate the precision of these correlations, bootstrap 95% confidence intervals (CI) were estimated through 1,000 replicates.

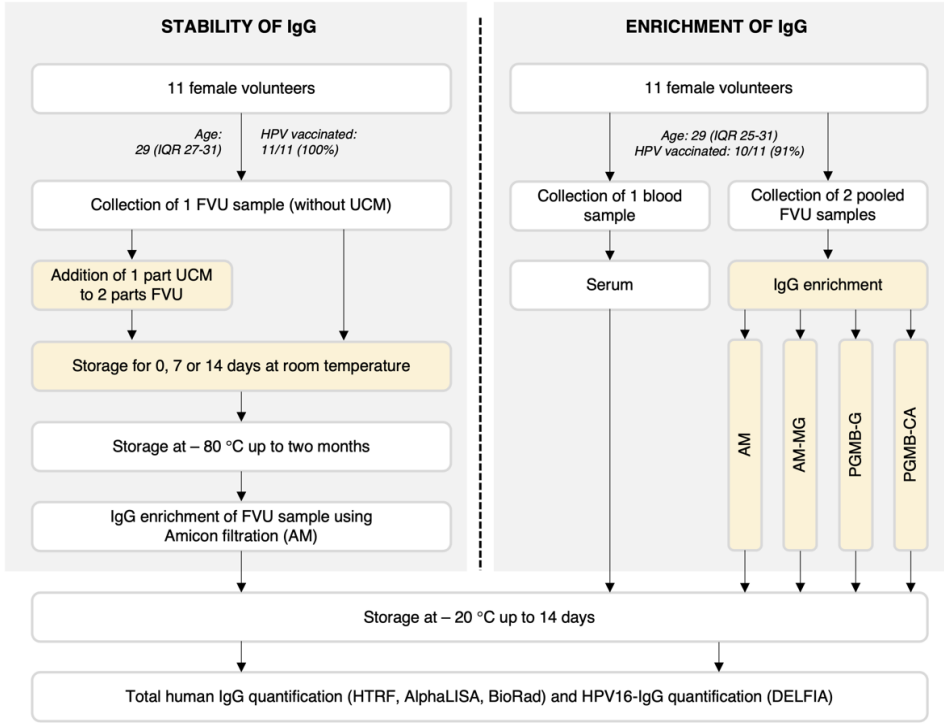


Figure 1: *Study design. To assess storage and stability of IgG in FVU, eleven women provided a FVU sample collected without UCM. To 10 ml of the FVU sample, UCM was added in a 1:2 UCM:FVU ratio. The samples were aliquoted in 4 ml samples and stored at RT for different durations whereafter they were stored at – 80 °C. All samples were processed using Amicon filtration. To evaluate different IgG enrichment methods, eleven women provided both blood samples and two pooled FVU samples. From the blood samples, serum was extracted, while the pooled FVU samples were aliquoted in 4 ml samples and processed using different protocols. The different IgG enrichment protocols were: Amicon filtration (AM), AM followed by melon gel purification (AM-MG), protein G magnetic bead purification using glycine elution (PGMB-G) and citric acid elution (PGMB-CA). All samples were analyzed for total human IgG using the HTRF and AlphaLISA technology from PerkinElmer, as well as the BioPlex isotyping assay from Bio-Rad. Additionally, HPV16-IgG concentrations were measured using the HPV16 DELFIA.*

3 Results

3.1 Population and sample characteristics

For stability and UCM compatibility analyses, 11 healthy female volunteers with a median age of 29 (IQR 27–31) provided two FVU samples, and 100% (11/11) reported being fully vaccinated with one of the HPV vaccines (Figure 1). Similarly, to compare IgG enrichment methods for FVU, 11 healthy female volunteers with a median age of 29 (IQR 25–31) provided paired FVU and serum samples, with 91% (10/11) of the participants

reporting being vaccinated against HPV (Figure 1). Notably, 6 out of the 11 participants provided samples for both analyses. All participants reported to not have had an HPV infection in the previous six months and the erythrocyte count in all samples was negative.

3.2 IgG storage and stability in FVU

We assessed the stability of IgG in FVU under various storage conditions and evaluated the compatibility of the UCM buffer with IgG storage. Samples were stored with or without UCM buffer at various durations at RT before freezing at – 80 °C. Three different quantification methods were compared to measure total human IgG concentrations, and HPV16-IgG levels were measured using the HPV16 DELFIA ((Supplementary Table 1, Figure 2).

Using the BioRad assay, total human IgG concentrations were significantly higher in samples stored with the UCM buffer than those without when samples were immediately stored at – 80 °C ($p = 0.005$). For the HTRF assay, significantly higher total IgG concentrations were quantified for samples stored with UCM buffer for all RT storage durations ($p \leq 0.002$). The AlphaLISA assay showed significantly lower IgG concentrations for samples stored with UCM (14.26 µg/ml) than those without UCM (15.37 µg/ml) for those directly stored at – 80 °C ($p = 0.005$) and significantly higher (10.92 µg/ml vs. 8.11 µg/ml) for samples stored at RT for 7 days ($p = 0.001$). Furthermore, significantly higher total human IgG concentrations were quantified with the AlphaLISA for UCM buffered samples directly stored at – 80 °C (14.26 µg/ml) compared to those stored at RT for 7 days before storage at – 80 °C (10.92 µg/ml) ($p = 0.02$) (Figure 2A).

The HPV16-IgG concentrations were significantly higher in samples stored with UCM buffer compared to those without UCM buffer, both for samples directly stored at – 80 °C ($p = 0.02$) and those stored at RT for 7 days before storage at – 80 °C ($p = 0.04$). Additionally, a significantly lower median HPV16-IgG concentration was observed for UCM buffered samples directly stored at – 80 °C (0.024 IU/ml) compared to those stored at RT for 14 days before storage at – 80 °C (0.026 IU/ml) ($p = 0.02$) (Figure 2B). The UCM control wells did not provide signals above the LOD for each total IgG measurement method.

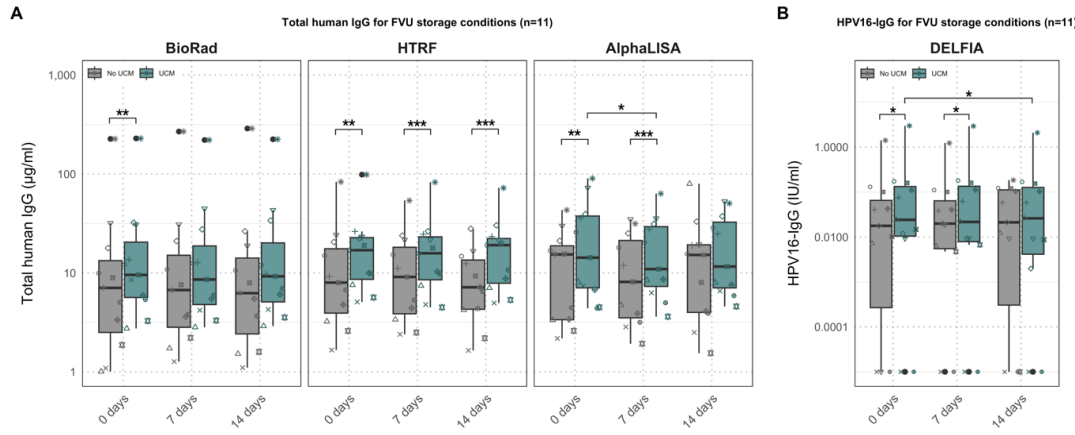


Figure 2: *Total IgG and HPV16-IgG results for different storage conditions of FVU. (A) Boxplots of total human IgG concentrations for 11 samples stored under different conditions using the BioPlex, the HTRF assay and the AlphaLISA. (B) Boxplots of HPV16-IgG concentrations. Significance levels are represented in the figure by an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Outliers in all figures represent the same ID.*

Significant Spearman rank correlations were found among the three total human IgG immunoassays, with overall coefficients of 0.84 (95% CI 0.66–0.94) or higher (Figure 3). The highest correlation coefficient was observed between BioRad and HTRF (Figure 3B). Additionally, Bland Altman plots indicate that largest absolute differences between total human IgG methods are found for samples with higher total human IgG concentrations (Supplementary Figure 1).

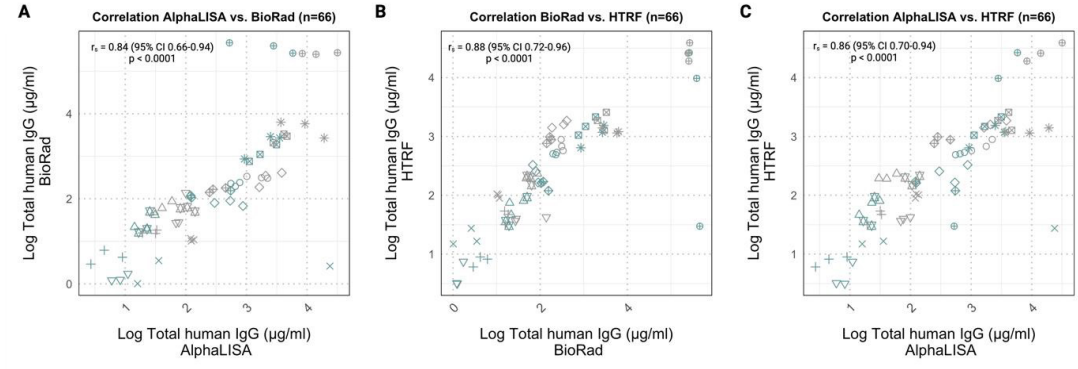


Figure 3: *Spearman correlation plots for all stability data combined (A) Correlation plot between the AlphaLISA and the BioRad assay. (B) Correlation plot between the BioRad assay and the HTRF assay. (C) Correlation plot between the AlphaLISA and the HTRF assay. Spearman's rank correlation coefficient presented in the figure are for all conditions combined. Different symbols represent IDs and different colors represent buffer conditions in the correlation plots. Outliers in all figures represent the same ID.*

3.3 IgG enrichment in FVU

We evaluated four different methods for enriching IgG in FVU samples. All processed samples were then analyzed using three distinct total human IgG kits, and we used the DELFIA assay for HPV16-IgG measurement (Figure 1, Supplementary Table 2). Results for PGMB-CA and PGMB-G enrichment methods tested using TRF based methods were reported as invalid due to non-specific binding. For the BioRad total IgG assay, significantly higher concentrations were found for AM compared to AM-MG ($p = 0.004$) and PGMB-CA ($p = 0.004$) (Figure 4). The HTRF kit provided significantly higher total IgG concentrations for AM compared to AM-MG ($p = 0.001$), PGMB-G ($p = 0.005$), and PGMB-CA ($p = 0.03$). However, no significant differences were observed between the enrichment methods for the AlphaLISA ($p \geq 0.06$) (Figure 4A). For the AlphaLISA, samples processed with the PGMB methods gave signals close to the LOQ but above LOD. No significant differences in HPV16-IgG concentrations were observed between the IgG enrichment methods ($p \geq 0.06$) (Figure 4B).

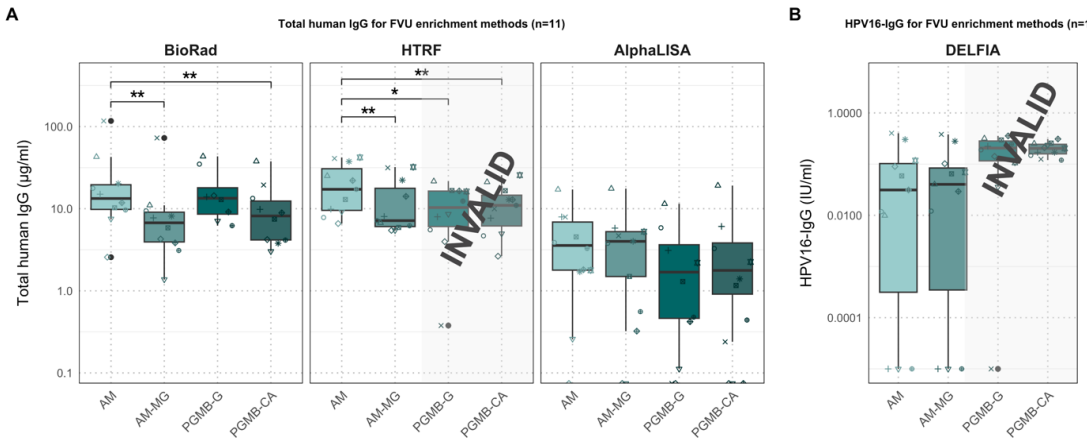


Figure 4: *Total IgG and HPV16-IgG results in FVU of the IgG enrichment cohort. (A) Boxplots of total human IgG concentrations after different enrichment protocols for 11 FVU samples using the BioPlex, the HTRF assay and the AlphaLISA. (B) Boxplots of HPV16-IgG concentrations after different enrichment protocols for 11 FVU samples. Significance levels are represented in the figure by an asterisk ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Different symbols represent different IDs. Results for TRF based methods (HTRF and DELFIA) are invalid for PGMB enrichment methods due to non-specific binding.*

For the unvaccinated volunteer, no HPV16-IgG was detected in the serum, nor in the AM and AM-MG purified FVU samples (Figure 5). Nevertheless, employing the PGMB methods, an HPV16-IgG titer above the median titer was assigned for the unvaccinated

volunteer. The Spearman rank correlations between enrichment methods were generally most significant when comparing AM to AM-MG for all quantification methods ($p \leq 0.001$), except for AlphaLISA, where the most significant correlation was observed between PGMB-G and PGMB-CA ($p \leq 0.0002$) (Supplementary Table 3).

Significant correlation coefficients were found for AlphaLISA vs. BioRad (0.67 (95% CI 0.02–0.96)) and BioRad vs. HTRF (0.72 (95% CI 0.08–0.99)) for AM enriched samples. AM-MG enriched samples showed significant correlations for total IgG AlphaLISA vs. BioRad (0.83 (95% CI 0.37–0.98)) and AlphaLISA vs. HTRF (0.61 (95% CI -0.03–0.97)). However, no significant correlations were observed between the total IgG quantification kits for the PGMB-G and PGMB-CA enriched samples ($p \geq 0.06$) (Table 1). Additionally, Bland Altman plots again indicate that largest absolute differences between total human IgG methods are found for samples with higher total human IgG concentrations (Supplementary Figure 2).

Table 1: *Correlation coefficients for the different total human IgG quantification methods for different FVU IgG enrichment methods and serum.*

	BioRad vs. HTRF		AlphaLISA vs. BioRad		AlphaLISA vs. HTRF	
	Spearman correlation (95% CI)	Adjusted *p*-value	Spearman correlation (95% CI)	Adjusted *p*-value	Spearman correlation (95% CI)	Adjusted *p*-value
FVU						
AM	0.72 (0.08-0.99)	0.0242	0.67 (0.02-0.96)	0.0394	0.23 (-0.53-0.81)	0.5031
AM-MG	0.61 (-0.08-0.97)	0.0802	0.83 (0.37-0.98)	0.0032	0.61 (-0.03-0.97)	0.04817
PGMB-G	0.50 (-0.40-0.96)	0.5517	0.65 (-0.16-1.00)	0.0568	0.49 (-0.38-0.94)	0.1425
PGMB-CA	0.38 (-0.27-0.90)	0.775	0.51 (-0.27-0.91)	0.1327	0.38 (-0.36-0.91)	0.2419
Serum	0.26 (-0.77-0.89)	0.4697	0.04 (-0.74-0.86)	0.9186	-0.14 (-0.69-0.65)	0.6935

Additionally, the correlations between the processed FVU samples and the serum samples for HPV16-IgG were examined (Figure 5). Highly significant Spearman correlation coefficients were observed between HPV16-IgG levels for AM-processed FVU and serum (0.94 (95% CI 0.72–1.00)) and between AM-MG processed FVU and serum (0.90 (95% CI 0.59–1.00)), whereas no significant correlations were found between PGMB-G or PGMB-CA processed FVU, and serum ($p \geq 0.2$). For the serum samples, no significant Spearman correlations were found for the three total human IgG kits ($p \geq 0.5$) (Table 1) and Bland Altman plots also show poor concordance between the kits (Supplementary Figure 3).

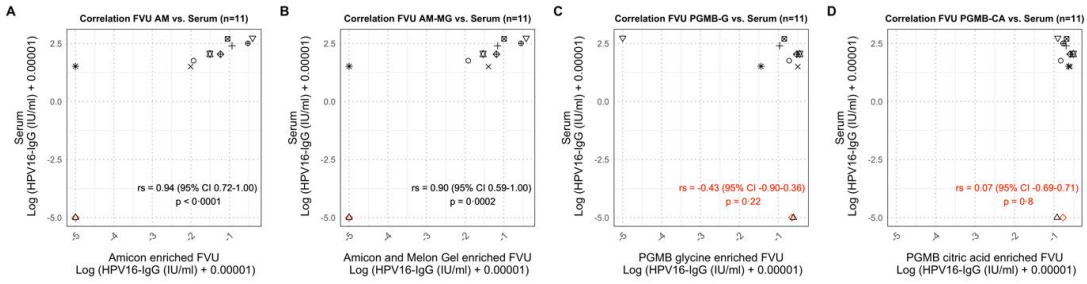


Figure 5: *Correlation plots for HPV16-IgG for differently processed FVU samples and serum samples. Note that the results of PGMB-G and PGMB-CA should be interpreted with caution due non-specific binding and insignificant correlations with serum. Different shapes represent different IDs. Vaccinated IDs are presented in black, unvaccinated in red.*

3.4 Non-working IgG enrichment methods

In addition to the four described IgG enrichment methods, four other elution steps were explored for the PGMB as described in the methods section (data not shown). However, despite these efforts, the samples did not yield quantifiable concentrations using any of the described immunoassays, leading us to exclude these methods from further evaluation.

4 Discussion

FVU as a valuable sample for vaccine and disease monitoring

It is well-established that IgG can be detected in urine samples, serving as a valuable tool for identifying specific IgG related to various infections and diseases [209–213]. FVU has the unique capability of capturing and concentrating FGT secretions, making it an ideal sample for assessing pathogen-specific IgG associated with the FGT [19,25].

In this study, we aimed to assess the stability of total human and HPV16-specific IgG in FVU, evaluate its compatibility with DNA preservation methods, and explore various methods for enriching and quantifying IgG. Our findings represent a significant step in exploring the potential of FVU as a non-invasive sample for monitoring HPV vaccine trials and epidemiological studies [51].

Impact of UCM buffer on IgG stability in FVU

Previous studies focusing on HPV DNA detection in FVU have demonstrated the effectiveness of the UCM buffer in preserving DNA [24,41,169]. To streamline non-invasive sampling for both follow-up of vaccination and infection, we investigated the influence of the UCM buffer, a PBS-based solution containing a chelating agent, a microbicide, a fungicide, and BSA, on IgG stability in FVU. Overall, our findings suggest that the UCM buffer positively affects the detection of total human IgG and HPV16-specific IgG. Only for one assay, the AlphaLISA, we did observe some variating effects of the UCM buffer on the IgG detection. BSA in the UCM buffer likely reduces IgG adhesion to tube surfaces, leading to detection of higher (HPV16-)IgG concentrations [169,214]. Moreover, we observed that IgG remains stable at RT for at least 14 days, with minimal alterations in total and HPV16-specific IgG concentrations over time, even after a freeze-thaw cycle.

Comparison of IgG enrichment methods for FVU

Although HPV-specific antibodies have shown to be adequately detectable in FVU samples enriched using Amicon filtration, lower HPV-antibody positivity rates have been reported compared to the standardly used serum samples [49–51]. To reduce sample impurities that could interfere with the immunoassays and achieve a concentrated IgG sample, we tested various purification methods for FVU samples, including two PGMB-based protocols and combined Amicon filtration with Melon Gel purification, referred to as AM-MG. The addition of the Melon Gel purification step resulted in approximately 50% lower total IgG concentrations for the BioRad and HTRF assays, as compared to using Amicon filtration alone. Notably, both enrichment methods yielded similar concentrations when measured with AlphaLISA for total IgG and HPV16-IgG, suggesting that the difference observed in the BioRad and HTRF assays may not be attributed to IgG loss but rather to non-specific binding when residual proteins are not removed. We observed consistently high and significant Spearman correlation coefficients between HPV16-IgG concentration for both enrichment methods and serum. The additional Melon Gel filtration step might thus be valuable when IgG purity is essential.

PGMB has been widely used for IgG purification from, i.e., serum and cell culture supernatant, but has not been evaluated for FVU samples [215]. In contrast to the first two enrichment methods, the PGMB IgG enrichment methods show limited significant

correlations between the IgG quantification methods. Furthermore, Spearman correlation coefficients between HPV16-IgG concentrations in serum and those obtained through PGMB-G or PGMB-CA are notably low and not statistically significant. Remarkably, the PGMB methods produced an HPV16-IgG titer for an unvaccinated FVU sample that was not detected by the other two FVU IgG processing methods or in serum. The HPV16-IgG DELFIA assay relies on time-resolved fluorescence using the lanthanide Europium (Eu^{3+}) [216,217] . Since PGMB contains magnetite (Fe_3O_4), and PGMB-G samples are eluted in glycine, both of which are known to interact with Eu^{3+}, potential non-specific binding and contamination of exogenous lanthanides may have affected the assay's reproducibility for samples enriched using the PGMB methods [218–221]. Therefore, the results of the PGMB for HPV16-IgG DELFIA and total IgG HTRF assays are reported as invalid [222–225]. However, it is worth noting that some correlations are observed for the PGMB enrichment when citric acid is used for elution instead of glycine, suggesting that there might be less interference with this assay for the PGMB-CA method.

Comparison of total human IgG quantification methods

Human IgG might be important in normalizing inter- and intra-individual variation in FGT secretions collected with FVU samples. Since no assays were validated for FVU samples, our study compared three distinct total human IgG quantification methods, each with different protocols and readouts. Homogeneous assays (HTRF and AlphaLISA) offered several advantages over the BioRad ELISA-based assay, including reduced hands-on time, shorter run times, and lower variability, even though they have a narrower dynamic range [226]. These assays operate on an energy transfer mechanism between fluorophores or beads, whereas the BioRad assay relies on Luminex technology [224,227]. Importantly, all the quantification methods we employed proved to be compatible with AM and AM-MG enriched FVU samples, demonstrating significant overall correlations. As mentioned earlier, the HTRF quantification method was reported as incompatible with the PGMB enrichment methods due to non-specific binding [216]. Surprisingly, the strong correlation between the IgG quantification methods did not extend to serum samples, suggesting potential challenges or distinctions between sample types. While differences in dynamic range, matrix interactions, and distance factors between donor and acceptor may contribute to the observed variations, it is worth noting that these discrepancies were exclusive to serum samples, and the exact reasons for this distinction remain under investigation.

Limitations

This study has certain limitations. Firstly, as this is a pilot study to evaluate protocols, the sample size is limited. Nevertheless, the obtained results provide valuable information that contributes to the evaluation of FVU as a non-invasive clinical and immunological sample. Additionally, it should be noted that HPV16-IgG levels were assessed using an in-house developed DELFIA assay, however our in-house experiments have shown strong correlations with established HPV-specific immunoassays like M9ELISA, M4ELISA, and GST-L1-MIA [51,142,164,166]. Further investigations, including prolonged storage periods at RT and – 20 °C and comparisons to unfrozen samples, could enhance our understanding of IgG stability. Moreover, it is essential to verify the outcomes of this study for samples containing naturally induced HPV16 antibodies.

Conclusions

In conclusion, this study provides essential results that will contribute to further research exploring FVU as a valuable sample for clinical and immunological studies. Our research demonstrates that IgG in FVU samples remains stable for at least 14 days at room temperature and that the use of the UCM buffer enhances IgG detection in FVU. Furthermore, our study shows that different total IgG quantification methods are compatible with FVU samples. However, it is crucial to note that not all tested IgG enrichment methods yield scientifically accurate results with all quantification methods, and careful consideration is necessary when selecting an appropriate method for specific applications. Both the AM and AM-MG enrichment methods can be used for all analyses, with slightly superior results observed for AM-MG.

5 Supplementary materials

Supplementary Table 1: *Overview of median (IQR) total human IgG and HPV16-specific IgG concentrations in Amicon filtered FVU for different storage conditions and with different immunoassays.*

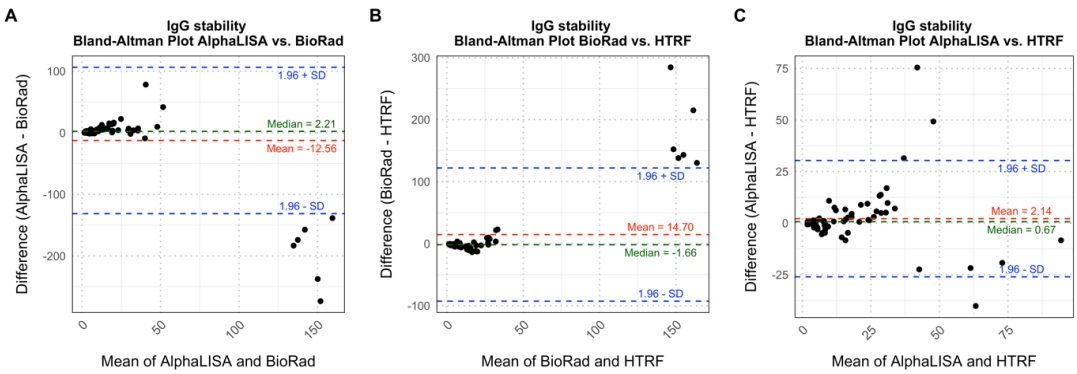
	Total Human IgG			HPV16-IgG
	BioRad (µg/ml)	HTRF (µg/ml)	AlphaLISA (µg/ml)	DELFIA (IU/ml)
No UCM				
0 days	7.05 (2.61-13.87)	7.98 (4.00-17.73)	15.37 (3.36-19.91)	0.018 (0.004-0.071)
7 days	6.70 (2.91-15.91)	9.10 (3.89-18.43)	8.11 (3.53-21.58)	0.020 (0.006-0.070)
14 days	6.24 (2.63-14.71)	7.16 (4.29-13.54)	15.19 (3.99-19.21)	0.021 (0.005-0.112)
All	6.47 (2.20-17.82)	7.57 (4.21-15.31)	10.00 (3.39-19.44)	0.020 (0.005-0.100)
UCM				
0 days	9.54 (5.65-22.21)	16.97 (8.69-22.75)	14.26 (7.06-37.62)	0.024 (0.011-0.135)
7 days	8.57 (4.83-20.13)	15.78 (8.60-23.19)	10.92 (7.37-29.40)	0.022 (0.008-0.136)
14 days	9.22 (5.16-22.89)	19.08 (7.90-22.30)	11.58 ((7.07-32.89)	0.026 (0.005-0.129)
All	9.22 (5.38-27.55)	16.97 (7.54-23.19)	11.58 (6.76-35.51)	0.024 (0.009-0.154)

Supplementary Table 2: *Overview of median (IQR) total human IgG and HPV16-specific IgG concentrations in FVU for different FVU enrichment methods and serum with different immunoassays. Results for TRF based methods (HTRF and DELFIA) are invalid for PGMB enrichment methods due to non-specific binding.*

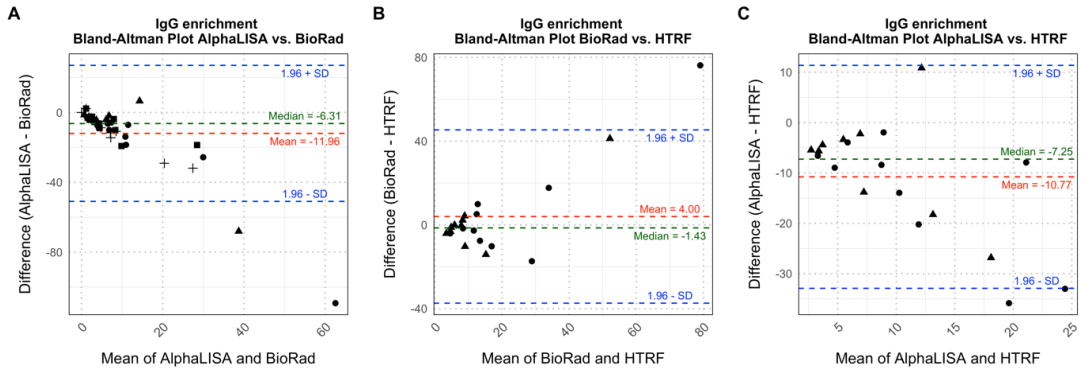
	Total Human IgG			HPV16-IgG
	BioRad (µg/ml)	HTRF (µg/ml)	AlphaLISA (µg/ml)	DELFIA (IU/ml)
FVU				
AM	13.42 (9.81-19.60)	17.23 (9.56-31.31)	3.28 (1.74-6.23)	0.031 (0.008-0.104)
AM-MG	6.79 (3.94-9.09)	7.16 (6.06-18.20)	3.79 (0.44-4.97)	0.040 (0.006-0.086)
PGMB-G	12.91 (7.10-14.42)	~~10.49 (6.14-16.39)~~	0.89 (0.19-2.88)	~~0.206 (0.117-0.284)~~
PGMB-CA	8.19 (4.16-12.48)	~~10.98 (6.32-14.73)~~	1.16 (0.12-2.76)	~~0.203 (0.159-0.245)~~
Serum	3711 (2750-4216)	4029 (3401-7056)	5979 (5276-37338)	109.4 (32.6-286.9)

Supplementary Table 3: *Spearman correlation coefficients between different IgG enrichment methods. Results for HTRF assay are invalid for PGMB enrichment methods due to non-specific binding.*

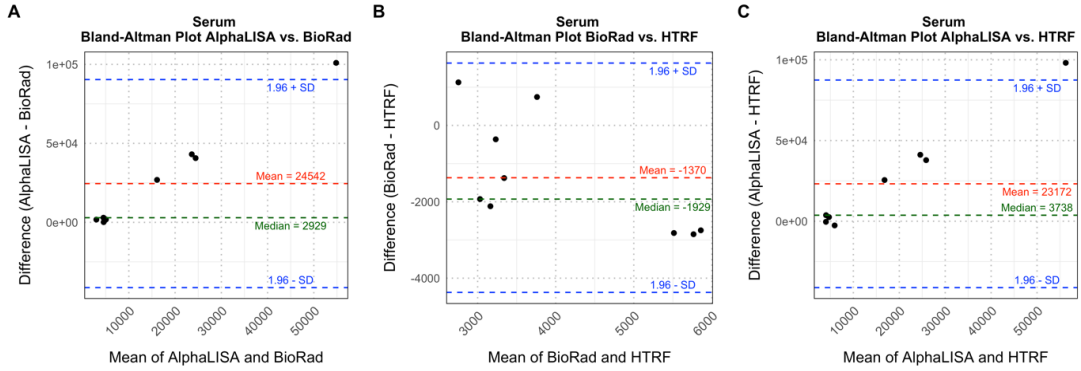
	Total Human IgG						HPV16-IgG	
	BioRad		HTRF		AlphaLISA		DELFIA	
	Spearman correlation (95% CI)	Adjusted p-value	Spearman correlation (95% CI)	Adjusted p-value	Spearman correlation (95% CI)	Adjusted p-value	Spearman correlation (95% CI)	Adjusted p-value
AM vs								
AM-MG	0.89 (0.46-1.00)	0.001	0.8 (0.29-1.00)	0.005	0.72 (0.13-1.00)	0.01	0.96 (0.77-1.00)	<0.0001
PGMB-G	0.12 (-0.73-0.98)	0.78	~~0.40 (-0.40-0.96)~~	~~0.26~~	0.57 (-0.16-1.00)	0.08	~~-0.33 (-0.92-0.50)~~	~~0.36~~
PGMB-CA	0.68 (-0.01-1.00)	0.04	~~0.79 (0.22-1.00)~~	~~0.006~~	0.65 (0.00-0.96)	0.03	~~-0.009 (-0.75-0.63)~~	~~0.97~~
AM-MG vs								
PGMB-G	0.33 (-0.53-1.00)	0.38	~~-0.0006 (-0.70-0.69)~~	~~1~~	0.72 (0.09-1.00)	0.02	~~-0.27 (-0.86-0.46)~~	~~0.44~~
PGMB-CA	0.77 (0.13-1.00)	0.01	~~0.46 (-0.27-0.92)~~	~~0.15~~	0.86 (0.40-1.00)	0.0006	~~0.04 (-0.73-0.67)~~	~~0.92~~
PGMB-G vs								
PGMB-CA	0.4 (-0.47-0.98)	0.29	~~0.79 (0.33-1.00)~~	~~0.01~~	0.92 (0.63-1.00)	0.0002	~~0.52 (-0.37-0.92)~~	~~0.13~~



Supplementary Figure 1: *Bland Altman plots for the different total human IgG quantification kits and the IgG stability cohort. The x-axis represents the mean of the measurements obtained from the two methods, while the y-axis represents the difference between the measurements. The red dashed line represents the mean of the differences, the green dashed line represents the median of the differences, and the upper and lower blue dashed lines represent the mean plus and minus 1.96 times the standard deviation (SD) of the differences, respectively.*



Supplementary Figure 2: *Bland Altman plots for the different total human IgG quantification kits and the IgG enrichment cohort. AM results are presented as dots, AM-MG results as triangles, PGMB-G results as squares and PGMB-CA results as plus signs. Results for the HTRF assay are invalid for PGMB enrichment methods due to non-specific binding and therefore excluded for those specific analyses. The x-axis represents the mean of the measurements obtained from the two methods, while the y-axis represents the difference between the measurements. The red dashed line represents the mean of the differences, the green dashed line represents the median of the differences, and the upper and lower blue dashed lines represent the mean plus and minus 1.96 times the standard deviation (SD) of the differences, respectively.*



Supplementary Figure 3: *Bland Altman plots for the different total human IgG quantification kits and the serum samples from the enrichment cohort. The x-axis represents the mean of the measurements obtained from the two methods, while the y-axis represents the difference between the measurements. The red dashed line represents the mean of the differences, the green dashed line represents the median of the differences, and the upper and lower blue dashed lines represent the mean plus and minus 1.96 times the standard deviation (SD) of the differences, respectively.*

CHAPTER

03–B

Time-resolved fluorescence (TRF) for total IgG and HPV16-specific antibody detection in first-void urine and serum: A comparative study.

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Submitted to Journal of Virological Methods.

Abstract

Recent studies demonstrated that human papillomavirus (HPV) specific immunoglobulins (IgG) are present and detectable in non-invasively collected first-void urine (FVU) samples. As IgG levels in urine are low, we evaluated the potential of a highly sensitive HPV16-specific assay based on time-resolved fluorescence, DELFIA, and compared it with three immunoassays, GST-L1-MIA, M4ELISA, and M9ELISA. A total of 225 paired serum and FVU samples from two cohorts of healthy female volunteers were analyzed. Strong Spearman rank correlations between HPV16-specific IgG results measured with DELFIA, M4ELISA, GST-L1-MIA, and M9ELISA were found for both sample types ($r_s > 0.80$). Additionally, total human IgG results, determined in all samples using HTRF human IgG kit and BioPlex Pro™ Human Isotyping Assay, were compared. Moderate correlations between total human IgG concentrations in FVU samples were found for the two total IgG assays ($r_s \geq 0.42$, $p < 0.0001$), while correlations for serum were non-significant. In conclusion, the HPV16-DELFIA assay is usable for detecting HPV16-specific antibodies in FVU and serum samples. As total human IgG remains an interesting parameter for the normalization of HPV-specific IgG in FVU, the accuracy of both assays needs to be validated further.

1 Introduction

Human papillomavirus (HPV) vaccination is a crucial step in primary cervical cancer prevention, given its direct association with nearly all cases of cervical cancer [228]. Currently, assessing the immunogenicity of HPV vaccines predominantly relies on serum samples [229]. Interestingly, recent studies have demonstrated that humoral immune responses specific to HPV can be assessed in first-void urine (FVU) [49–51]. Considering that cervical cancers after HPV infection predominantly develop at the cervical transformation zone, evaluating antibody-mediated immune responses at the cervix, and thus local protective immunity is important [123,230].

Vaccine-induced circulating antibodies reach the female genital tract (FGT) through transudation or exudation at microlesion sites. This results in a higher presence of immunoglobulins (IgG) originating from serum, with fewer locally produced IgG and secretory IgA (sIgA) [30,231]. These HPV-related immunological markers, together with discharged mucus and (debris from) exfoliated cells from the female genital organs, accumulate around the urethral opening and the small labia, and are subsequently washed away during urination. The initial part of a urine sample, known as first-void urine contains a higher concentration of FGT secretions compared to the subsequent part. This includes HPV DNA, HPV-type-specific antibodies, and other HPV biomarkers [19,25,40,44,46,232]. The non-invasive nature of this sample type makes it highly acceptable and allows women to collect it at home, eliminating the need for a blood draw at a medical facility [35].

As a correlate of protection for HPV infections remains undetermined, the detection of HPV-specific antibodies at the site of infection could emerge as an important immunological monitoring tool [118,230,233–235]. Recent studies have demonstrated the quantifiability of HPV-specific IgG in FVU samples, revealing a significant difference in HPV-type-specific antibody concentrations between FVU samples from vaccinated and unvaccinated individuals [50,51]. Given that HPV type-specific antibody concentrations are approximately three logs higher in serum compared to FVU samples, the concentrations in FVU often reach the detection limit of existing immunoassays, which are exclusively validated for serum. Furthermore, to account for the lower HPV-specific antibody concentrations present in FVU, less diluted samples must be used, potentially leading to more non-specific background compared to serum. To improve HPV-specific antibody detection in FVU a highly sensitive immunoassay is required.

Previous studies were already able to show HPV-specific antibody positivity for HPV16 in more than 80% of FVU samples from vaccinated women [50,51]. This was achieved using the bead-based assay (Glutathione S-transferase-L1 multiplex serology assay, GST-L1-MIA) or multi-spot VLP-based ELISAs (M4ELISA, M9ELISA) which are validated and routinely employed for detecting HPV-type specific antibodies in serum samples [164–166]. For both assays, results for FVU were relatively close to the detection limit. Consequently, further steps such as sample purification, assay optimization, or the development of a new immunoassay using highly sensitive technology are deemed necessary to enhance assay sensitivity.

Our research group has developed an HPV16 type-specific Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA). This innovative assay combines the principles of time-resolved fluorescence (TRF) with the properties of lanthanide fluorescent compounds, resulting in a potentially more sensitive assay [217]. A major benefit to the use of lanthanides is the delay in measurement between excitation and emission, resulting in a large Stokes shift and a reduction in self-quenching [217]. The technology offers a high sensitivity, making it suitable for the detection of low-abundance analytes, and has a wide dynamic range to cover a broad range of analyte concentrations.

In this study, we assessed the efficacy of the TRF-based assay and compared its outcomes for HPV16-specific antibody concentrations to those obtained from the GST-L1-MIA, M4ELISA, and M9ELISA. To achieve this, we analyzed paired FVU and serum samples collected from two distinct cohorts of women.

Because of the heterogeneous nature of a urine sample, both within and between individuals, fluctuations in detectable biomarkers can arise. These fluctuations may stem from differences in time of collection, collection volume, pre-collection washing, concentration of FGT secretions, and potential changes over the menstrual cycle [204]. To ensure accurate comparisons in longitudinal studies, involving samples from the same individual, or between different individuals, normalization and standardization are imperative. A potential normalization parameter for HPV-specific IgG in FVU is the total amount of IgG present in a FVU sample. However, since no validated total human IgG quantification assay exists for FVU, we conducted a comparison between the bead-based BioPlex Pro™ Human Isotyping Assay (Bio-Rad, USA) and the Homogeneous Time-Resolved Fluorescence (HTRF®) assay (PerkinElmer, Waltham, MA, USA) [49,51].

2 Methods

2.1 Sample collection and processing

In this study, a total of 225 paired FVU and blood samples were used, originating from two cohorts of female volunteers (Figure 1). The first cohort, referred to as the single timepoint cohort (ST cohort), comprised 54 female volunteers of which 17 unvaccinated and 37 vaccinated with either a bivalent (2vHPV) or quadrivalent (4vHPV) HPV vaccine (NCT02714114). From each participant paired FVU and serum samples were obtained, resulting in a total of 54 paired samples. The FVU samples were self-collected, while the serum samples were taken by a clinician. The second cohort, referred to as the multiple timepoint cohort (MT cohort), consisted of 57 female volunteers, all of whom collected paired FVU and serum samples at three different time points: before vaccination, 7 months after initial 9vHPV vaccination, and approximately 43 months (3.5 years) after initial 9vHPV vaccination (NCT03542227). This resulted in a total of 171 paired samples. Informed consent was obtained from all volunteers and data was coded to ensure privacy of the participants.

All FVU samples were self-collected by the volunteers using a 20 ml Colli-Pee™ collection device (Novosanis, Wijnegem, Belgium). Participants were instructed not to thoroughly wash their genitals and not to urinate for at least 1 h before FVU sample collection. For MT cohort samples, the device was pre-filled with 6.33 ml urine conservation medium (UCM, Novosanis, Wijnegem, Belgium) whereas for the ST cohort, 20 ml FVU samples were collected without preservative. After collection, all samples were immediately placed on ice or refrigerated at 4 °C before being aliquoted and stored at − 80 °C until further pre-analytical processing and testing. The median time span between FVU collection and aliquot storage at − 80 °C was 3 h 50 min (IQR: 0:22–5:19). At all timepoints in each cohort, a paired 10 ml blood sample was collected from each participant using BD Vacutainer serum tubes (Becton-Dickinson, Benelux). The blood samples were allowed to clot for 30–60 min and centrifuged at 1000 × *g* for 10 min at 20 °C. The resulting serum was collected, aliquoted, and stored at − 80 °C until further testing. The median time span between blood collection and aliquot storage at − 80 °C was 58 min (IQR: 0:49–1:05).

2.2 Pre-analytical processing

FVU samples were concentrated prior to antibody testing. Aliquots of ST cohort samples were thawed and diluted with UCM in a 1:2 ratio (UCM:FVU) before processing. For MT cohort samples, UCM was integrated during the collection process. Subsequently, 4 ml buffered aliquots from each cohort were subjected to centrifugation at 3820 × *g* for 10 min at 20–21 °C using an Amicon Ultra-4 50K filter. The concentrate on the Amicon filter was diluted with dPBS (Dulbecco's phosphate buffered saline, Gibco, United Kingdom) to achieve a final volume of 500 µl. This concentrate was stored at – 80 °C until testing.

2.3 HPV(16)-specific IgG detection

2.3.1 M4- and M9ELISA

M4- and M9ELISA testing protocols and corresponding results for ST cohort and MT cohort samples, respectively, have been described previously [50,51]. Antibodies in paired FVU and serum samples were quantified using these multi-spot Virus-Like Particle (VLP)-based assays, as described by Panicker et al. [164,166]. Specifically, ST cohort samples underwent testing with the M4ELISA, assessing HPV type-specific antibody responses to the HPV types included in the 4vHPV vaccine (HPV6, 11, 16, 18). MT cohort samples were subjected to the M9ELISA, which identifies HPV type-specific antibody responses to the HPV types included in the 9vHPV vaccine (4vHPV types plus HPV31, 33, 45, 52, 58). HPV-specific antibody concentrations for all included types were reported in arbitrary units/ml (AU/ml) except HPV16 and 18, which were reported in international units (IU/ml). Arbitrary units were used for all types except HPV16 and 18 because of the absence of an international reference for these types at the time of reporting. The HPV16 (05–134) and HPV18 IU standards (10–140) were obtained from the National Institute for Biological Standards and Controls. Only HPV16-IgG results were used in this comparison study. All concentrations were calculated using the parallel line (PLL) method, described in the World Health Organization (WHO) HPV Labnet Manual 2009 [207]. Notably, for HPV16-IgG concentrations measured by the M9ELISA for the MT cohort, the Relative Light Unit (RLU) signal of a bovine serum albumin (BSA) spot was subtracted before PLL calculation. For all other samples, the raw RLUs were employed. For serum samples, the lower limit of quantification (LLOQ) was employed as described previously (LLOQ = 0.3 IU/ml) [51]. For FVU samples, cut-offs were calculated using the mean plus three standard

deviations of the unvaccinated samples of the ST cohort for the M4ELISA (cut-off = 0.0048 IU/ml) and of the MT cohort for the M9ELISA (cut-off = 0.0021 IU/ml).

2.3.2 GST-L1-MIA

Results of the GST-L1-MIA were obtained from all ST cohort samples in a previous study [49,50]. For this analysis, FVU samples underwent a 1:4 dilution, while serum samples were diluted 1:100 before testing. The HPV16-specific antibody results were expressed as Mean Fluorescence Intensity (MFI) values, derived from the analysis of at least 1000 beads per bead set. Cut-offs were determined by calculating the mean plus three standard deviations of the unvaccinated samples. Specifically, for FVU samples, the cut-off was set at 75.8 MFI, while for serum samples, the cut-off was established at 9580.8 MFI.

2.3.3 HPV16-DELFIA

In this study, all paired FVU and serum samples from cohorts 1 and 2 underwent additional testing using the in-house developed HPV16-DELFIA assay. Yellow 96-well DELFIA plates (Revvity, Waltham, MA, USA) were coated with 100 µl/well of 0.5 µg/ml in-house produced HPV16 pseudovirions (PsV), produced following the protocol by Buck et al., 2005 [236]. After an overnight incubation at 4 °C, the plates were blocked by adding 1 × dPBS + 1% DTPA-purified BSA (Revvity, Waltham, MA, USA) to each well and incubated at room temperature (RT) on a plate shaker (300 rpm) for at least 1 h. Subsequently, 100 µl of four serial 1:2 dilutions of serum and FVU samples were added to the wells, starting from 1:400 for serum and 1:1 for FVU. Negative control wells contained only assay buffer. After three washes, Europium-anti-human antibodies, specific for the Fc-part of human IgG, were added to bind to the HPV16-specific antibodies bound to the coated PsV. Following a 60–min incubation at RT and 300 rpm, the plates underwent six washes, and enhancement solution (Revvity, Waltham, MA, USA) was added. The enhancement solution was incubated for at least 30 min, and plates were read using the Victor Nivo multimode plate reader with TRF settings (PerkinElmer, Waltham, MA, USA). Concentrations were calculated using the PLL method. HPV16-specific antibody concentrations were reported in international units (IU/ml) by using the HPV16 (05–134) standard obtained from the National Institute for Biological Standards and Controls.

LLOQ values for both sample types were generated using a dilution curve. For FVU samples, the LLOQ was defined as the lowest concentration where the coefficient of variation (%CV) of counts was <15%, and accuracy was between 80 and 120% (LLOQ =

0.0026 IU/ml). For serum, the LLOQ was determined using the formula $10*SD_{intercept}$/slope of the standard calibration curve (LLOQ = 0.0325 IU/ml).

2.4 Total human IgG detection

2.4.1 Bio-Rad BioPlex Luminex assay

Total human IgG results using the BioPlex Pro™ Human Isotyping Assay (Bio-Rad, USA) were obtained previously for both serum and FVU samples [49,51]. Briefly, 50 µl of pre-processed, diluted FVU (1:128; 1:256; 1:512) or serum (1:40,000) was mixed with captured antibodies coupled to fluorescent-labelled, magnetic polystyrene beads, detecting total human IgG using the LX200 platform (Luminex, Austin, Texas, USA). Antibody concentrations (µg/ml) were quantitated from the median fluorescence intensity (MFI) values using a five-parameter logistic regression, within a working range of 0.003–30.27 µg/ml. Total human IgG in FVU was reported as the average of three dilution-corrected concentrations.

2.4.2 HTRF homogenous assay

In this study, total human IgG concentrations were additionally assessed using the Homogeneous Time-Resolved Fluorescence (HTRF) assay (Revvity, Waltham, MA, USA), following the manufacturer's instructions. The FVU samples were diluted 1:300 and the serum samples 1:500,000. Measurements were conducted with the Victor Nivo multimode plate reader (Revvity, Waltham, MA, USA) at wavelengths 665 nm and 620 nm. The analysis was performed using Graphpad Prism version 10.0.3 (Dotmatics, Boston, MA, USA). A four-parameter logistic equation was used to calculate antibody concentrations based on the ratio of 665 nm and 620 nm fluorescence emission values. The reported total IgG concentrations represent the average of two dilution-corrected concentrations.

2.5 Statistical analysis

All analyses were performed using R statistical software version 4.3.1 (packages: tidyverse, readxl, ggbeeswarm, ggpubr, RColorBrewer, ROCR, cutpointr). Data were tested for normality using the Shapiro-Wilk test. Since all data was not normally distributed, a non-parametric Wilcoxon signed-rank test was performed to determine significant differences

between antibody titers between assays and between sample types. We used the Spearman rank test to calculate the correlation between the different assays or sample types. Statistical significance was defined as p-adjusted < 0.05 (using Holm-Bonferroni method for p-value adjustment). Figures were made using antibody titers or $\log_{10}(x)$ transformed data where zero values were assigned as 0.0001.

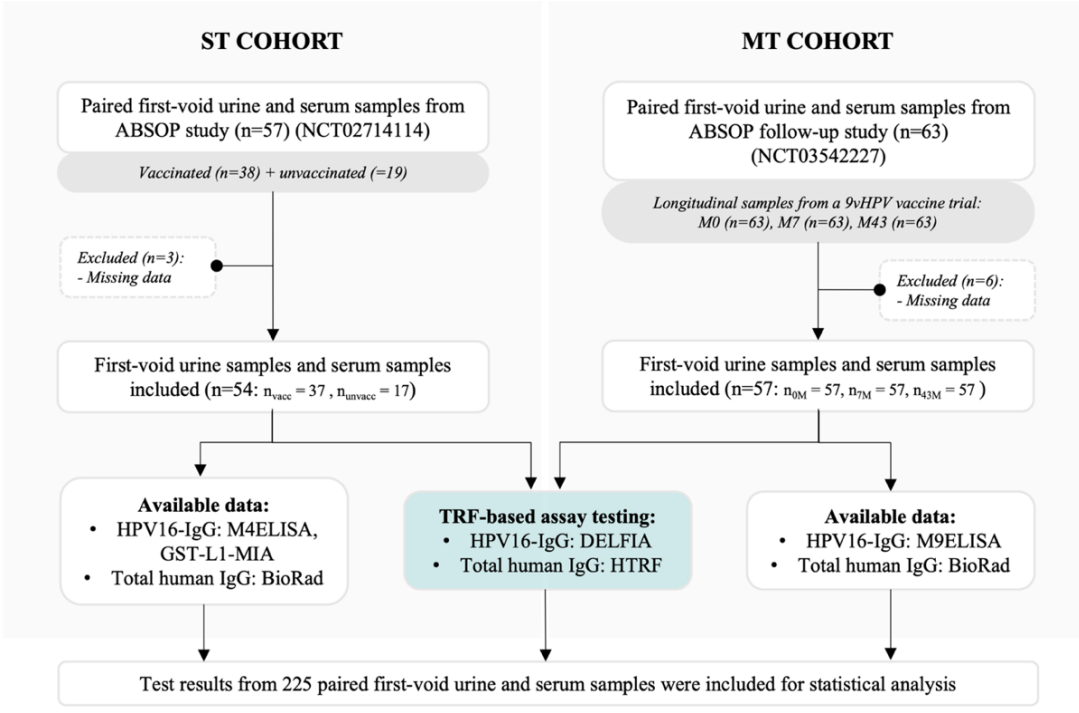


Figure 1: *Flow diagram of the study. This study included 54 paired FVU and serum samples from the ST cohort, along with 171 paired samples from the MT cohort. The samples underwent testing for HPV16-IgG utilizing the HPV16 DELFIA assay, alongside assessment for total human IgG using the HTRF assay. Comparative analyses for HPV16-IgG results were done between DELFIA and M4ELISA, as well as GST-L1-MIA results for the ST cohort. For the MT cohort, the comparisons were made between DELFIA and M9ELISA HPV16-IgG results. Additionally, the HTRF-generated total human IgG results were compared to previously generated results with the Bio-Rad.*

3 Results

3.1 Population characteristics

Data from paired FVU and serum samples of 54 participants in the ST cohort and 57 participants in the MT cohort were included for statistical analysis (Figure 1). The median

age at enrollment was 22 years (IQR: 20–24) for the ST cohort and 35 years (IQR: 27–41) for the MT cohort.

In the ST cohort, out of the 54 female volunteers, 37 were vaccinated, and 17 were unvaccinated. All vaccinated women had received three doses of an HPV vaccine, with 32/37 receiving the 4vHPV vaccine, 4/37 the 2vHPV vaccine, and 1/37 a combination of both. The vaccination status of these female volunteers was self-reported. For the ST cohort, paired FVU and serum samples collected at a single timepoint were included for analysis (n = 54). Median time between having received a first vaccine dose and collection of the samples is 7 years (IQR: 5–9).

All participants in the MT cohort received three doses of the 9vHPV vaccine in total, i.e. one dose at timepoints 0M, 1M, and 6M within the HPV V503-004 study (EudraCT NUMBER: 2015–005093–38). For the MT cohort, paired FVU and serum samples collected before vaccination, and at 7M and 43M after initial 9vHPV vaccination, were included for analysis (n = 171).

3.2 Evaluation of HPV16-IgG detection using novel TRF assay

Before testing all samples, we established the LLOQ for the DELFIA assay on Amicon-filtered FVU samples and serum samples through a triplicate dilution series (Appendix Figure 1). For FVU, the LLOQ was determined by identifying the lowest concentration where the %CV among the three measurements (counts) was below 15%, and the recovery fell within the range of 80 to 120%. For serum, the LLOQ was calculated using the formula $10*SD_{intercept}$/slope of the calibration curve. Additionally, the %CV of the negative control wells across the tested plates was 13.38%, indicating robust assay reproducibility. Furthermore, the %CV of the control samples was 29.06%, which is considered acceptable.

HPV16-specific antibody detection using the TRF-based DELFIA assay was performed on all samples from the ST cohort and the MT cohort (Table 1). For FVU samples, 40/54 (74%) ST cohort samples and 118/171 (69%) MT cohort samples had detectable antibody concentrations. Among the vaccinated individuals in the ST cohort, 33/37 (89%) had detectable antibody concentrations, while in the MT cohort, samples collected at the two time points post-vaccination, at 7M and 43M after receiving the first 9vHPV vaccine dose, 107/114 (94%) tested positive for HPV16-IgG. The median (IQR) HPV16-IgG

concentrations for FVU samples from vaccinated volunteers were 0.03 (0.01–0.13) IU/ml for the ST cohort and 0.04 (0.02–0.18) IU/ml for the MT cohort. The geometric mean titer (GMT) for all ST cohort HPV16-IgG positive FVU samples was 0.33 (95% CI 0.01–0.65) IU/ml, while the GMT for MT cohort FVU samples was 0.28 (95% CI 0.15–0.41) IU/ml. Among FVU samples from unvaccinated volunteers or collected before vaccination, 7/17 (41%) ST cohort samples and 11/57 (19%) MT cohort samples were HPV16-IgG positive. The geometric mean titer (GMT) for all HPV16-IgG positive ST cohort unvaccinated FVU samples was 0.02 (95% CI 0.00–0.04) IU/ml, while the GMT for MT cohort FVU samples was 0.05 (95% CI (–0.02) –0.13) IU/ml.

For serum samples, 42/54 (78%) ST cohort samples and 119/171 (70%) MT cohort samples had detectable antibody concentrations. Among the vaccinated individuals in the ST cohort, 36/37 (97%) had detectable antibody concentrations while in the MT cohort, samples collected at the two time points post-vaccination showed 113/114 (99%) testing positive for HPV16-IgG. The total median (IQR) HPV16-IgG concentrations for serum were 130.48 (61.37–202.45) IU/ml for the ST cohort and 126.05 (27.41–437.94) IU/ml for the MT cohort. The geometric mean titer (GMT) for all ST cohort HPV16-IgG positive serum samples was 196.07 (95% CI 125.78–266.35) IU/ml, while the GMT for MT cohort serum samples was 319.98 (95% CI 240.56–399.40) IU/ml. Among serum samples from unvaccinated volunteers or collected before vaccination, 6/17 (35%) ST cohort samples and 6/57 (11%) MT cohort samples were HPV16-IgG positive. The geometric mean titer (GMT) for all HPV16-IgG positive ST cohort unvaccinated serum samples was 54.84 (95% CI 42.43–67.25) IU/ml, while the GMT for MT cohort serum samples was 63.66 (95% CI 31.11–96.20).

Comparing HPV16-specific median antibody levels in FVU to those in serum measured by DELFIA, the ratios of FVU to serum levels were 0.03% for the ST cohort and 0.05% for the MT cohort. Significant differences were found between median antibody levels based on vaccination status in both FVU and serum for both cohorts ($p < 0.001$). Significant Spearman ank correlation coefficients were obtained between paired FVU and serum samples for both cohorts ($r_s \geq 0.76$, $p < 0.001$) (Figure 2A).

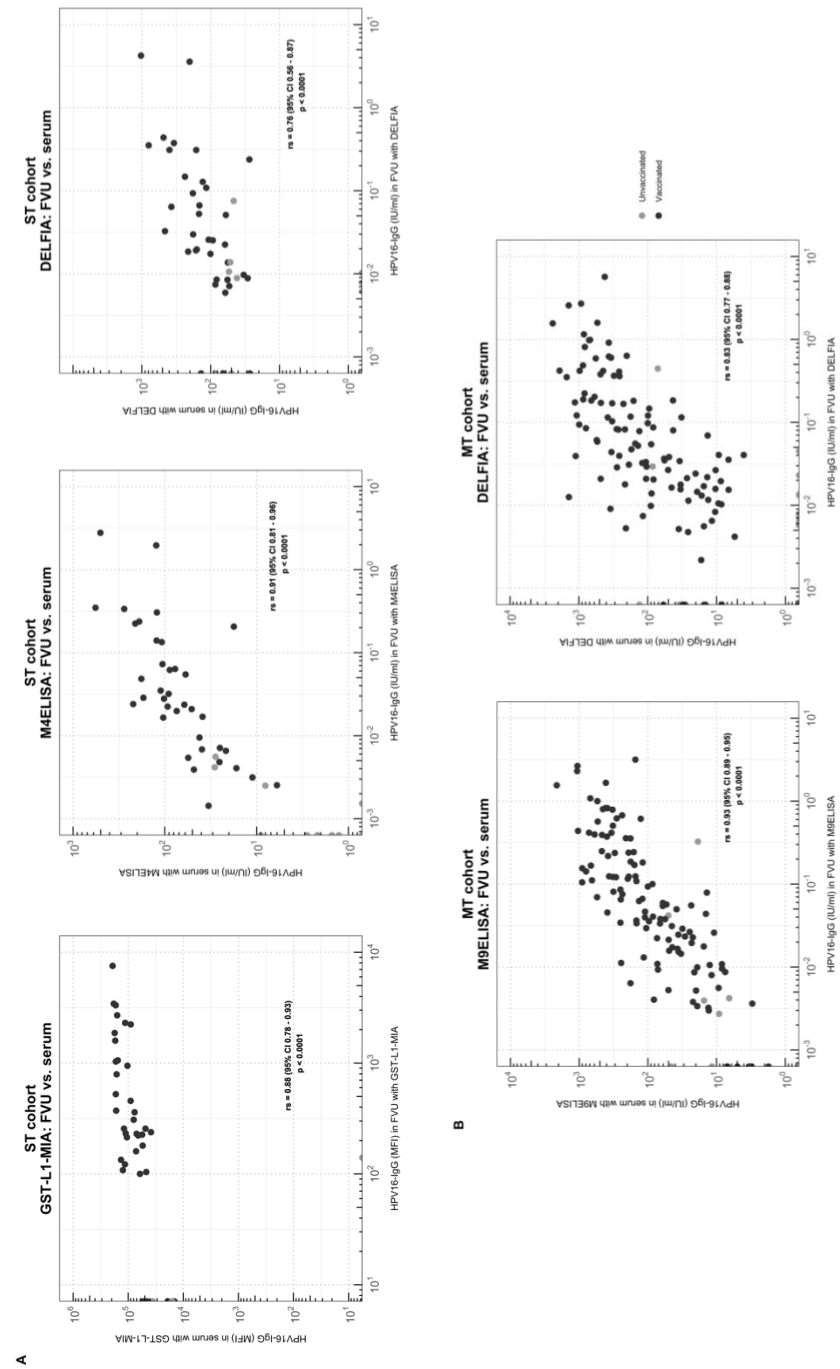


Figure 2: *Comparison between FVU and serum antibody results using the (A) GST-L1-MIA, M4ELISA and DELFIA for the ST cohort, and the (B) DELFIA and M9ELISA for the MT cohort. Spearman rank correlation coefficients (95% CI) are presented in the figure. Black dots represent the vaccinated volunteers, gray dots represent the unvaccinated volunteers.*

Table 1: *HPV16-IgG detection (n, %) and concentrations (median, IQR) for all FVU and serum samples from both cohorts using the GST-L1-MIA, M4ELISA and DELFIA for the ST cohort and the M9ELISA and DELFIA for the MT cohort. Concentrations are reported in IU/ml for M4-M9ELISA and DELFIA and in MFI for GST-L1-MIA. FVU/serum ratio are antibody levels in first-void urine (FVU) divided by serum levels, denoted as the median %, plus IQR. P-values (Wilcoxon signed rank test) report significant differences between median antibody yield between vaccinated and unvaccinated women for FVU or serum.*

ALL SAMPLES						
		FVU		Serum		FVU/Serum % (IQR) ratio
	Assay	Antibody detection	Median (IQR)	Antibody detection	Median (IQR)	
ST cohort						
	GST-L1-MIA (MFI)	33/54 (61%)	625 (0 - 150)	38/54 (70%)	58,575 (0 - 118,262)	0.33 (0.17 - 0.67)
	M4ELISA (IU/ml)	41/54 (76%)	0.01 (0.00-0.05)	46/54 (85%)	40.65 (6.51-105.50)	0.03 (0.02-0.07)
	DELFIA (IU/ml)	40/54 (74%)	0.01 (0.00-0.07)	42/54 (78%)	63.56 (27.79-161.45)	0.03 (0.01-0.07)
MT cohort						
	M9ELISA (IU/ml)	114/171 (67%)	0.02 (0.00 - 0.11)	124/171 (73%)	35.63 (0.00 - 184.50)	0.06 (0.03 - 0.011)
	DELFIA (IU/ml)	118/171 (69%)	0.02 (0.00 - 0.09)	119/171 (70%)	33.27 (0.00 - 259.29)	0.05 (0.02 - 0.12)
FVU SAMPLES						
		Vaccinated		Unvaccinated		*p*-value
	Assay	Antibody detection	Median (IQR)	Antibody detection	Median (IQR)	
ST cohort						
	GST-L1-MIA (MFI)	32/37 (86%)	256 (134 - 1,032)	1/17 (6%)	0 (0 - 0)	< 0.001
	M4ELISA (IU/ml)	37/37 (100%)	0.03 (0.01 - 0.13)	4/17 (24%)	0.00 (0.00-0.00)	< 0.001
	DELFIA (IU/ml)	33/37 (89%)	0.03 (0.01 - 0.13)	7/17 (41%)	0.00 (0.00 - 0.01)	< 0.001
MT cohort						
	M9ELISA (IU/ml)	109/114 (96%)	0.05 (0.02 - 0.21)	5/57 (9%)	0.00 (0.00 - 0.00)	< 0.001
	DELFIA (IU/ml)	107/114 (94%)	0.04 (0.02 - 0.18)	11/57 (19%)	0.00 (0.00 - 0.00)	< 0.001
SERUM SAMPLES						
		Vaccinated		Unvaccinated		*p*-value
	Assay	Antibody detection	Median (IQR)	Antibody detection	Median (IQR)	
ST cohort						
	GST-L1-MIA (MFI)	36/37 (97%)	103,375 (55,925 - 158,675)	2/17 (12%)	0 (0 - 0)	< 0.001
	M4ELISA (IU/ml)	37/37 (100%)	88.40 (39.40 - 123.00)	9/17 (53%)	0.92 (0.00 - 2.83)	< 0.001
	DELFIA (IU/ml)	36/37 (97%)	130.48 (61.37 - 202.45)	6/17 (35%)	0.00 (0.00 - 46.31)	< 0.001
MT cohort						
	M9ELISA (IU/ml)	114/114 (100%)	116.96 (31.79 - 320.75)	10/57 (18%)	0.00 (0.00 - 0.00)	< 0.001
	DELFIA (IU/ml)	113/114 (99%)	126.05 (27.41 - 437.94)	6/57 (11%)	0.00 (0.00 - 0.00)	< 0.001

3.3 Comparison between HPV16-IgG immunoassays

HPV16 IgG results using the DELFIA assay were compared to previously obtained results of the M4ELISA and GST-L1-MIA for the ST cohort and to M9ELISA for the MT cohort. Antibody positivity in samples collected from all vaccinated female volunteers was comparable between all three assays. Specifically, for the vaccinated volunteers, the M4ELISA yielded the most HPV16-IgG positive FVU samples for the ST cohort (37/37, 100%), while this was the M9ELISA for the MT cohort (109/114, 96%). GST-L1-MIA detected HPV16-specific antibodies in 32/37 (86%) and 36/37 (97%) of vaccinated FVU and serum samples, respectively. For the unvaccinated female volunteers, larger variations in antibody positivity were observed. The DELFIA provided the highest FVU positivity for both cohorts (≥ 19%), while the M4- or M9ELISA yielded the highest serum positivity (≥ 18%) for both cohorts (Table 1). GST-L1-MIA detected HPV16-specific IgG in 1/17 (6%) unvaccinated FVU samples and in 2/17 (12%) unvaccinated serum samples. Good to excellent correlations between FVU and serum samples were obtained for all assays ($r_s \geq 0.76$, $p < 0.001$) (Figure 2A and 3A). Additionally, the ratio of HPV16-specific antibody concentrations in FVU compared to serum in the ST cohort was 0.03% for both the M4ELISA and the DELFIA. For the MT cohort, the FVU-serum ratio was 0.06% for the M9ELISA and 0.05% for the DELFIA. In the ST cohort, Spearman Rank correlations between the assays ranged from 0.80 to 0.87 for FVU and from 0.89 to 0.95 for serum (Figure 3A-C). The Spearman rank correlation coefficients between the DELFIA and the M9ELISA for the MT cohort were 0.92 for FVU and 0.97 for serum (Figure 3B-D).

For the MT cohort, the antibody concentrations for vaccinated and unvaccinated volunteers were plotted alongside the antibody concentrations at the three different timepoints, using both assays and for both sample types (Figure 4). The results underscore the usability of both assays in monitoring vaccine induced HPV16 humoral immune response.

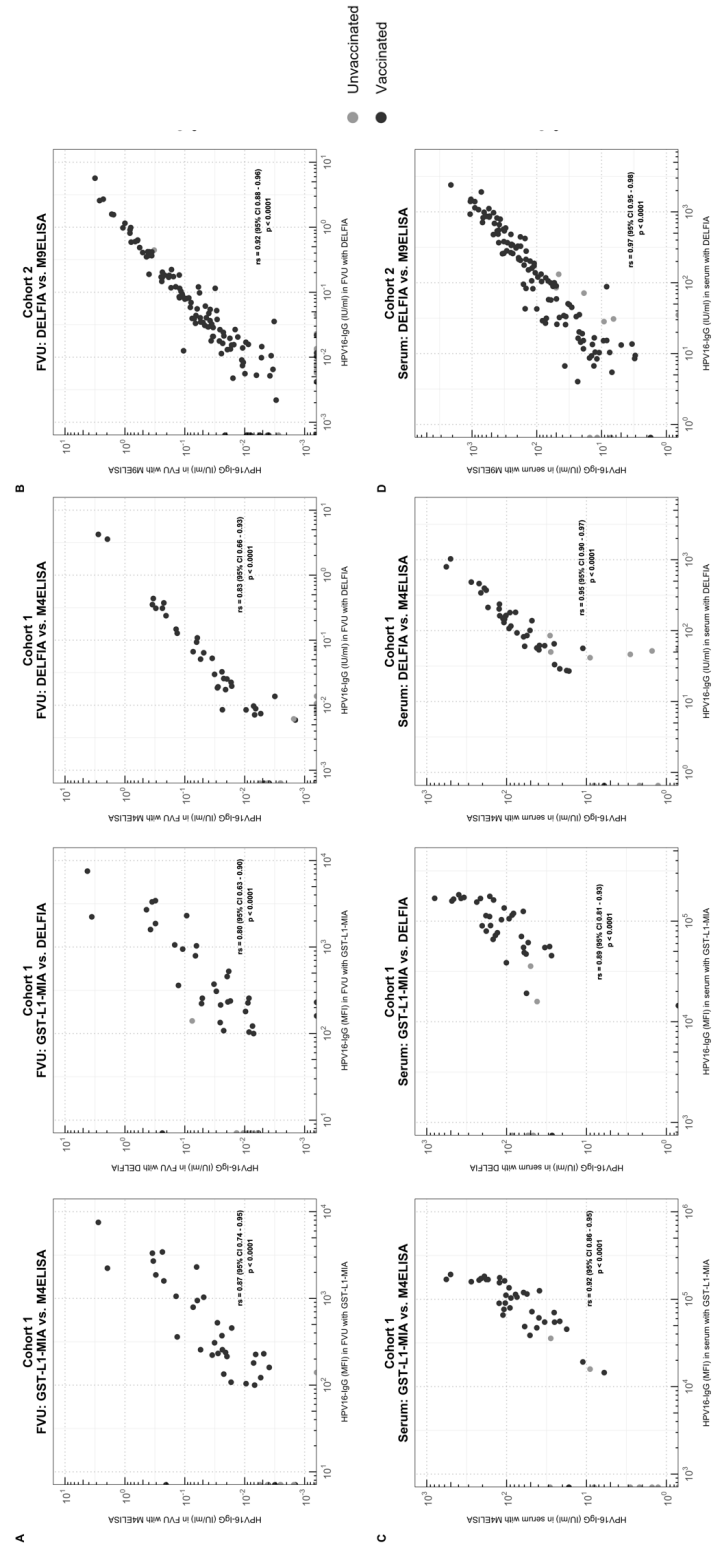


Figure 3: *Correlations between assays. Comparison between GST-L1-MIA, M4ELISA and DELFIA HPV16-IgG results for (A) FVU samples and (C) serum samples from the ST cohort. Comparison between M9ELISA and DELFIA HPV16-IgG results for (B) FVU samples and (D) serum samples from the MT cohort. Spearman rank correlation coefficients (95% CI) are presented in the figure. Black dots represent the vaccinated volunteers, gray dots represent the unvaccinated volunteers.*

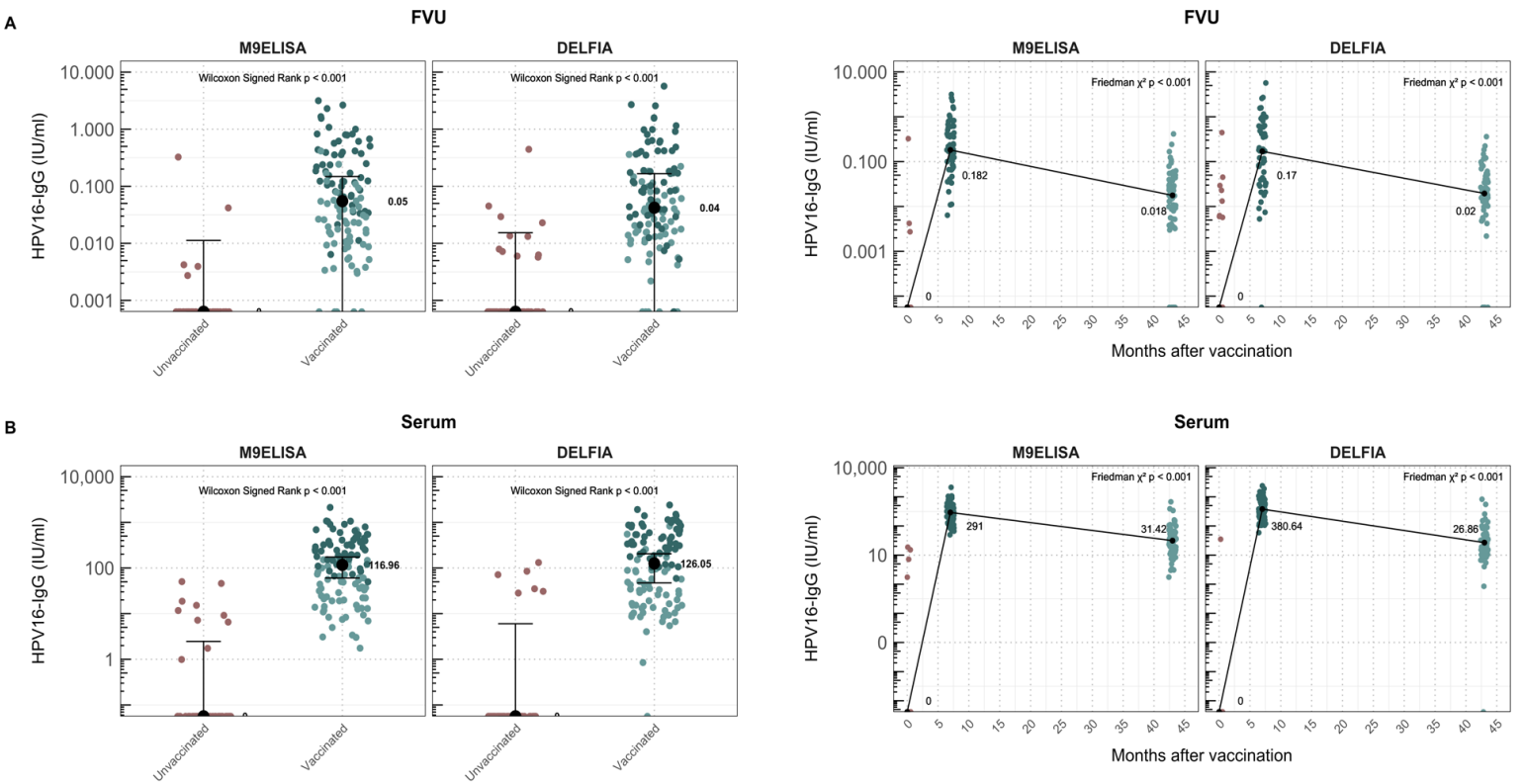


Figure 4: *M9ELISA and DELFIA HPV16-IgG concentrations for unvaccinated and fully 9vHPV vaccinated samples or at all three different timepoints of the MT cohort. Results are presented for (A) FVU and (B) serum samples. The median antibody concentrations are shown in the figure. Wilcoxon Singed Rank test results are reported between median antibody levels in unvaccinated and vaccinated samples. Friedman test results are reported for repeated-measures analysis between all different time points. Pink dots represent the results at 0M timepoints, dark blue dots at 7M and light blue dots at 43M.*

3.4 Comparison between Bio-Rad and HTRF total IgG assay

In addition to measuring HPV16-IgG concentrations, we quantified the total human IgG concentrations in all samples. The HTRF results were compared to the Bio-Rad total IgG concentrations for all samples (Figure 5). In FVU samples, a moderate correlation between total IgG concentrations was observed for both cohorts, being 0.50 ($p < 0.0001$) and 0.42 ($p < 0.0001$) for the ST and the MT cohort, respectively. However, for serum samples, only a weak correlation was observed between Bio-Rad and HTRF total IgG concentrations in the ST cohort ($r_s = 0.28$, $p = 0.04$), and for the MT cohort serum samples, no significant correlation was observed ($p = 0.34$).

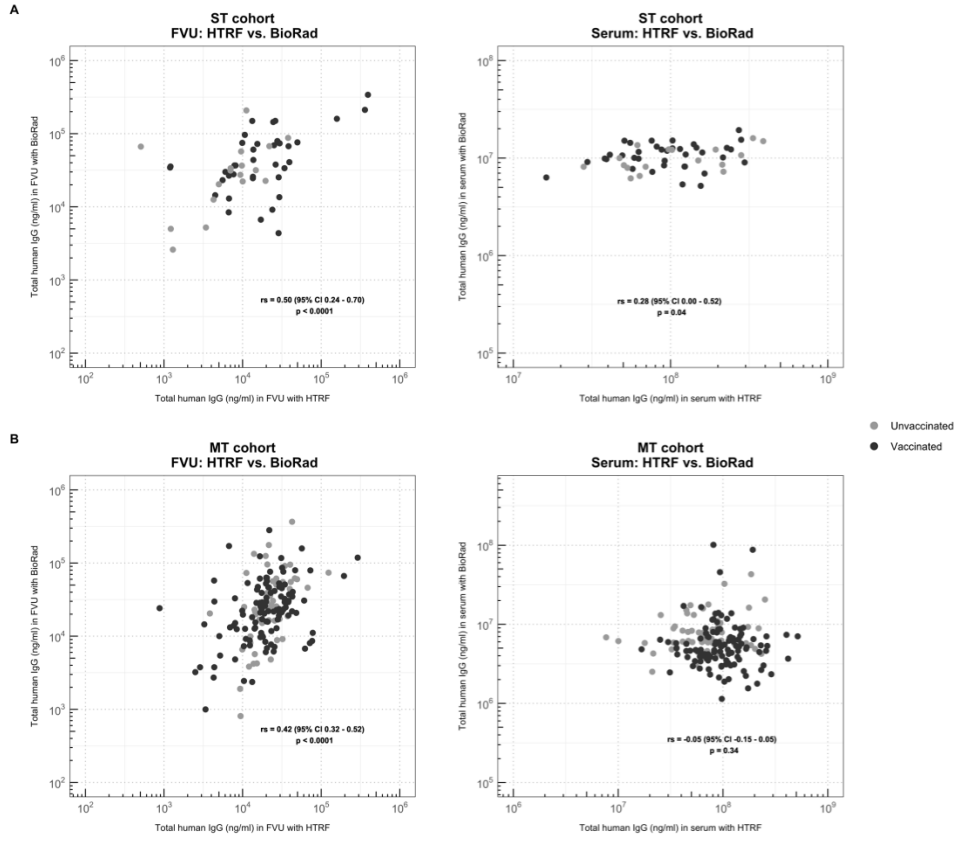


Figure 5: *Comparison between HTRF and Bio-Rad total human IgG results for (A) all ST cohort samples and (B) all MT cohort samples. Spearman rank correlation coefficients (95% CI) are presented in the figure. Black dots represent the vaccinated volunteers, gray dots represent the unvaccinated volunteers.*

4 Discussion

The use of FVU for the detection of HPV-specific antibodies offers several advantages. Other than being non-invasive, easy-to-collect, and giving the option for at-home self-sampling, this sample is particularly interesting in vaccine- and epidemiological trials where lost-to-follow-up and cost are limiting factors.

Using FVU as a non-invasive sample of the urogenital tract has already provided promising results for follow-up of HPV-related biomarkers [51]. Humoral immune responses, specifically HPV-specific antibodies, have demonstrated to be present and detectable in FVU [33,49–51]. As expected, and in line with previous research, antibody concentrations after vaccination were considerably lower in FVU than those in serum, encompassing less than 1% of HPV16-specific median antibody levels in serum [49–51]. To be able to adequately define this HPV-specific antibody response in FVU, it is essential to identify assays capable of effectively monitoring these lower-level antibody concentrations.

This study evaluated the performance of a newly developed HPV16 DELFIA assay for the detection and quantification of HPV16-specific antibodies in FVU and serum. A total of 225 paired FVU and serum samples from two different cohorts of female volunteers were tested using the HPV16 DELFIA assay. Results were compared to previously reported M4ELISA and GST-L1-MIA results for the ST cohort (n = 54) or M9ELISA results for the MT cohort (n = 171) [49–51].

Very strong correlations between DELFIA and M4ELISA, M9ELISA, or GST-L1-MIA for both sample types ($r_s > 0.80$) were found. In addition, the Spearman rank correlation between FVU and serum for the HPV16 DELFIA was strong ($r_s = 0.76$) to very strong ($r_s = 0.83$) for the ST cohort and the MT cohort, respectively.

Overall, the DELFIA had high sensitivity in both FVU and serum samples, 93% and 99%, respectively. The sensitivity was determined using all results from vaccinated female volunteers and based on the vaccination status vs. the observed HPV16-specific antibody concentration. This was done using the formula: true positive/(true positive + false negative). GST-L1-MIA had a sensitivity of 86% for FVU, while the two VLP-based assays had a sensitivity of 96% (M9ELISA) and 100% (M4ELISA). For serum, the sensitivity of the immunoassays ranged from 97% (GST-L1-MIA) to 100% (M4ELISA/M9ELISA). Based on these results we can conclude that for detection of vaccine-induced HPV16-

specific antibodies present in FVU, DELFIA is more sensitive than GST-L1-MIA (>86%), and less sensitive than M9ELISA (<96%) and M4ELISA (<100%). For vaccine-induced antibodies in serum samples, DELFIA is more sensitive than GST-L1-MIA (>97%) and less sensitive than both VLP-based ELISAs (<100%).

For FVU samples, the number of samples with a detectable HPV16-IgG before vaccination was highest using the DELFIA assay in both cohorts. While this might suggest greater sensitivity in identifying naturally induced antibodies, there is only an average agreement with corresponding serum samples or FVU samples testing positive with other immunoassays. In the ST cohort, DELFIA detected HPV16-specific antibodies in 7/17 unvaccinated FVU samples and 6/17 unvaccinated serum samples. Two participants showed exclusive HPV16-IgG positivity in FVU using the DELFIA assay. For the MT cohort, eight samples showed exclusive HPV16-specific antibody positivity with the DELFIA assay (Appendix table 1). Results reported by Gaudet et al. suggest that humoral immunity in the cervix is comprised of a local and unique spectrum of B-cells [237]. When natural HPV infection occurs, a local immune response is elicited. The local environment could thus have a presence of Ig-expressing B-cells, which is what the DELFIA might have detected in the unvaccinated FVU samples. Moreover, a study by Scherer et al. suggests that natural HPV infection elicits a low-avidity and less qualitative serum antibody response than HPV vaccination [238], which might also be the case for locally-produced antibodies after natural infection. Thus, antibodies that are locally produced after natural infection potentially have a low avidity, for which DELFIA could prove sufficiently sensitive for detection. These hypotheses may explain the average agreement ($r_s < 0.52$) between sample types in the non-vaccinated cohort. It will be interesting to further explore this in the future.

For serum samples, the overall performance of the DELFIA assay was comparable to that of M4- or M9ELISA, showing average to good agreement between HPV16-IgG positive samples. Notably, the sensitivity for naturally induced antibody detection in serum appeared to be lower when using the GST-L1-MIA. These results showed that the developed DELFIA assay can accurately be used for the detection of HPV16-specific antibodies in both FVU and serum samples.

As mentioned previously, the number of samples with a detectable HPV16-IgG before vaccination was highest using the DELFIA assay in both cohorts for FVU samples. While the limited overlap with corresponding serum samples may indicate presence or detection

of locally produced antibodies after natural infection, it might also be caused by aspecific binding to the DELFIA assay plate. Aspecific binding could be overcome by an extra purification step for the PsV coating antigens, for example by gradient removal, or by adding additional controls to the assay. These steps would benefit the sensitivity as well because lower results would be expected to be detected in negative control wells. Furthermore, there are two FVU and two serum samples that had a concentration near the LLOQ we described for this assay. This possible non-specific binding in the DELFIA assay might be the result of Europium contamination and must be further investigated. In this study, we have used the DELFIA as a singleplex assay. Nevertheless, this technique has the potential to be multiplexed to create a competitive multiplex HPV-specific DELFIA using other lanthanide chelates (Europium, Samarium, Terbium, and Dysprosium).

The DELFIA principle uses a TRF-based readout from lanthanide fluorescence, while M4/M9ELISA employs electrochemiluminescence technology, and GST-L1-MIA is a bead-based assay that uses suspension array technology [164–166,217]. The performance of the three ELISA assays (DELFIA, M4ELISA, M9ELISA) is expected to be comparable, being direct ELISAs. However, a major benefit to the use of lanthanides is the delay in measurement between excitation and emission, resulting in a reduction in self-quenching [217]. This should make DELFIA suitable for the detection of low-abundance analytes, where for the other assays HPV-type-specific antibodies in FVU were near the limit of the detection [49,50]. GST-L1-MIA, which employs the Luminex technology, has an intrinsic problem of direct binding of antibodies from human sera to the beads, resulting in high non-specific background [239]. Furthermore, there is a difference in used antigens between the assays. For DELFIA, plates are coated with PsV, M4- and M9ELISA use virus-like L1 and L2 particles (VLP), and GST-L1-MIA uses glutathione S-transferase HPV-L1-fusion protein antigens. The difference between PsV and VLP is minor. Both are produced by transfection of Human Embryonic Kidney 293TT cells with plasmid constructs for the desired HPV subtype. PsV, however, incorporate an additional reporter plasmid within its structure. This difference does not affect any of the outcomes of the assay set-ups, as they are based on the binding between HPV L1 and the antibodies present in the tested sample. However, GST-L1-MIA utilizes viral antigens specific for the HPV subtype, bacterially expressed as GST fusion proteins, which do not reflect the HPV capsid in its true three-dimensional conformation. This difference may result in poor or no detection of conformation-dependent neutralizing epitopes by the GST-L1-MIA.

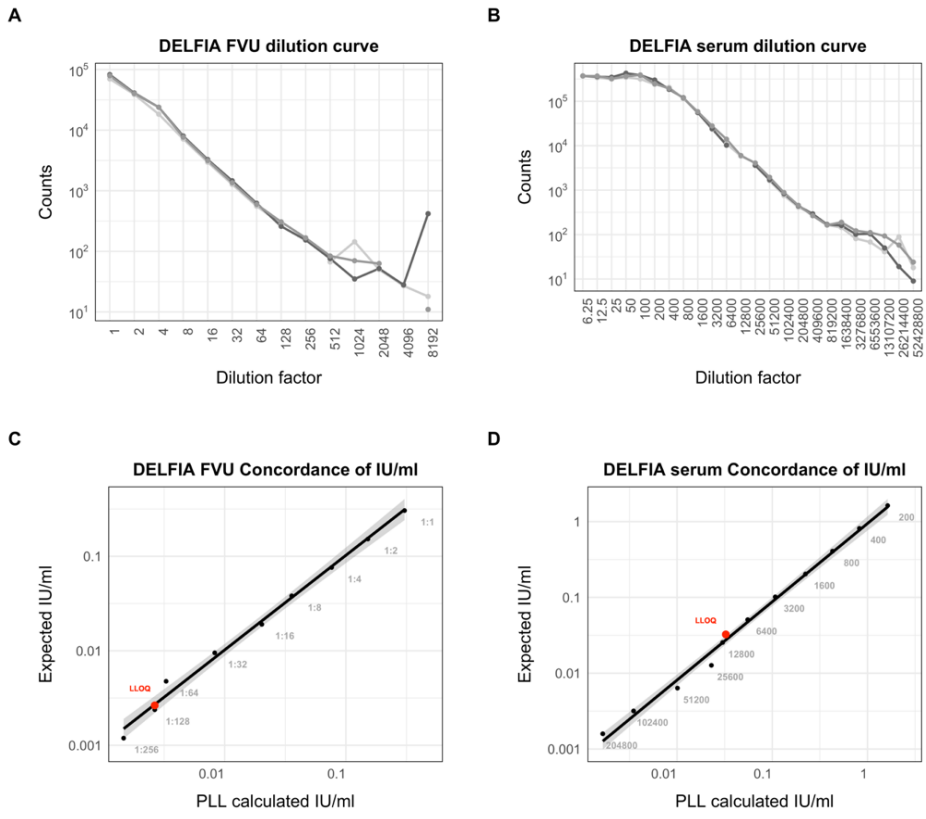
Our DELFIA assay quantifies antibody concentrations in international units, employing the established International Standards for HPV serological methods [168]. While these standards have been accessible for HPV16 and HPV18 for over a decade, they are now also available for the remaining 9vHPV vaccine types (HPV6, 11, 31, 33, 45, 52, 58). As we explore potential expansion of this assay into a multiplex format for detecting various HPV vaccine types, we will continue to use these units. The implementation of a uniform unit system among laboratories facilitates the standardization of assays, contributing to the evaluation of antibody levels in both surveillance and vaccine efficacy studies.

This study has some limitations. First, not all assays had a defined LLOQ for both FVU and serum samples. Since the previously used assays were exclusively validated for serum samples, no LLOQ was established for FVU samples. As the matrix of FVU and serum is different, separate LLOQ needs to be defined for each sample type. Where there was no predefined LLOQ, the empirical rule formula was used, which states that 99.7% of observations fall within the first three standard deviations of the mean. While the use of various cut-off values may impact the results, not using any cut-off values could lead to comparisons with nonspecific outcomes. Although the total sample size is sufficient for this comparative study, the number of samples for the ST cohort comparison is limited. Within this study, we only report on the detection of HPV16-specific antibody responses. Further development of a multiplex platform, detecting additional HPV vaccine-types, of this DELFIA assay is required for HPV vaccine impact or surveillance studies. Moreover, results of the total IgG immunoassays should be interpreted with caution, as no significant correlation between the assays was established. Further studies diving into the possible reasons for these differences must be performed to identify which assay yields the most robust total IgG concentration for both FVU and serum samples. Lastly, normalization of the HPV16 specific antibody response quantified in FVU should be further explored. Despite these limitations, we believe that the main outcomes of this study remain unaffected, and a robust assay evaluation and comparison could be made.

In conclusion, we have proven that the HPV16 DELFIA assay is a suitable immunoassay for the detection of HPV16 antibodies. Using this assay, good correlations between HPV16-IgG concentration in FVU and serum were demonstrated. Our developed DELFIA-based assay provided a higher sensitivity for HPV16-IgG detection in vaccinated female volunteers than the GST-L1-MIA and a comparable sensitivity to the M4- and M9ELISA. Interestingly, using the DELFIA assay, more FVU samples had detectable HPV16 IgG before vaccination, potentially elicited by natural infection, and produced locally. With

this study, we provide data on a novel detection system for HPV-specific antibodies, possibly able to adequately detect low antibody concentrations, as required for the analysis of HPV-related humoral immune response in FVU samples.

5 Supplementary materials



Appendix Figure 1: *DELFIA dilution curves of three replicates for A) Amicon filtered FVU and B) serum samples. The concordance between the PLL calculated IU/ml and expected IU/mL concentration (based on known start concentration and dilutions) are presented for C) FVU and D) serum samples. LLOQ values are shown as red dots.*

Appendix table 1: *Unvaccinated FVU or serum samples that have a HPV16-specific IgG titer using DELFIA. (+) indicates HPV16-specific detectable antibodies; (-) indicates no detectable HPV16-specific antibodies. For the ST cohort, two FVU samples (ID: 1.1, 1.9) are exclusively positive using DELFIA. For the MT cohort, eight FVU samples (ID: 2.3, 2.6, 2.7, 2.8, 2.9, 2.11, 2.12, 2.15) and one serum sample (ID: 2.13) are exclusively positive using DELFIA.*

HPV16-IgG positivity in unvaccinated samples						
ST cohort						
	FVU			Serum		
Sample	GST-L1-MIA	M4ELISA	DELFIA	GST-L1-MIA	M4ELISA	DELFIA
1.1	-	-	+	-	-	-
1.2	+	+	+	-	+	+
1.3	-	+	-	+	+	+
1.4	-	-	+	-	-	+
1.5	-	-	+	-	+	+
1.6	-	-	+	+	+	+
1.7	-	+	-	-	+	+
1.8	-	+	+	-	-	-
1.9	-	-	+	-	-	-
MT cohort						
	FVU			Serum		
Sample	M9ELISA	DELFIA		M9ELISA	DELFIA	
2.1	+	+		+	+	
2.2	-	+		+	-	
2.3	-	+		-	-	
2.4	+	+		+	+	
2.5	+	-		+	+	
2.6	-	+		-	-	
2.7	-	+		-	-	
2.8	-	+		-	-	
2.9	-	+		-	-	
2.10	+	-		+	+	
2.11	-	+		-	-	
2.12	-	+		-	-	
2.13	-	-		-	+	
2.14	-	-		+	+	
2.15	-	+		-	-	

CHAPTER

03-C

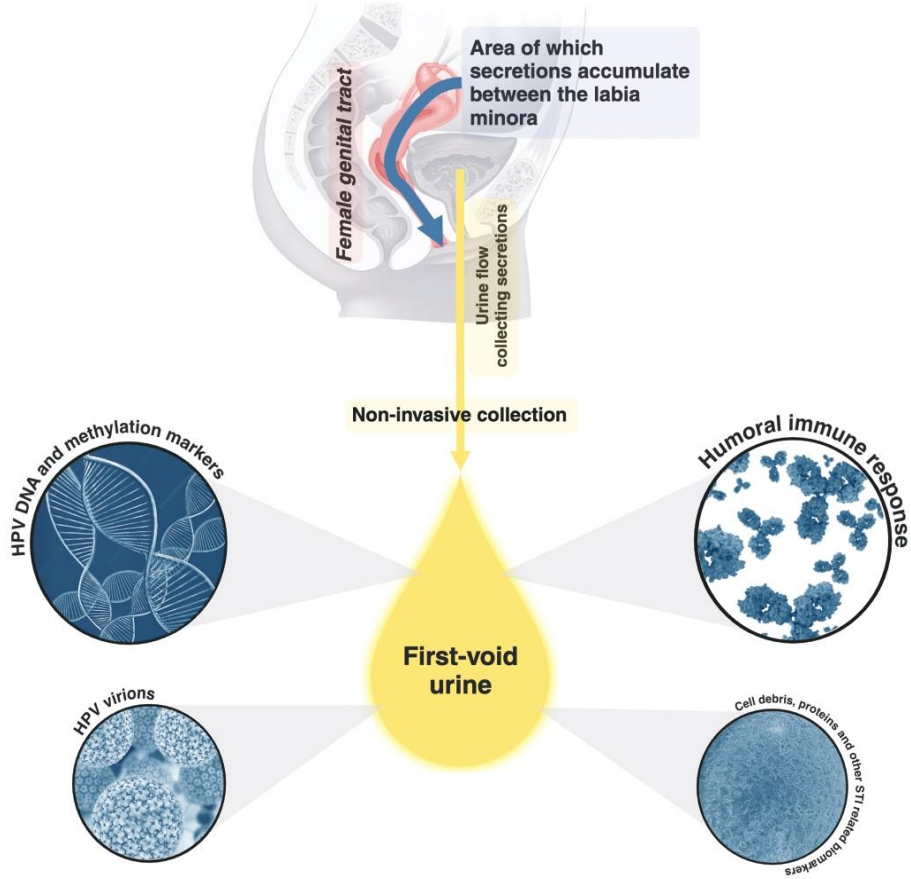
Follow-up of humoral immune response after HPV vaccination using first-void urine: A longitudinal cohort study.

Laura Téblick, Jade Pattyn, Severien Van Keer, Annemie De Smet, Ilse De Coster, Wiebren A.A. Tjalma, Ira Rajbhandari, Gitika Panicker, Elizabeth R. Ünger, and Alex Vorsters.

Published in Journal of Medical Virology, August 2023.

JOURNAL OF
MEDICAL
VIROLOGY

Volume 95, Number 10 October 2023



WILEY

ISSN 0146-6615

Cover Image, Volume 95, Number 10, October 2023

The cover image is based on the Research Article *Follow-up of humoral immune response after HPV vaccination using first-void urine: A longitudinal cohort study* by ***Téblick et al.***

Abstract

Assessment of humoral immune responses following human papillomavirus (HPV) vaccination currently relies on invasive blood sampling. This longitudinal cohort study explores the usability of first-void urine as a non-invasive alternative sample for antibody detection. In this study, 58 women receiving three doses of the 9vHPV vaccine within a Gardasil9 (9vHPV) Phase III randomized controlled trial were included. Participants provided paired first-void urine and blood samples before vaccination (M0), 1 month after the third dose (M7), and ~3 years after the third dose (M43). Type-specific antibody responses to the 9vHPV types were analyzed in 174 first-void urine and 172 serum samples using a virus-like particle-based IgG multiplex enzyme-linked immunosorbent assay. Additionally, total human IgG concentrations were determined using the BioPlex assay. At M7, 1 month after complete 9vHPV vaccination, 95%–100% of first-void urine and 100% of serum samples had detectable concentrations, varying by HPV type. At M43, 84%–100% of first-void urine and 98%–100% of serum samples had HPV-specific antibody concentrations. Results show significant Spearman rank correlations between type- specific HPV-antibody concentrations for paired first-void urine and serum at all time points. This study confirms the potential feasibility of utilizing first-void urine as a non-invasive immunological sample within HPV vaccine trials.

1 Introduction

The possibility of using a single non-invasive, home-collected sample to monitor human papillomavirus (HPV) infection and vaccination is an interesting prospect. This study focuses on investigating the potential of the initial stream of urine, defined as first-void urine, as a means to achieve this objective [171]. A first-void urine sample contains genital secretions that accumulate between the labia minora and urethra opening. These secretions can include HPV DNA, transudated antibodies, methylation markers, and other HPV-related biomarkers (Figure 1) [40,44,46,100,232]. Our research group at the University of Antwerp, Belgium, recently confirmed that transudated HPV vaccine-induced antibodies are detectable in the first-void urine of young women. In this study, significantly higher HPV-specific antibody levels were found in the first-void urine of vaccinated compared with unvaccinated women. Moreover, positive correlations were observed between antibodies for the HPV types included in the quadrivalent HPV vaccine in first-void urine and paired sera using two different immunoassays not optimized for first-void urine samples [49,50].

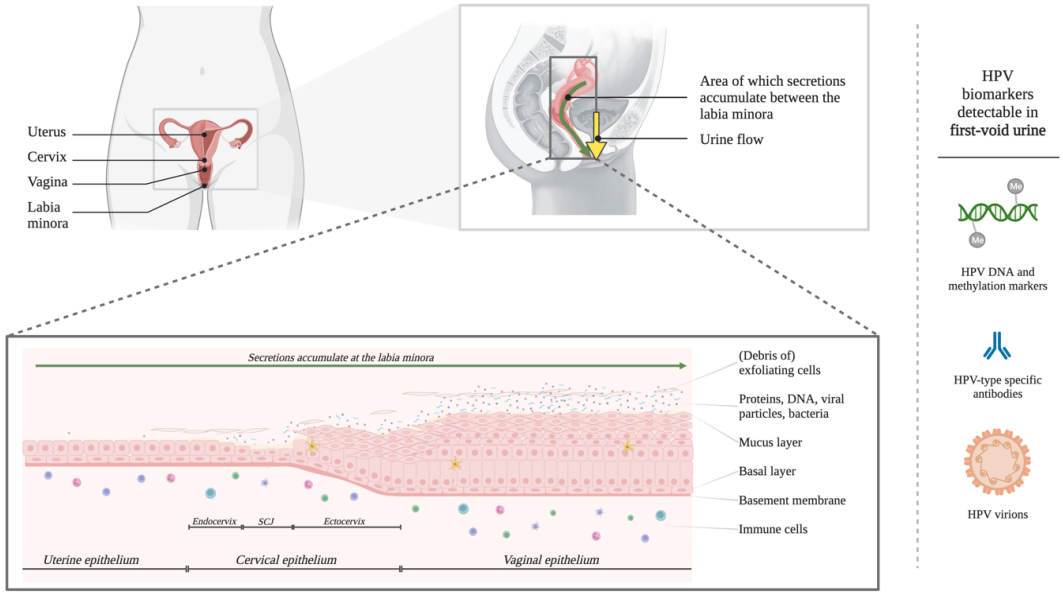


Figure 1: *Overview of female genital secretions captured in first-void urine. Secretions from the uterine, cervical, and vaginal epithelium transfer to the labia minora, where they accumulate. When the woman urinates, accumulated secretions are captured in the first fraction of the urine (e.g., first-void urine). Various proteins, DNA, viral particles, and bacteria are present in the vaginal epithelium, less in the cervical epithelium, and to the lowest degree in the uterine epithelium. SCJ, squamocolumnar junction.*

Cervical cancer can be prevented most effectively by combining HPV vaccination with cervical cancer screening. In the absence of a correlate of protection, it is generally accepted that the HPV type-specific antibodies produced after vaccination or natural infection are indicators of protection against HPV infection [118,233,240,241]. Screening and immunomonitoring during HPV vaccine trials have relied mainly on serology and cervical samples [13]. First-void urine could be ethically acceptable to determine the prevaccination HPV DNA status in girls too young to be exposed to a gynecological examination or to take a vaginal self-sample, as already demonstrated in the HPV vaccine monitoring programs in Bhutan and Rwanda [157,158,205,242]. Furthermore, non-invasive, easily obtained, at-home sampling can simplify the operational management and logistics of HPV vaccine trials, therefore potentially leading to fewer lost-to-follow-up, lower trial costs, and increased sampling frequency if needed [35].

As limited data are available on the evaluation of first-void urine as an alternative sample for HPV antibody detection, this longitudinal cohort study, nested in a randomized controlled vaccine trial, was set up. This study is the first to assess if first-void urine is a suitable tool for monitoring HPV vaccination with the nonavalent HPV vaccine (9vHPV) in women, in combination with HPV DNA detection.

2 Methods

2.1 Study design and participants

This longitudinal cohort study was nested in a Phase III randomized controlled trial in which volunteers were vaccinated with 9vHPV (Merck & Co. Inc.) at 0, 1, and 6 months (HPV V503-004 study; EudraCT NUMBER: 2015-005093-38) [243]. Recruitment occurred in Belgium at the Centre for the Evaluation of Vaccination (CEV), University of Antwerp (UA) and Antwerp University Hospital (UZA) from December 2017 to February 2018 (Visit 1, M0), with additional visits in June–October 2018 (Visit 2, M7), and August–September 2021 (Visit 3, M43) (Figure 2). Key exclusion criteria included incomplete 9vHPV vaccination (three doses), cervical cancer, or precancerous treatment in the last 6 months, and participation in any other interventional clinical trial.

In this substudy, women participating in the Phase III clinical trial (HPV V503-004) were asked if they were willing to provide first-void urine samples in addition to blood samples

at M0 and M7. Women willing to participate were sent the information brochure, informed consent form, and practical instructions by e-mail. They were asked to read these documents thoroughly before participating in the study. Approximately 3.5 years after initial inclusion, women were contacted again and asked if they were willing to provide another first-void urine sample and a blood sample (M43). All volunteers were asked to complete a questionnaire at each visit, including information on the menstruation cycle and contraception. At M43, additional information on demographics and their sexual status was collected. Informed consent was obtained from all volunteers and data were coded to ensure privacy of the participants. The institutional review board of the Biobank and the ethical committee of UZA/UA (B300201734258) approved all study procedures, and the study is registered with clinicaltrials.gov (NCT03542227).

2.2 Sample collection

Before each first-void urine sample collection, we asked the women to not wash their genitals thoroughly, not to use a tampon, and not to urinate at least 2 h before collection. All first-void urine samples were collected using the Colli-Pee® 20 mL device (Novosanis) prefilled with a urine conservation medium. Samples were collected at the CEV or UZA, refrigerated for up to 4 h, vortexed, aliquoted, and stored at – 80 °C (Biobank Antwerpen; ID: BE 71030031000) before further analysis [189]. Before aliquoting, the presence of erythrocytes in first-void urine was determined using Hemastix® reagent strips for urinalysis (Siemens Healthcare Diagnostics Inc.). Blood samples were collected at M0 and M7 as part of the 9vHPV Phase III clinical trial [243]. and at M43 as part of the substudy. We obtained 200 µL aliquots of serum from the M0 and M7 samples from Merck & Co. Inc. These aliquots were stored at – 80 °C until further use. For the M43 samples, blood was collected using 10 mL BD Vacutainer® Serum Tubes without anticoagulant (BD Benelux N.V) and allowed to clot for 30–60 min. The samples were then centrifuged at 1000*g* for 10 min at 20 °C and the serum was divided into aliquots before being stored at – 80 °C.

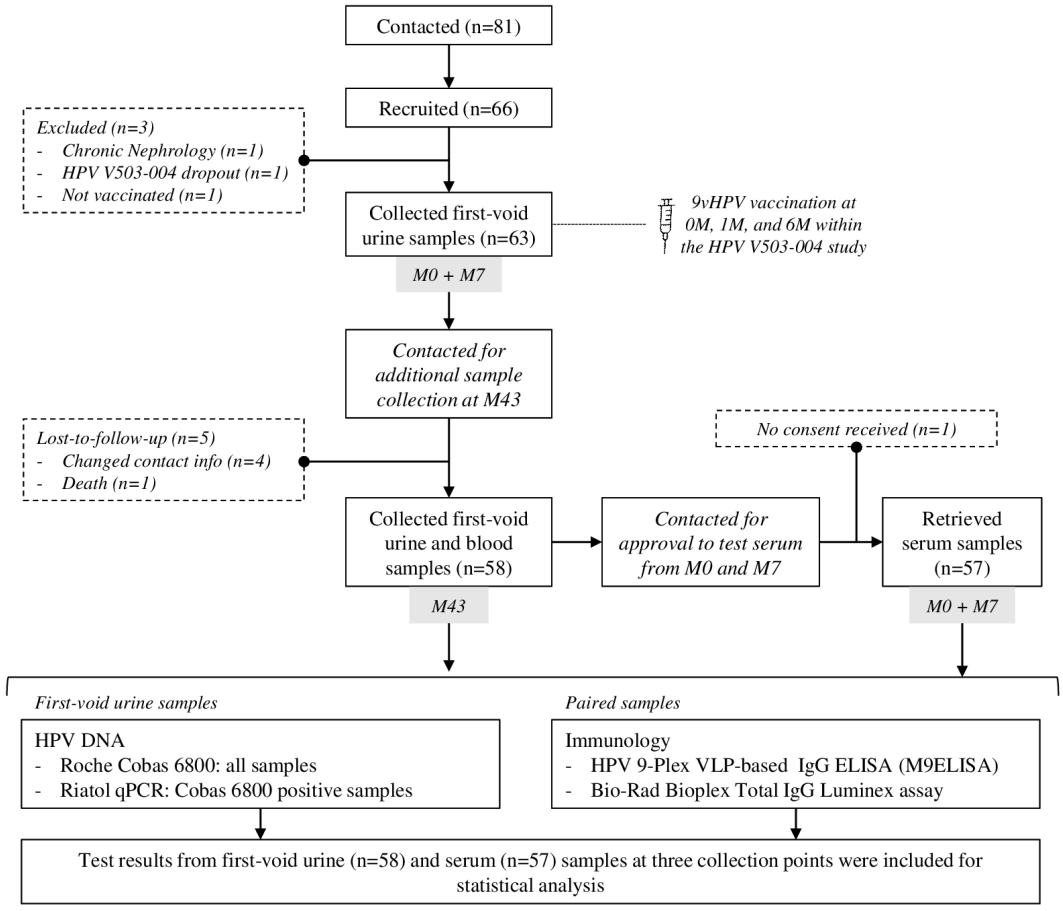


Figure 2: *Study flowchart. Sixty-six women were willing to provide first-void urine samples at M0 and M7, of which 63 eligible women were included. All 63 women collected first-void urine samples before vaccination (M0) and 1 month after the third 9vHPV vaccine dose (M7). We contacted all 63 women 3.5 years after the initial inclusion date to invite them for a follow-up visit. Fifty-eight women were reached and provided paired first-void urine and blood samples ~3 years after complete vaccination (M43). In addition, we contacted the women again to ask for their consent to test the serum samples collected at M0 and M7 as part of the human papillomavirus (HPV) V503-004 study. The presence of HPV DNA was determined for each first-void urine sample using Roche Cobas 6800, and the HPV DNA-positive samples were genotyped using the Riatol quantitative reverse-transcription polymerase chain reaction HPV genotyping assay. All samples were analyzed for HPV-type-specific antibodies (HPV 9-plex virus-like particle-based IgG enzyme-linked immunosorbent assay [M9ELISA]) and total IgG (BioRad). Data from 58 first-void urine and 57 serum samples were included for analysis.*

2.3 HPV DNA testing

HPV DNA testing was performed on first-void urine samples. Buffered aliquots were thawed before sample preparation. HPV DNA testing for HPV16, HPV18, and 12 other high-risk (HR)-HPV types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) was

performed at the Centre for Medical Analysis on 1 mL of buffered first-void urine using Cobas 6800 (Roche Molecular System) and the clinical cutoff for cervical samples. If positive on Cobas 6800, we genotyped the sample. For this, DNA was extracted using an in-house protocol [169]. Briefly, 4 mL of first-void urine was centrifuged at 3820*g* for 20 min at 20 °C in an Amicon Ultra-4 50 K filter device (Merck Millipore). Next, 2 mL NucliSENS Lysis Buffer (bioMérieux Benelux) was added to the concentrate retained on the filter. After incubation at room temperature, all material was transferred to the NucliSENS Lysis buffer vial for DNA extraction. DNA was eluted in 55 µL, and 35 µL was transferred to a vial with ultrapure water, resulting in a total volume of 75 µL DNA extract for HPV DNA genotyping. HR-HPV DNA-positive samples were genotyped using the Riatol quantitative reverse-transcription polymerase chain reaction (qPCR) HPV genotyping assay. This assay quantifies beta-globin and 18 HPV genotypes (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, and 68) as described by Depuydt et al. [244].

2.4 HPV-specific antibody testing

For antibody testing, first-void urine samples were concentrated. Thawed 4 mL aliquots were centrifuged at 3820*g* for 10 min at 20 °C using an Amicon Ultra-4 50 K filter device (Merck Millipore). 1× Dulbecco's phosphate-buffered saline (Gibco) was added to the concentrate retained on the filter to reach a final volume of 0.5 mL. First-void urine concentrates were stored at – 80 °C until further testing.

HPV-type-specific antibodies were quantified using the HPV 9-plex virus-like particle-based IgG enzyme-linked immunosorbent assay (M9ELISA) as described by Panicker et al. [164]. First-void urine samples were tested starting undiluted, whereas serum samples (including standards and controls) testing started at 1:100 dilution or higher to ensure that the generated signal was within the linear range of the assay. A minimum of three dilutions were tested for each sample. The plates were read on the MESO® Quickplex SQ120 (Meso Scale Diagnostics LLC). HPV antibody concentrations were determined using raw relative light units (RLUs) for serum samples and background subtracted RLUs (signal from bovine serum albumin [BSA] spot subtracted) for concentrated first-void urine samples using the parallel line method (PLL) described in the World Health Organization HPV Labnet Manual 2009 [207,245]. A serum or first-void urine sample passed PLL conditions if (1) the correlation between the three selected dilutions was ≥ 0.9; (2) the absolute value of the

slope was ≥ 0.4; (3) the ratio of the slope of the standard and the test sample was ≥ 0.5; and (4) if there was not more than one data point out of three points outside the linear range. We report antibody concentrations in arbitrary units/mL (AU/mL) for all types, except HPV16 and 18, which are reported in International Units (IU/mL). The HPV16 (05–134) and HPV18 IU standards (10–140) were obtained from the National Institute for Biological Standards and Controls. A serum sample was given a concentration if it passed all PLL conditions and if the concentration was at or above the lower limit of quantification (LLOQ, only defined for serum). The LLOQ values for serum are as ≥ 0.2 AU/mL for HPV6, 0.4 AU/mL for HPV11, 1.0 IU/mL for HPV16, 0.3 IU/mL for HPV18, 1.6 AU/mL for HPV31, 3.0 AU/mL for HPV33, 2.3 AU/mL for HPV45, 1.6 AU/mL for HPV52, and 2.2 AU/mL for HPV58. A first-void urine sample was given a concentration if it titrated linearly and passed all PLL conditions, as no LLOQs were defined yet.

2.5 Total human IgG testing

Total human IgG concentrations were determined using the BioPlex Pro™ Human Isotyping Assay for total human IgG (Bio-Rad) according to the manufacturer's instructions. We diluted concentrated first-void urine samples at 1:128 and 1:512, and serum samples at 1:10 000 and 1:100 000. Measurements were performed using the LX200 platform (Luminex). The software calculated concentrations from the median fluorescence intensity values using a five-parameter logistic regression. Total human IgG concentrations were calculated as the average of two dilution-corrected concentrations.

2.6 Statistical analysis

We performed statistical analysis using R statistical software version 4.2.2 (packages: rstatix, tidyverse, irr, RVAideMemoire). If the data were normally distributed (Shapiro–Wilk test and Q–Q plot), significant differences in mean antibody concentrations between the three visits were examined using a one-way repeated-measures analysis of variance. Otherwise, a nonparametric Friedman test was used. This was followed by a posthoc paired *t* test (parametric) or Wilcoxon signed-rank test (nonparametric) for multiple paired comparisons. We used the Spearman rank test to calculate the correlation between antibody levels in different samples and bootstrap 95% confidence intervals (95% CIs)

were calculated using 1000 reps. Cohen's κ and the corresponding 95% CIs were calculated to assess the antibody agreement between sample types ($\kappa \leq 0.20$, poor; $0.21 \leq \kappa \leq 0.40$, fair; $0.41 \leq \kappa \leq 0.60$, moderate; $0.61 \leq \kappa \leq 0.80$, good; and $\kappa \geq 0.81$, excellent agreement). For analyses, statistical significance was defined as p-adjusted < 0.05 (using Holm–Bonferroni method for p adjustment). We performed all analyses on untransformed data. Figures were made using antibody concentrations or log10(x) transformed data where zero values were assigned as 0.0001.

3 Results

3.1 Population characteristics

Between December 11, 2017, and October 12, 2018, 63 out of 81 contacted women participating in the Phase III randomized controlled trial consented to be enrolled in this substudy (Figure 2). From August 26, 2021, to September 30, 2021, all 63 women were contacted again, and 58 participants were reached. We obtained first-void urine at all time points from all 58 included women. From one woman, we did not receive consent to test serum samples collected at M0 and M7 (Figure 2). Median time between the M0 and M7 visit was 6.4 months (interquartile range [IQR]: 6.2–7.1), and the median time between the M0 and M43 visit was 43.4 months (IQR: 42.7–44.0). Median age at enrollment was 35 years (IQR: 27–41). Of all included women, 97% (56/58) had been sexually active before enrollment, with a median age at first sexual encounter of 17 years (IQR: 16–19). The median number of sexual partners was 4 (IQR: 4–6). Eighty-six percent of the women had provided a cervical sample in the past (50/58) and the median age of providing the first cervical sample was 20 years (IQR: 18–25). Twenty-two percent of the women (13/58) had a smoking history and 69% (40/58) of the women had given birth at least once. The most common contraceptive used was an intrauterine device, 47% (27/58), and 4% (2/58) used a condom (Table 1).

Table 1: *Population characteristics and demographic data.*

Characteristic	Number
Total	58 (100%)
Age, years	35 (27–41)
Time after previous urination, min	120 (90–165)
Sexually active	56 (97%)
Age at first sexual encounter, years	17 (16–19)
Number of sexual partners	4 (2–6)
Smoker	13 (22%)
History of cervical sample	50 (86%)
Age at first cervical sample collection, years	20 (18–25)
Already given birth	40 (69%)
Oral contraceptives	13 (22%)
Intrauterine device	27 (47%)
No contraceptives	16 (28%)
Condom	2 (4%)

Note: Data are n (%) and median (IQR). Two women never had a sexual encounter, one volunteer did not provide details on the number of sexual partners, six volunteers did not know whether they had a cervical sample, eight volunteers did not know when they got the first cervical sample or did not provide a cervical sample yet, one volunteer used both oral contraceptives and condom at M7. Abbreviation: IQR, interquartile range.

3.2 HPV DNA results

Using the Cobas 6800 assay, we identified 8/58 (14%) women with an HR-HPV infection in first-void urine before vaccination (Table 2). At M7, 14% (8/58) of the women had an HR-HPV infection, two M0 infections cleared, and two new infections were detected. At M43, 7% (4/58) of the women tested HPV DNA positive, six M7 infections were cleared, and two new infections were detected.

All samples that were HPV DNA positive on the Cobas 6800 assay were genotyped using the Riatol qPCR. A total of five women had infections with HPV types included in the 9vHPV vaccine (HPV16, 31, 33, 52, 58). One woman had HPV16 and HPV33 DNA detected at all visits, and one had detectable HPV58 DNA at M43.

Table 2: *HPV DNA results in first-void urine samples.*

ID	M0	M7	M43
	Riatol qPCR	**Riatol qPCR**	**Riatol qPCR**
14	HPV59	HPV59	HPV58, HPV59
19	None	None	/
24	/	HPV66	/
28	/	HPV52	/
31	HPV33, HPV59	/	/
35	HPV51	HPV51	/
37	HPV56	HPV56	/
45	/	/	HPV51
48	/	/	HPV67
51	HPV16, HPV33, HPV51	HPV16, HPV33, HPV51, HPV56, HPV66	HPV16, HPV33
60	HPV31, HPV51	HPV35, HPV51	/
62	HPV51	/	/

Note: HPV types of which DNA was detected using the Riatol qPCR is presented here. Only samples that had a positive result for HR-HPV DNA other, HPV16, and/or HPV18 by Roche Cobas 6800 were tested using Riatol qPCR. One ID (ID19) had a positive result for HR-HPV DNA other using the Roche Cobas 6800 at M0 and M7 but was negative for the Riatol qPCR. Abbreviations: HPV, human papillomavirus; HR-HPV, high-risk human papillomavirus; qPCR, quantitative reverse-transcription polymerase chain reaction.

3.3 Detection of HPV-specific antibodies

HPV-specific antibody detection and median antibody concentrations were determined for first-void urine and serum samples (Table 3). Before vaccination, we detected HPV-specific antibodies in 0% (0/58, HPV52) to 16% (9/58, HPV6) of the first-void urine samples. One month after the third 9vHPV vaccine dose, 95% (55/58, HPV45) to 100% (58/58, HPV11–16–31–33–58) of first-void urine samples had detectable antibody concentrations, and 84% (49/58, HPV52) to 100% (58/58, HPV16) at M43. In serum, we detected HPV-specific antibodies in 9% (5/57, HPV33) to 37% (21/57, HPV6–18) samples collected before vaccination. One month after the third dose of the 9vHPV vaccine, antibody detection in serum was 100% (57/57) for all HPV types, and 98% (57/58, HPV52) to 100% (57/57, all other HPV types) at M43. For all samples, there were significant differences in HPV-specific antibody concentrations between all different collection points for each HPV type (Figure 3). HPV-specific antibody concentrations were nearly 1700 times higher in serum than first-void urine for all samples at each time point and were about 10-fold lower for all samples at M43 than 1 month after complete vaccination (M7). Total IgG antibody concentrations in serum samples were ~200-fold higher compared with first-void urine. Significant positive Spearman rank correlations

were found between serum and first-void urine HPV-specific antibody concentrations. Correlations coefficients ranged between 0.36 (95% CI: 0.002–0.64) (HPV18) and 0.79 (95% CI: 0.46–1.00) (HPV33) for M0, between 0.54 (95% CI: 0.30–0.75) (HPV11) and 0.71 (95% CI: 0.51–0.83) (HPV45) for M7, and between 0.64 (95% CI: 0.41–0.79) (HPV11) and 0.82 (95% CI: 0.71–0.89) (HPV33) for M43 (Table 3) (Supporting Information: Appendix Figure 1) [246].

The antibody detection agreement (k) between serum and first-void urine was moderate to excellent and ranged between 0.68 (95% CI: 0.56–0.81) (HPV6) and 0.93 (95% CI: 0.87–0.99) (HPV16 and HPV33) (Table 3). Finally, we combined the data on HPV detection and antibody concentrations for the women with HPV DNA detected at least at one of the visits (Supporting Information: Appendix Figure 2). Only four women had HPV DNA from HPV types included in the 9vHPV vaccine in their first-void urine sample.

For two of these women, we detected a naturally induced HPV-specific antibody response in the first-void urine samples to the specific type before vaccination. In addition, we provide an overview of elicited antibody concentrations for all samples at all visits (Supporting Information: Appendix Figure 2).

***Table 3**: Summary of antibody detection and concentrations after 9vHPV vaccination.*

Type	Months	First-void urine		Serum		First-void urine vs Serum concentration		First-void urine vs Serum detection
		Antibody detection	Antibody concentration (AU-IU/ml or µg/ml)	Antibody detection	Antibody concentration (AU-IU/mL or µg/mL)	Spearman Correlation (95% CI)	Adjusted *p*-value	Cohen's Kappa (95% CI)
HPV6	0	9/58 (16%)	0.000 (0.000–0.000)	21/57 (37%)	0.000 (0.000–0.380)	0.44 (0.12–0.68)	< 0.0001	
	7	57/58 (98%)	0.057 (0.022–0.203)	57/57 (100%)	92.60 (56.30–137.00)	0.63 (0.42–0.79)	< 0.0001	0.68 (0.56–0.81)
	43	57/58 (98%)	0.006 (0.002–0.013)	58/58 (100%)	10.01 (4.89–18.13)	0.70 (0.52–0.83)	< 0.0001	
HPV11	0	4/58 (7%)	0.000 (0.000–0.000)	7/57 (12%)	0.000 (0.000–0.000)	0.56 (-0.03–0.87)	< 0.0001	
	7	58/58 (100%)	0.058 (0.029–0.199)	57/57 (100%)	106.0 (56.60–158.0)	0.54 (0.30–0.75)	< 0.0001	0.92 (0.85–0.98)
	43	57/58 (98%)	0.007 (0.003–0.016)	58/58 (100%)	10.72 (5.24–17.93)	0.64 (0.41–0.79)	< 0.0001	
HPV16	0	7/58 (12%)	0.000 (0.000–0.000)	10/57 (18%)	0.000 (0.000–0.000)	0.76 (0.51–0.96)	< 0.0001	
	7	58/58 (100%)	0.184 (0.077–0.552)	57/57 (100%)	291.0 (178.0–468.0)	0.57 (0.35–0.75)	< 0.0001	0.93 (0.87–0.99)
	43	58/58 (100%)	0.019 (0.008–0.045)	58/58 (100%)	32.17 (13.32–64.67)	0.72 (0.54–0.85)	< 0.0001	
HPV18	0	6/58 (10%)	0.000 (0.000–0.000)	21/57 (37%)	0.000 (0.000–0.440)	0.36 (0.002–0.64)	0.0056	
	7	56/58 (97%)	0.058 (0.031–0.274)	57/57 (100%)	102.0 (44.00–210.0)	0.68 (0.49–0.80)	< 0.0001	0.69 (0.56–0.81)
	43	52/58 (90%)	0.005 (0.002–0.015)	58/58 (100%)	6.54 (2.07–17.35)	0.78 (0.64–0.86)	< 0.0001	
HPV31	0	4/58 (7%)	0.000 (0.000–0.000)	8/57 (14%)	0.000 (0.000–0.000)	0.63 (0.37–0.87)	< 0.0001	
	7	58/58 (100%)	0.145 (0.050–0.573)	57/57 (100%)	195.0 (118.0–541.0)	0.68 (0.48–0.82)	< 0.0001	0.85 (0.77–0.94)
	43	55/58 (95%)	0.016 (0.004–0.036)	58/58 (100%)	21.28 (10.48–49.17)	0.77 (0.60–0.87)	< 0.0001	
HPV33	0	3/58 (5%)	0.000 (0.000–0.000)	5/57 (9%)	0.000 (0.000–0.000)	0.79 (0.46–1.00)	< 0.0001	
	7	58/58 (100%)	0.238 (0.078–0.573)	57/57 (100%)	322.0 (197.0–677.0)	0.63 (0.40–0.79)	< 0.0001	0.93 (0.87–0.99)
	43	56/58 (97%)	0.020 (0.004–0.051)	58/58 (100%)	31.92 (13.36–69.07)	0.82 (0.71–0.89)	< 0.0001	
HPV45	0	2/58 (3%)	0.000 (0.000–0.000)	7/57 (12%)	0.000 (0.000–0.000)	0.53 (0.32–0.83)	< 0.0001	
	7	55/58 (95%)	0.202 (0.061–0.586)	57/57 (100%)	336.0 (140.0–562.0)	0.71 (0.51–0.83)	< 0.0001	0.87 (0.79–0.95)
	43	52/58 (90%)	0.016 (0.003–0.050)	58/58 (100%)	16.37 (8.63–63.59)	0.79 (0.60–0.88)	< 0.0001	
HPV52	0	0/58 (0%)	0.000 (0.000–0.000)	11/57 (12%)	0.000 (0.000–0.000)	NA	NA	
	7	57/58 (98%)	0.110 (0.035–0.507)	57/57 (100%)	297.0 (151.0–600.0)	0.65 (0.45–0.80)	< 0.0001	0.76 (0.66–0.87)
	43	49/58 (84%)	0.017 (0.003–0.046)	57/58 (98%)	28.27 (11.45–65.31)	0.80 (0.68–0.87)	< 0.0001	
HPV58	0	4/58 (7%)	0.000 (0.000–0.000)	9/57 (16%)	0.000 (0.000–0.000)	0.70 (0.38–0.90)	< 0.0001	
	7	58/58 (100%)	0.359 (0.079–0.849)	57/57 (100%)	365.0 (222.0–790.0)	0.64 (0.44–0.77)	< 0.0001	0.83 (0.74–0.92)
	43	53/58 (91%)	0.026 (0.010–0.078)	58/58 (100%)	39.81 (15.65–97.34)	0.81 (0.68–0.89)	< 0.0001	
Total IgG	0	58/58 (100%)	23.77 (16.57–56.07)	58/58 (100%)	6,660 (5,843–9,262)	0.23 (0.14–0.31)	< 0.0001	
	7	58/58 (100%)	22.40 (9.79–45.29)	57/57 (100%)	4,958 (3,505–6,599)	-0.014 (-0.11–0.08)	0.75	
	43	58/58 (100%)	23.21 (12.50–38.03)	57/57 (100%)	4,717 (3,870–7,108)	0.074 (-0.009–0.15)	0.092	

Note: Data are median (IQR) or n (%). One volunteer did not provide consent to test serum samples at M0 and M7. Spearman rank correlation coefficients between first-void urine and serum samples at all time points were calculated. Kappa values between the antibody detection results in first-void urine and serum were calculated. Total IgG concentrations are reported in µg/mL for both sample types. AU (HPV6, 11, 31, 33, 45, 52, 58); IU (HPV16, 18). Abbreviations: AU, arbitrary units; IU, international units; NA, not applicable.

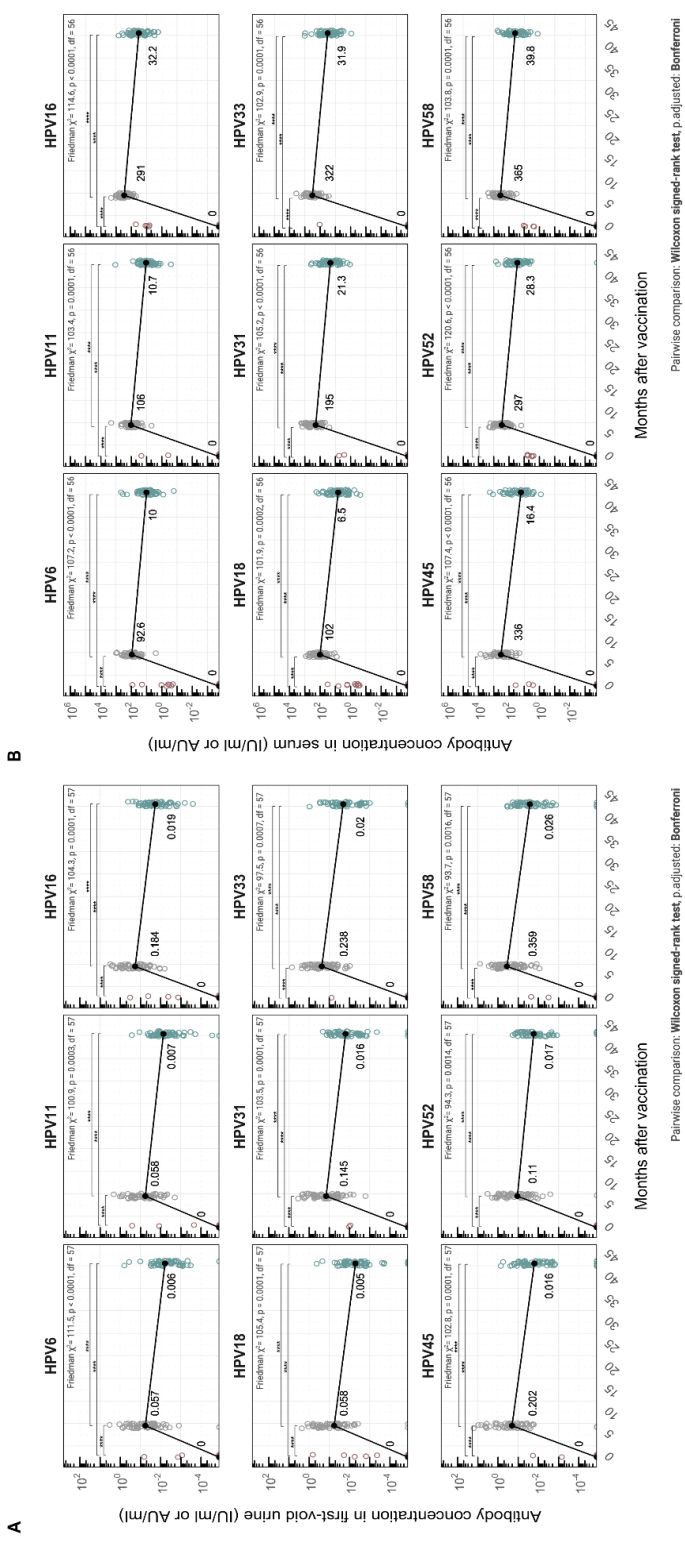


Figure 3: *Human papillomavirus (HPV)-specific antibody concentration at M0 (pink), M7 (gray), and M43 (blue) measured in first-void urine samples (A) and serum samples (B). The median antibody concentrations are shown in the figure. Concentrations are expressed as IU/mL for HPV16 and HPV18, and as AU/mL for all other HPV types. A Friedman test was used for repeated-measures analysis, followed by a Wilcoxon signed-rank test to analyze pairwise significant differences. Significant differences were found between all different time points for all HPV types and all sample types (****p-adjusted < 0.0001). AU, arbitrary units; IU, international units.*

4 Discussion

In this longitudinal cohort study, we provide the first data supporting the potential of first-void urine to monitor HPV-specific antibody response in a vaccine trial. The data show that we can detect HPV-specific antibodies to all 9vHPV vaccine types at peak levels and that they remain detectable in first-void urine for at least 3 years after vaccination. We were also able to measure HPV DNA status and detect naturally induced immunity using this sample type. Antibody concentrations after vaccination in first-void urine correlated well with serum concentrations for all HPV types at M7 and M43. In addition, we found significant correlations between serum and first-void urine antibody levels before vaccination (M0). As serum antibody levels are a good measure of protection in the absence of a correlate of protection, being able to measure the antibody levels at the site of infection could be helpful in the search for a correlate of protection against HPV infections and associated diseases.

With first-void urine, we collect the female genital secretions from, for example, the uterus, cervix, and vagina that accumulated between the labia minora. As the main Ig class in the genital tract is IgG, it was expected that these antibodies would also be abundant in the genital secretions captured with first-void urine [30,31].

Although antibodies were detected in most first-void urine samples at both M7 (>95%) and M43 (>84%), some samples had undetectable concentrations using the current M9ELISA. Since the assay was developed for serum, using a concentrated first-void urine sample could affect the background and sensitivity of the assay specifically optimized for diluted serum samples. For the first-void urine samples, HPV-specific antibody concentrations were calculated after subtracting the signal in the control spot (BSA) to mitigate the impact of non-specificity arising from the concentrated sample. The laboratory protocol for serum M9ELISA does not use background subtraction in concentration calculations, as backgrounds are generally low. However, occasionally samples do show high backgrounds. In the current study, two serum samples had high values in the BSA control spot, suggesting potential non-specificity. The effect of BSA will be further evaluated during assay optimization. Undetectable HPV-specific antibodies in first-void urine compared to serum could also be due to incorrect sampling. As first-void urine samples were collected at the clinical trials center instead of at home, women might be

more tempted to wash their genitals, resulting in fewer secretions being captured in the first void of urine.

Most of the HPV infections detected in the first-void urine samples were from HPV types not included in the 9vHPV vaccine. Looking at the limited combined data of the HPV DNA detection and HPV-specific antibody response, we observed naturally induced antibody responses before vaccination. The latter was expected in this sexually active cohort as serology is a marker of past or present infection. As first-void urine has detectable HPV-specific antibody concentrations after natural infection, detecting antibodies after one-dose HPV vaccination might be feasible since these concentrations are known to be two to four times higher than after natural infection [116].

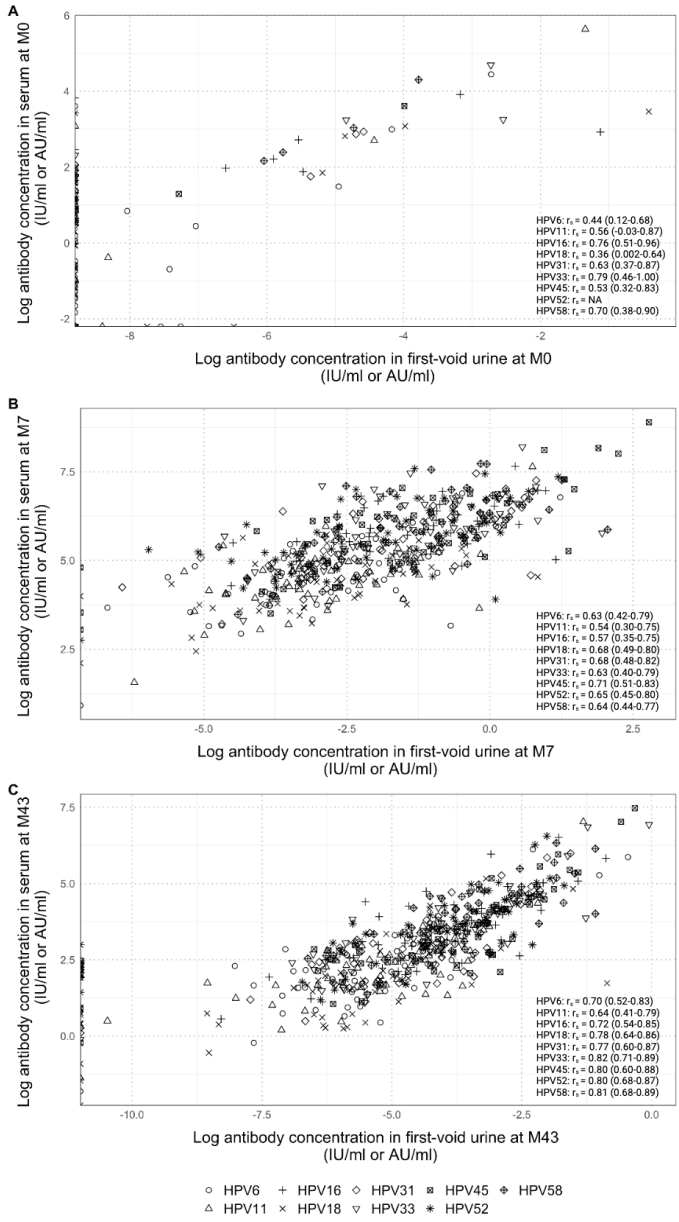
The use of first-void urine sampling for primary and secondary cervical cancer prevention offers a number of advantages such as non-invasiveness, not requiring trained personnel, ease of collection outside a clinical setting, and reducing lost-to-follow-up and cost for vaccine and epidemiological trials. This approach is particularly important for monitoring the vaccine effectiveness and efficacy of current vaccination programs and one-dose HPV vaccination, [116,247] reducing issues such as lost-to-follow-up and increasing compliance [246,248]. This alternative method could also be used to evaluate new prophylactic or therapeutic HPV vaccines [249]. Furthermore, first-void urine sampling could be suitable for detecting other sexually transmitted infections, for example, *Chlamydia trachomatis*, and for the evaluation of corresponding vaccines under development [250].

This pilot study has certain limitations. Due to the absence of existing data on HPV-specific antibody detection in first-void urine, a formal sample size calculation was not feasible. Nevertheless, our longitudinal analysis involved a total of 346 samples (174 first-void urine and 172 serum samples) and results for nine different HPV types. This sample size provides robust results to adequately describe the potential of a first-void urine sample for vaccination follow-up. We only included female participants in this study since first-void urine sampling only applies to women. Since the amount and origin of genital tract secretions differ between males and females, the secretions captured with first-void urine are also different. Optimizing the existing assays and further concentrating the urine samples may improve the detection of HPV-type-specific antibodies in first-void urine. In addition, normalization for IgG fluctuations over the menstrual cycle, [204] and hormonal influence on the accumulation of vaginal mucus are being investigated. As first-void urine data using total IgG normalization did not provide better results compared to non-

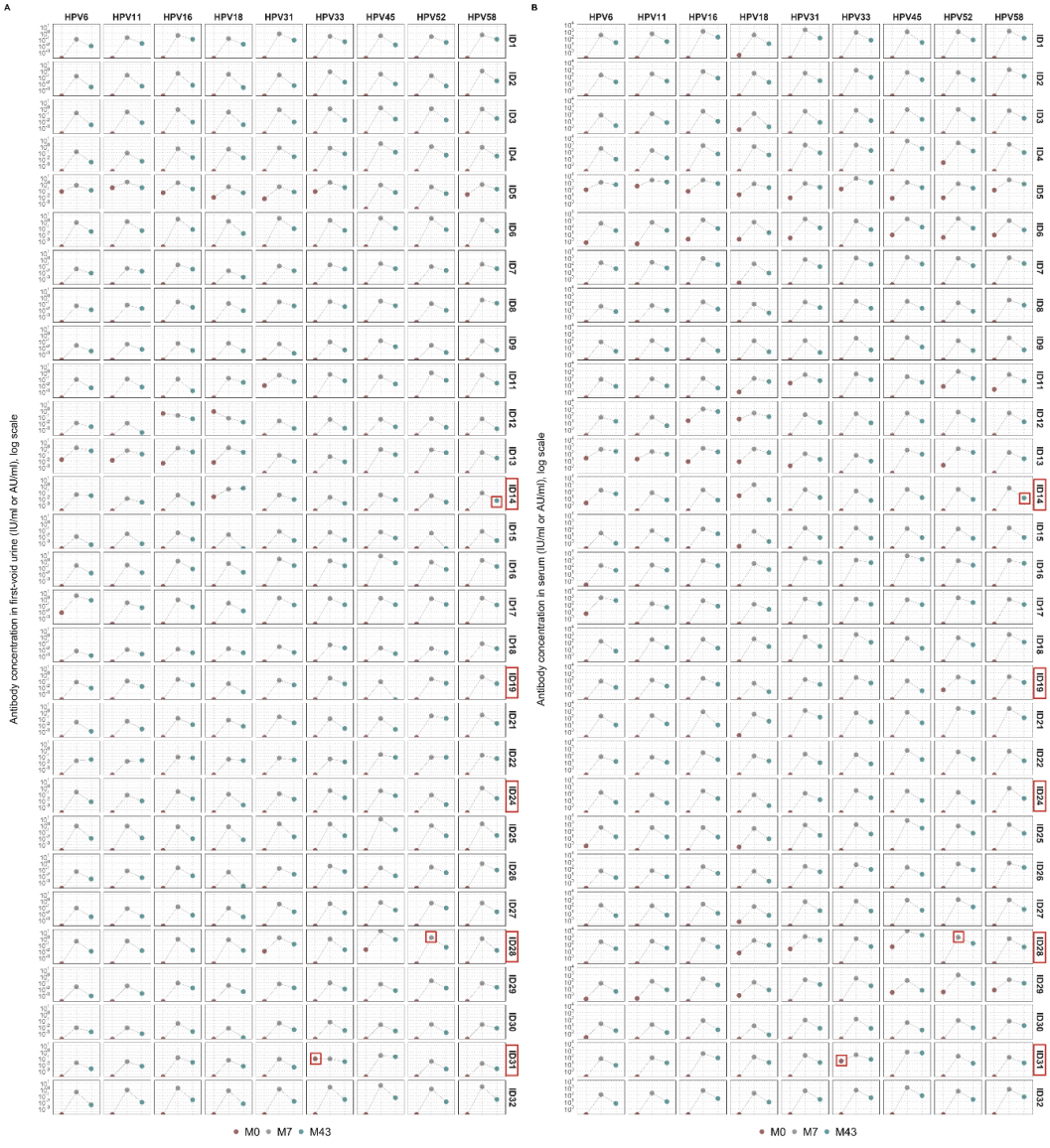
normalized data, we utilized non-normalized data in this manuscript. Nevertheless, we could still provide robust results even without assay optimization or normalization of the first-void urine data.

In conclusion, we are the first to demonstrate that first-void urine can be used in HPV vaccine trials to monitor HPV infection and immune responses, thereby replacing a clinician-taken cervical sample or vaginal self-sample and a blood sample with a non-invasive self-collected sample. Once validated, it can be a suitable sample for surveillance and follow-up of vaccine trials.

5 Supplementary materials



Appendix Figure 1*: Correlations between HPV-specific antibody concentrations in first-void urine and serum samples. Correlation plots are presented for different collection points; A) M0, B) M7, and C) M43. The symbols indicate HPV-type specific Log antibody concentrations. Spearman rank correlation coefficients (rs) and 95% CI are indicated in the figure, and p-values are listed in Table 2. Concentrations are expressed as Log IU/mL for HPV16 and HPV18 and in Log AU/mL for other HPV types. AU=Arbitrary Units. IU=International Units.*



Appendix Figure 2-A: *HPV-type specific antibody concentrations for sample IDs 1-32. Results are presented for first-void urine (A) and serum (B) samples before vaccination (M0), one month after full vaccination (M7), and approximately three years after full vaccination (M43). For ID51, we did not receive consent to test serum samples collected at M0 and M7. Red squares indicate the samples that were HPV DNA positive for that specific HPV type at that particular visit. For ID29 and ID57, we observed non-specific binding of anti-BSA similar to signal observed for all HPV-type specific spots. AU=Arbitrary Units. IU=International Units.*

Appendix Figure 2-B: *HPV-type specific antibody concentrations for sample IDs 33-62. Results are presented for first-void urine (A) and serum (B) samples before vaccination (M0), one month after full vaccination (M7), and approximately three years after full vaccination (M43). For ID51, we did not receive consent to test serum samples collected at M0 and M7. Red squares indicate the samples that were HPV DNA positive for that specific HPV type at that particular visit. For ID29 and ID57, we observed non-specific binding of anti-BSA similar to signal observed for all HPV-type specific spots. AU=Arbitrary Units. IU=International Units.*

CHAPTER

04

First-void urine to investigate the added value of HPV vaccination

CHAPTER

04–A

HPV-specific antibodies in female genital tract secretions captured via first-void urine retain their neutralizing capacity.

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Published in Human Vaccines & Immunotherapeutics, April 2024.

Abstract

Human papillomavirus (HPV) vaccines, primarily relying on neutralizing antibodies, have proven highly effective. Recently, HPV-specific antibodies have been detected in the female genital tract secretions captured by first-void urine (FVU), offering a minimally invasive diagnostic approach. In this study, we investigated whether HPV16-specific antibodies present in FVU samples retain their neutralizing capacity by using pseudovirion-based neutralization assays. Paired FVU and serum samples (vaccinated n = 25, unvaccinated n = 25, aged 18–25) were analyzed using two orthogonal pseudovirion-based neutralization assays, one using fluorescence microscopy and the other using luminescence-based spectrophotometry. Results were compared with HPV16-specific IgG concentrations and correlations between neutralizing antibodies in FVU and serum were explored. The study demonstrated the presence of neutralizing antibodies in FVU using both pseudovirion-based neutralization assays, with the luminescence-based assay showing higher sensitivity for FVU samples, while the fluorescence microscopy-based assay exhibited better specificity for serum and overall higher reproducibility. High Spearman correlation values were calculated between HPV16-IgG and HPV16-neutralizing antibodies for both protocols (r_s: 0.54–0.94, $p < .001$). Significant Spearman correlations between FVU and serum concentrations were also established for all assays (r_s: 0.44–0.91, $p < .01$). This study demonstrates the continued neutralizing ability of antibodies captured with FVU, supporting the hypothesis that HPV vaccination may reduce autoinoculation and transmission risk to the sexual partner. Although further protocol optimizations are warranted, these findings provide a foundation for future research and larger cohort studies that could have implications for the optimal design, evaluation, and implementation of HPV vaccination programs.

1 Introduction

Human papillomavirus (HPV) is a highly prevalent sexually transmitted virus causing diseases like genital warts and cervical cancer [68,89,251]. Current HPV vaccines are highly effective in preventing HPV infections and associated diseases [79,252–254]. The important role of neutralizing antibodies (nAbs) for protection against HPV infection and disease is well-established [110,111,255]. However, only limited data is available on the neutralizing capacity of HPV-specific nAbs in the genital tract, the primary site of HPV infection.

First-void urine (FVU) has been proposed as a noninvasive sample for assessing HPV-specific antibodies originating from the genital tract [49–51]. Current methods for measuring HPV-specific nAbs involve blood draws, which can be uncomfortable for patients and may not accurately reflect the neutralizing capacity of Abs at the site of infection. With FVU, the initial part of the urine stream is collected, serving as a rinsing liquid to capture the female genital tract secretions that have accumulated around the labia minora [19]. FVU collection is a simple and convenient procedure that individuals can perform in their homes and does not cause any trauma at the site of infection [33,35,256].

To date, HPV vaccination programs only target young individuals, and although studies indicate some benefits for people living with HPV-associated disease, vaccinating at an older age is not routinely considered [123,253,254,257–260]. While current prophylactic vaccines are not expected to provide therapeutic effects, the induced nAbs could potentially interact with newly produced virions, preventing the spread of HPV within the genital tract and transmission to the sexual partner [151]. With female genital tract sampling using FVU, antibodies at the site of infection are captured, making it an ideal source to investigate the virion-antibody interaction.

Pseudovirion (PsV)-based neutralization assays (PBNA) measure nAbs against HPV using cell lines that express a reporter gene in response to HPV PsV infection [261] . While widely used for testing serum samples and in some cases for cervical samples, its application in FVU samples is unexplored [262–265]. Due to lower concentrations of HPV-specific antibodies in FVU, enrichment is likely needed [51,266]. As FVU contains a concentrated amount of proteins, DNA, viral particles, bacteria, and (debris of) exfoliating cells, compared to the subsequent urine fractions, the odds of contaminating cell growth or interference with PsV infection within the PBNA is expected to be high. Hence, purification of the samples will also be required.

Most of the HPV PBNA protocols use Gaussia luciferase (Gluc), nanoluciferase (NLuc), or secreted alkaline phosphatase (SEAP) due to their simplicity and compatibility with 96-well or 384-well plate formats [142,160,163,164,261]. Gluc PBNAs yield comparable results to SEAP while significantly reducing hands-on and detection time. NLuc, as a reporter, offers additional advantages over Gluc, including a longer lifetime, smaller size, and thermal stability [163,267,268]. Another explored option is utilizing enhanced green fluorescent protein (EGFP) [163,261,269–271]. However, current methods using PsV with an EGFP reporter in PBNA require collection and/or lysis of the infected cells for measurement using a flow cytometer, making it a less commonly used method due to its time- and labor-intensive nature. Earlier comparisons of the different readouts showed good significant correlations between the different PBNA readout methods, with EGFP providing the least nAb positive samples [163].

In this study, we are the first that investigated the neutralizing capacity of HPV16-specific antibodies in non-invasively collected FVU. This was done using two different PBNA methods: a widefield microscopy-based PBNA (based on EGFP) and luminescence-based PBNA (based on Nluc), and the results were compared to HPV16-specific IgG concentrations. We analyzed FVU samples from both HPV-vaccinated and unvaccinated women, aiming to identify the most sensitive and reliable PBNA method for measuring HPV-specific nAbs in FVU. Additionally, we evaluated the correlation between FVU and serum nAb concentrations quantified by both PBNA methods. Our study contributed to understanding HPV infection protection mechanisms by exploring the neutralizing capacity of FVU antibodies, providing insights into preventing HPV infection and disease at the infection site.

2 Materials and methods

2.1 Study population

Fifty healthy females aged 18-25, comprising 25 unvaccinated and 25 vaccinated with an HPV vaccine, provided paired FVU and serum samples between May and July 2020 (clinicaltrials.gov ID: NCT04391647). The institutional review board of the Biobank and the ethical committee of Antwerp University Hospital (UZA)/UAntwerp, Belgium (B300201734258) approved all study procedures, and we obtained informed consent from all women before sample collection.

2.2 Sample collection and storage

Women were notified about the study via e-mail or social media and interested participants registered online through the Centre for the Evaluation of Vaccination (CEV) webpage. Using the R package tidyverse, the study randomly selected 25 vaccinated and 25 unvaccinated women with a normal age distribution, who received detailed study information via e-mail, including an information brochure and an informed consent form. The women were then asked to plan their appointment at the CEV, where the study team explained the study in detail. To ensure consistent collection of FVU samples, women were instructed not to wash their genitals thoroughly, use a tampon, or urinate at least two hours before FVU sample collection. All FVU samples were collected using the Colli-Pee® 20 ml FV-5020 device (Novosanis, Belgium) prefilled with a urine conservation medium (UCM). Samples were immediately placed in the refrigerator (up to 4 hours post collection), aliquoted, and stored at – 80 °C (Biobank Antwerpen, Antwerp, Belgium; ID: BE 71030031000) before further analysis. Hemastix® reagent strips were used to assess erythrocyte presence (Siemens Healthcare Diagnostics Inc., Belgium). Blood samples were collected using 10 ml BD Vacutainer® Serum tubes without anticoagulant (Becton-Dickinson, Benelux) and allowed to clot for 30–60 min. After clotting, blood samples were centrifuged at 1000 × *g* for 10 min at 20 °C, and serum was divided into aliquots before storage at – 80 °C. All volunteers were asked to complete a questionnaire including information on the menstruation cycle and contraception, demographics, and their sexual status.

2.3 Sample processing

FVU samples were purified using an in-house enrichment and purification method. Briefly, we centrifuged a 4 ml aliquot at 4000 × *g* for 20 min at 21 °C in an Amicon Ultra-4 50 K filter device (Merck Millipore, Belgium). Subsequently, the concentrate (>50 µl) was collected with sterile 1X Dulbecco's phosphate buffered saline (dPBS, Thermo Fisher Scientific) to reach a final volume of 500 µl, followed by a second centrifugation at 1000 × *g* for 10 minutes at 21 °C. The resulting supernatant was stored at – 20 °C in clean 1.5 ml Axygen® MAXYMum Recovery® microtubes (Corning, Inc., U.S.A.) until further analysis. We performed no purification step for serum samples.

2.4 DELFIA assay

We measured HPV16-specific IgG concentrations in all FVU and serum samples using an in-house developed HPV16 immunoassay based on time-resolved fluorescence (DELFIA). Dilution series determined optimal sample dilutions and lower limit of quantification (LLOQ). Samples were then tested using a serial 2-fold dilution starting at 1:2 for enriched FVU and 1:800 for serum. A minimum of four dilutions were tested for each sample. Plates were coated with 100 µl of 0.5 µg/ml HPV16 PsV, incubated overnight at 4 °C, and washed with 300 µl wash solution (Revvity, Lier, Belgium) before blocking with dPBS (Thermo Fisher Scientific) containing 1% DTPA-purified Bovine Serum Albumin (BSA) (Revvity, Lier, Belgium). After a 1-hour incubation, 100 µl of the sample was added to the plate and left to incubate for 2 h at ambient temperature and with shaking at 300 rpm. Following incubation, plates were washed three times, and 100 µl of 200 ng/ml Eu-anti-human antibody was added to each well for a 1-hour incubation at ambient temperature with shaking at 300 rpm. Plates were washed six times to remove all unbound Eu-labeled reagents, and 200 µl of enhancement solution (Revvity, Lier, Belgium) was added to each well. After 30 min incubation at ambient temperature and shaking at 300 rpm, plates were read using the Viktor Nivo multimode plate reader (Revvity, Lier, Belgium). On each plate, we added a negative and positive control and four dilutions of a serum sample with a known international units (IU)/ml. The parallel line method (PLL) was used to calculate HPV16-specific IgG concentrations for each sample [207].

2.5 Pseudovirion based neutralization assay (PBNA)

Since we performed a PBNA using two different reporters, we produced two PsV stocks, one with an enhanced green fluorescent protein (EGFP) reporter and the other with a Nanoluciferase (NLuc) reporter. All HPV16 PsV were produced using the protocol by Buck et al. [236] with minor adaptations. Briefly, 293TT HEK cells were cotransfected with the HPV16 L1L2 genes contained in the p16sheLL plasmid and either reporter plasmid phsNuc for NLuc or pCIneoEGFP for EGFP. pCIneoEGFP and p16sheLL were gifts from John Schiller and phsNuc was a gift from Christopher Buck [272,273]. The PsV maturation time for the EGFP PsV was 24 h and 48 h for NLuc PsV. After production, the PsV were titrated and specificity for HPV16 was confirmed with H16.V5 monoclonal antibodies kindly gifted by Dr. Neil Christensen [274].

For the PBNA, 293TT HEK cells were plated in a 96-well plate in 100 µl neutralization buffer (DMEM w/o phenol red, 10% heat-inactivated Fetal Bovine Serum (FBS), 1% glutamax, 1% nonessential amino acids, 1% Antibiotic-Antimycotic) approximately 4 hours before infection. The amount of preplated cells was 20,000 cells/well in a Cellvis black polystyrene glass bottom plate (Cellvis, California, U.S.A.) for EGFP PBNA and 30,000 cells/well in a tissue culture-treated flat bottom plate (Corning-Costar, New York, U.S.A) for NLuc PBNA. Outer wells were excluded to avoid evaporation and edge effects. Serum samples were 2-fold diluted starting at dilution 1:40 and enriched FVU samples were diluted 1:1, 1:2, 1:3, 1:4, 1:5, and 1:6 using neutralization buffer. Optiprep purified EGFP PsV stocks were diluted 1:480 to reach a minimum of 30% EGFP infection and NLuc PsV stocks were diluted 1:48000 to fall between the linear range of the multimode plate reader. 96 µl of diluted PsV stocks were combined with 24 µl of diluted samples and incubated on ice for 1 h. Additionally, positive neutralization controls (serum from a vaccinated volunteer and heparin (1 mg/ml)) and a negative control (serum from an unvaccinated volunteer that had no measurable HPV16 antibodies) were added to each plate. After incubation, the PsV antibody mixtures were incubated 5 more minutes at room temperature (RT) and 100 µl was added to the preplated cells. The plates were incubated at 37 °C and 5% CO_2 for 68-72 h.

2.5.1 High-throughput automated widefield microscopy – EGFP method

For EGFP measurement, 100 µl of supernatant was removed, and the cells were fixated for 20 minutes with 100 µl 4% paraformaldehyde (PFA) before immunofluorescent staining. Cells were washed twice with 200 µl 1X dPBS, permeabilized for 8 minutes using 100 µl of 0.5% Tergitol and washed once with 200 µl 1X dPBS. They were then incubated for 30 minutes at RT with 100 µl 0.02% cyanine5 (Cy5) in a 50% HI-FBS and 50% 1X dPBS mixture. After incubation, cells were washed twice with 200 µl 1X dPBS and incubated for 15 minutes at RT with 100 µl of 1 µg/ml 4′, 6-diamidino-2′-phenylindole, dihydrochloride (DAPI) in 1X dPBS. Cells were washed once more with 200 µl 1X dPBS and 200 µl PBS-NaN_3 was added before storing the plates at 4 °C until imaging. Imaging was performed on a fully automated Nikon Ti Eclipse inverted widefield fluorescence microscope, equipped with a Perfect Focus System and LED-based illumination source. For each well, 20 regions were scanned using a 20×/0.75 Plan Fluor dry lens. The illumination was fine-tuned to ensure minimal intensity fluctuation (<5%) across the entire field of view. For excitation of DAPI, EGFP, and Cy5, we employed 395/25 nm, 470/24 nm, and 640/30 nm LED illumination (Lumencor), respectively. Detection was

accomplished using a quadruple dichroic in conjunction with 435/26 nm, 510/40 and 705/72 nm bandpass filters, respectively, employing a DS-Qi2 CMOS camera.

FIJI image analysis freeware version 2.9.0, with the De Vos lab image analysis pipeline (https://github.com/DeVosLab/CellBlocks) was used for image analysis [275–277]. Infected cells were identified by applying a user-defined intensity threshold to the raw EGFP intensity or the EGFP intensity normalized to the average of control cells per independent experimental replicate. Based on this, the ratio of infected cells to the total number of cells was calculated using R statistical software version 4.2.2 (packages dplyr, car, tidyverse). The percentage of infected cells was used to calculate the 50% neutralization activity (effective concentration, EC50) using the 4-parameter curve fit for serum and linear fit for FVU in GraphPad Prism version 9.5.1. Samples were given a concentration if R^2 was above 0.85. Serum samples were considered positive if the HPV type-specific neutralization titer was ≥ 100, as described previously [164]. For FVU samples, only the curve fit and R^2 were used since no cutoff has been established yet.

2.5.2 Luminescence – Nluc method

For the luminescence method, after the 68-72 h plate incubation, 25 µl of the supernatant was used for further analysis using the Nano-Glo® Luciferase Assay kit (Promega, Madison,WI) and results were read on the Viktor Nivo multimode plate reader (Revvity, Lier, Belgium). The antibody concentrations showing 50% neutralization activity were calculated using the same method as described for the EGFP method.

2.6 HPV DNA detection

FVU samples were subjected to HPV DNA testing. HPV DNA testing was performed on 1 ml UCM buffered FVU using the Cobas 6800 (Roche Molecular System, Pleasanton, CF, USA) and the clinical cutoff for cervical samples at the Centre for Medical Analysis (CMA, Herentals, Belgium). If the Cobas 6800 test showed HR-HPV DNA positivity, they were genotyped using the Riatol qPCR HPV genotyping assay [278].

2.7 Statistical analysis

We used R statistical software version 4.2.2 to analyze the data. The data was first checked for their normality using Shapiro–Wilk test. If the data were normally distributed,

significant differences between parameters were examined using paired *t*-tests. Otherwise, non-parametric Wilcoxon signed-rank testing was used. Statistical significance was defined as *p*-adjusted <0.05 (using Holm–Bonferroni method for *p*-value adjustment). Interactions between demographic parameters and test outcomes (HPV DNA, HPV16-specific Abs, and HPV16-specific nAbs) were calculated using the 'lm' function. Receiver operating characteristic (ROC) analyses were done to determine the lowest DELFIA concentration expected to provide a nAb titer (if present) using the specific PBNA. Spearman rank correlation coefficients were calculated between different assays and sample types.

3 Results

3.1 Impact of patient characteristics on virological and immunological endpoints.

We collected paired FVU and serum samples from 50 healthy female volunteers, 25 vaccinated with an HPV vaccine and 25 unvaccinated (Figure 1). The overall median age was 23 (IQR 23–24). Participants received their first vaccine dose at a median age of 14 (IQR 12–14), and the median time between the first vaccine dose and sample collection was 10 (IQR 9–11) years. No interaction was observed between the time since vaccination and the antibody detection ($p \geq .81$). Among the vaccinated volunteers, 12% (3/25) received the 2vHPV vaccine, 84% (21/25) the 4vHPV vaccine, and 4% (1/25) the 9vHPV vaccine, with no significant differences in antibody concentrations based on vaccine type ($p \geq .07$).

Of all participants, 6% (3/50) were current smokers, 20% (10/50) had a smoking history, and 74% (37/50) had never smoked. No interaction was observed between smoking history and antibody concentrations ($p \geq .43$), but a positive association between smoking history and HPV DNA presence was found ($p = .01$). Overall, 64% (32/50) used oral contraceptives, 2% (1/50) used condoms, 18% (9/50) used a hormonal intrauterine device (IUD), 4% (2/50) used a local IUD, and 12% (6/50) used no contraceptive method. No significant interactions were observed between contraceptive use and HPV DNA or antibody presence ($p \geq .23$). Among participants, 62% (31/50) had undergone cervical sample collection, starting at a mean age of 18 (SD 0.96) years. All women (100%) reported being sexually active but had not given birth before inclusion. The mean number of lifetime sexual partners was 3 (IQR 1–6), with a highly significant interaction seen with

HPV DNA presence ($p \leq .0001$), while no interaction was observed with HPV16- specific antibodies ($p \geq .63$).

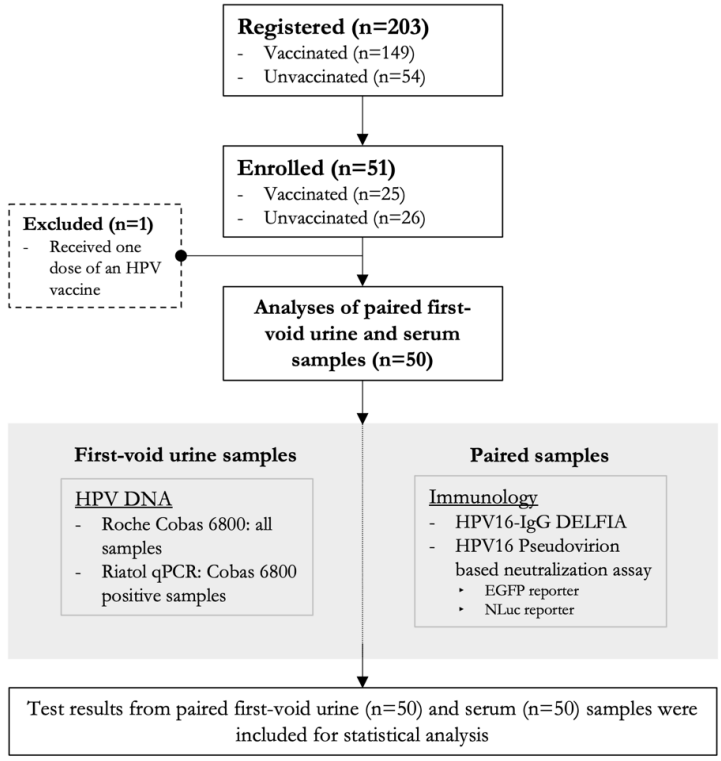

Figure 1: *Flow diagram of the study. Test results from 50 paired first-void urine and serum samples were included.*

The Cobas 6800 assay identified high-risk HPV (HR-HPV) infection in 22% (11/50) of women, including 24% (6/25) of vaccinated and 20% (5/25) of unvaccinated women. Subsequent genotyping using the Riatol qPCR revealed a 18% (9/50) HR-HPV infection rate, including 20% (5/25) of vaccinated and 16% (4/25) of unvaccinated women. Detected HR-HPV types were HPV51, 56, 58, 59, 66, 67, and 68 (Table 1), with no infections from HPV types included in the 2vHPV or 4vHPV vaccines.

Table 1: *Study population characteristics according to vaccination status.*

Characteristic	Total	Vaccinated	Unvaccinated
N (%)	50 (100)	25 (100)	25 (100)
Age (median, IQR)	23 (23-24)	23 (23-24)	24 (23-25)
Age of 1st vaccination (median, IQR)	13.5 (12.2-14.2)	13.5 (12.2-14.2)	
Vaccine type (n, %)			
2v HPV	3 (6)	3 (12)	
4v HPV	21 (42)	21 (84)	
9v HPV	1 (2)	1 (4)	
Ever smoked (n, %)			
Current yes	3 (6)	1 (4)	2 (8)
Past yes	10 (20)	5 (20)	5 (20)
No	38 (76)	19 (76)	18 (72)
Contraceptive use (n, %)			
Oral	32 (64)	18 (72)	14 (56)
condom	1 (2)	0 (0)	1 (4)
Hormonal IUD	9 (18)	4 (16)	5 (20)
Local IUD	2 (4)	1 (4)	1 (4)
None	6 (12)	2 (8)	4 (16)
Ever had a cervical smear (n, %)	31 (62)	17 (68)	14 (56)
Age at first cervical smear (mean, SD)	18 (0.96)	18 (0.71)	17 (1.41)
Number of sexual partners (median, IQR)	3 (1-6)	2 (2-6)	3 (1-6)
Cobas 6800 HPV DNA pos (n, %)	11 (22)	6 (24)	5 (20)
Riatol HPV DNA pos (n, %)	9 (18)	5 (20)	4 (16)
Detected HPV types	51, 56, 58, 59, 66, 67, 68	51, 59, 66, 67, 68	51, 56, 58, 67

3.2 HPV16-specific antibody titers in FVU correlate well with those in serum

We used an in-house HPV16 DELFIA assay to quantify HPV16-specific antibody concentrations. Establishing the LLOQ involved a dilution series (Supplementary Figure S1). The FVU LLOQ, set at 0.00803 IU/mL, was determined based on the lowest concentration with coefficient of variation (%CV) of counts <15%, and accuracy (measured as concordance between expected and PLL-calculated concentration) within the acceptable 80 to 120% range. All Amicon-filtered FVU samples were tested with 1:2 to 1:16 dilutions to precisely detect low antibody levels without excessive background interference. For serum, dilutions of 1:200 to 1:3200 were employed. The serum LLOQ, calculated using the formula $10^{*}SD_{Intercept}$/slope of the standard calibration curve, was 0.0325 IU/mL [279].

Out of all FVU samples, 62% (31/50) showed detectable HPV16 antibody concentrations (Table 2). Among the unvaccinated cohort, 24% (6/25) were antibody positive, while 100% (25/25) of vaccinated women demonstrated antibody positivity. The median (IQR) FVU HPV16 antibody concentration post-HPV vaccination was 0.047 (0.025–0.104)

IU/ml, and the geometric mean titer (GMT) for all positive samples was 0.14 (95% CI 0.03–0.24) (Figure 2). For serum samples, 58% (29/50) had detectable antibody concentrations, with 16% (4/25) of unvaccinated and 100% (25/25) of vaccinated women testing positive. The median (IQR) concentration of HPV16 antibodies in serum post-HPV vaccination was 86.5 (47.2–167.9) IU/ml, and the GMT for all positive samples was 174.04 (95% CI 31.50–317.00). Median concentrations and GMT observed in FVU were approximately 0.07% and 0.08% of those in serum, respectively.

Excellent correlations were established between HPV16-IgG concentration in serum and FVU for the overall cohort (r_s = 0.91) and the vaccinated cohort (r_s = 0.81). Furthermore, HPV16-IgG in FVU and serum remain detectable over ten years after HPV vaccination (Supplementary Figure S2). Additionally, a good correlation between serum and FVU HPV16-IgG levels was obtained for the unvaccinated cohort (r_s = 0.63) (Figure 3). Of the six HPV16-IgG positive FVU samples from the unvaccinated female volunteers, four also had detectable HPV16-IgG in serum and one had detectable HPV DNA for HPV51.

Table 2: *Results for HPV16-IgG using the DELFIA assay and HPV16-nAbs using the EGFP- and Nluc-based PBNAs for both FVU and serum samples. The percentage of samples containing detectable concentrations and the median (IQR) concentration are presented.*

Assay	Outcome	Total			Unvaccinated		Vaccinated	
		FVU	Serum	Ratio FVU/serum % (IQR)	FVU	Serum	FVU	Serum
DELFIA	Positive (%)	31/50 (62%)	29/50 (58%)		6/25 (24%)	4/25 (16%)	25/25 (100%)	25/25 (100%)
	HPV16-IgG (IU/mL)	0.012 (0.000-0.046)	19.6 (0.0-86.1)	0.07 (0.04-0.12)	0.000 (0.000–0.000)	0.0 (0.0–0.0)	0.047 (0.025–0.104)	86.45 (47.2–167.9)
EGFP PBNA	Positive (%)	10/50 (20%)	27/50 (54%)		2/25 (8%)	2/25 (8%)	8/25 (32%)	25/25 (100%)
	HPV16-nAbs EC50	0.00 (0.00-0.00)	197 (0.00-1457)	0.22 (0.09-0.33)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00-6.04)	1573 (774-4887)
Nluc PBNA	Positive (%)	19/50 (38%)	28/50 (56%)		1/25 (4%)	3/25 (12%)	18/25 (72%)	25/25 (100%)
	HPV16-Abs EC50	0.00 (0.00-6.65)	211 (0.00-2030)	0.34 (0.15-0.51)	0.00 (0.00–0.00)	0.000 (0.00–0.00)	5.69 (0.00-18.69)	1616 (520-6981)

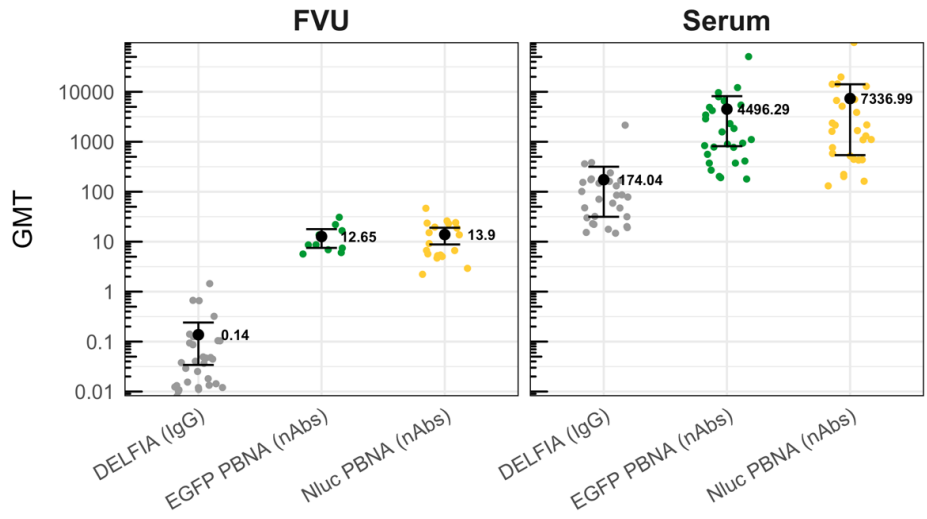

Figure 2: *HPV16 IgG (IU/mL) and nAb (EC50) geometric mean titers (GMT) and confidence intervals for only antibody positive samples are presented. Colored dots represent the concentration of one sample.*

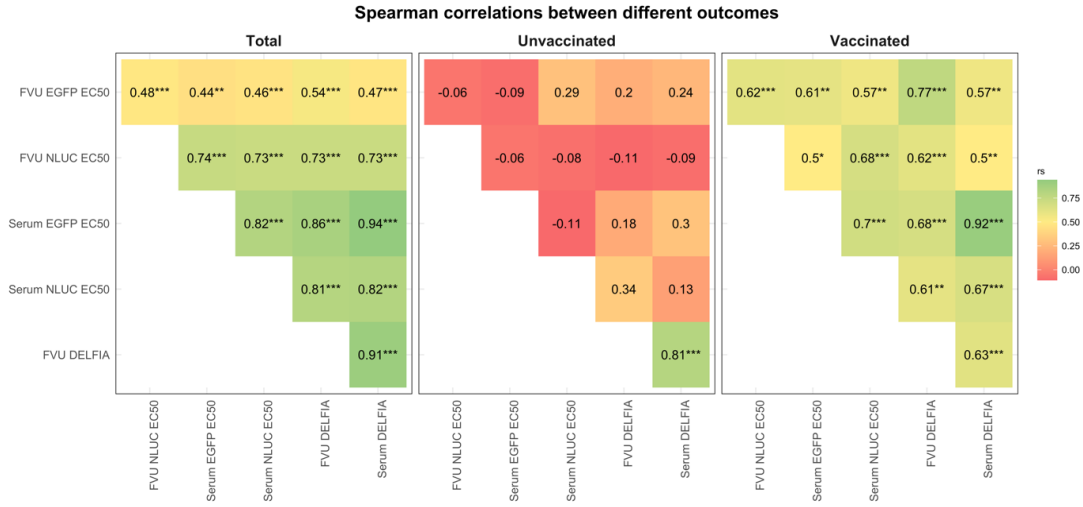

Figure 3: *Spearman rank correlations (r_s) between HPV16 antibody concentrations for all different assays and sample types. Significance levels are represented in the figure by an asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

3.3 Both PBNA assays show good reproducibility

To measure the neutralizing capacity of the HPV16-specific antibodies, we conducted two PBNA assays: one using fluorescence microscopy (EGFP method) and the other using spectrophotometry (NLuc method). Visual inspection of the EGFP method images revealed that increasing dilutions of samples from vaccinated individuals progressively increased the number of EGFP-positive cells (Figure 4). This quantitatively translated in infection and neutralization rates. We also evaluated the differences in nuclear count over all FVU and serum plates separately, resulting in a %CV of 28.9% for FVU and 26.9% for serum. As an internal control, we calculated the average nuclear count for each sample dilution and type, resulting in a %CV of 6.7% for FVU and 14.6% for serum.

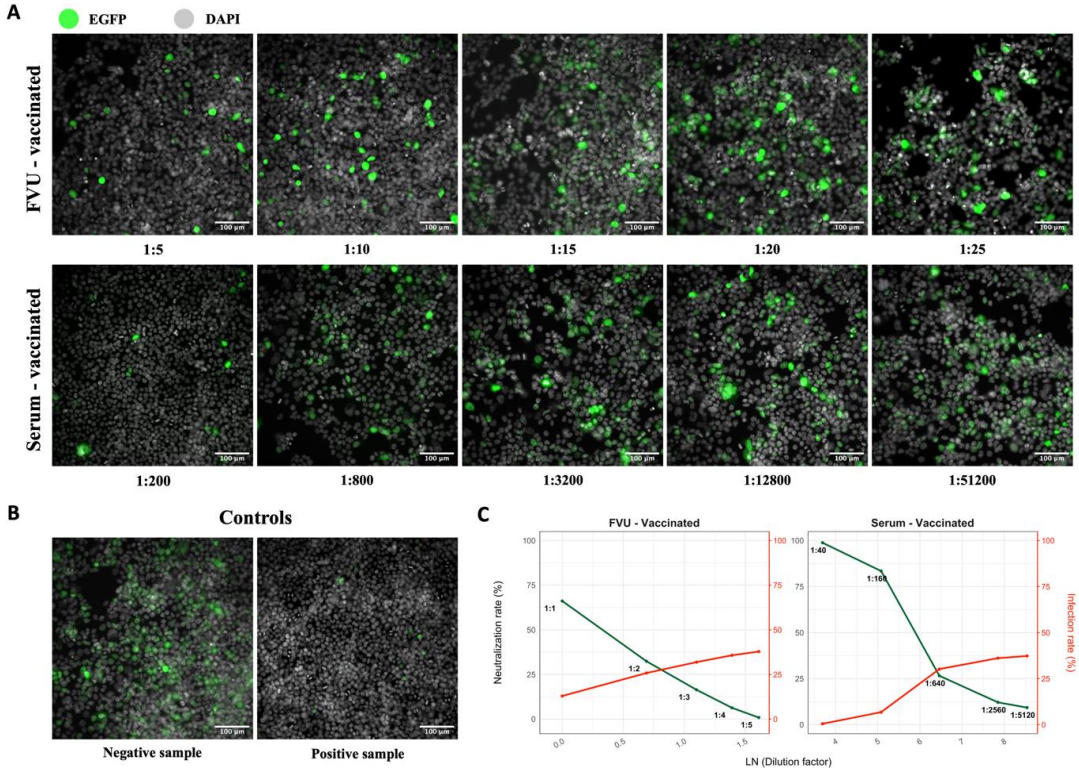

Figure 4: *(A) EGFP-based PBNA microscopic images for five dilutions of a FVU and serum sample from one vaccinated female volunteer and (B) control images of a serum sample with HPV16-specific nAbs (positive sample) and without (negative samples). The overlay images show EGFP signal (green) after HPV16-PsV infection and cell nuclei (gray). (C) Neutralization rate (%) and infection rate (%) for the specific sample is presented for each dilution. Neutralization rate was calculated by comparing the infection rate to the infection rate of the positive control. Infection rate was calculated by dividing the number of cells expressing EGFP by the number of cell nuclei.*

On each PBNA plate, four wells were allocated for control samples used to determine the neutralizing titers. Median %CV among the controls on individual plates was 8.5% for the EGFP method and 7.4% for Nluc (Figure 5(A)). The %CV for the average of the controls across plates was 3.5% for the EGFP method and 35.4% for the Nluc method (Figure 5(B)). Median neutralization percentage for each dilution for positive samples were plotted (Figure 5(C,D)).

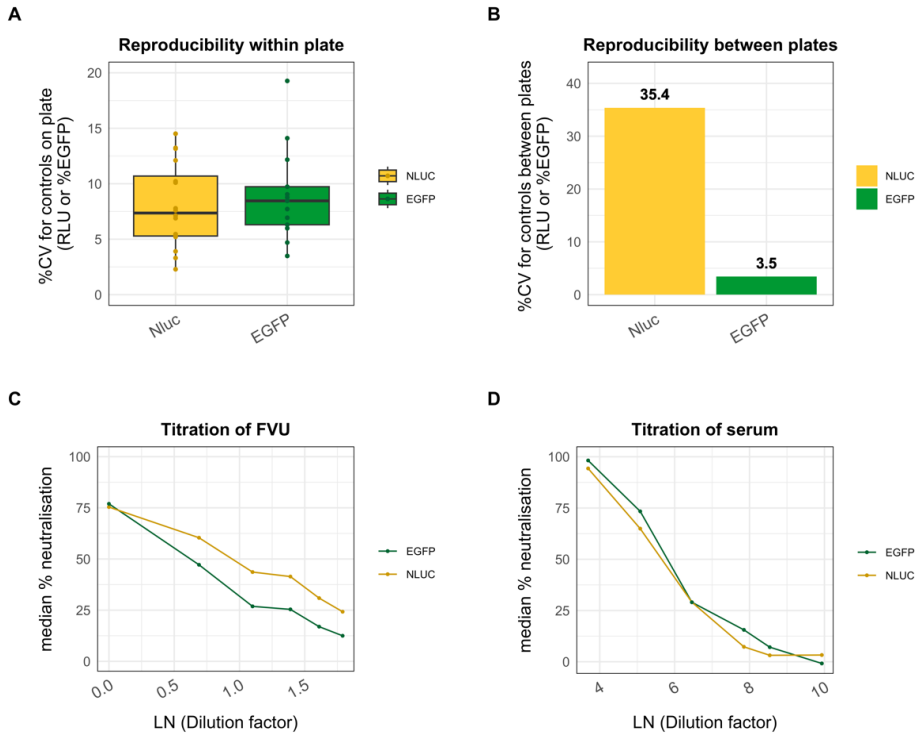

Figure 5: *PBNA reproducibility and titration results for both readouts. (A) Reproducibility of controls within plates and (B) between plates. The number of plates tested for using Nluc PsV was 18 and for EGFP 13 plates were analyzed. (C, D) Median values of each point on the titration curves for FVU and serum samples are presented for both PBNA readouts.*

3.4 PBNA protocols identify neutralizing antibody capacity with varying sensitivity and specificity

For serum samples, the EGFP method detected EC50 concentrations in 54% (27/50) of samples, while the Nluc-based assay detected concentrations in 56% (28/50) of samples (Table 2). Notably, 8% (2/25) of unvaccinated and 100% (25/25) of vaccinated women exhibited detectable titers in their serum samples using the EGFP method, and 12% (3/25) of unvaccinated and 100% (25/25) vaccinated women using the Nluc method. The median

(IQR) EC50 titer after vaccination for serum samples was 1573 (774–4,887) for EGFP and 1,616 (520–6,981) for Nluc. The GMT for all positive serum samples was 4496 (95% CI 816–8177) for EGFP and 7,337 (95% CI 540–14,134) for Nluc.

Using the EGFP method, 20% (10/50) of FVU samples had EC50 titers, while the Nluc method detected titers in 38% (19/50). Among positive samples, 7 showed positivity with both methods. Specifically, 8% (2/25) of unvaccinated and 32% (8/25) of vaccinated women exhibited detectable titers in FVU samples using the EGFP method, and 4% (1/25) of unvaccinated and 72% (18/25) vaccinated women using the Nluc method. After vaccination, the median (IQR) EC50 titer for FVU samples using the EGFP method was 0.00 (0.00–6.04), and 5.69 (0.00–18.69) for the Nluc method. The GMT for all positive FVU samples was 12.65 (95% CI 7.51–17.80) for EGFP and 13.90 (95% CI 8.80–19.00) for Nluc.

Comparing FVU to serum samples, using the EGFP method, the median EC50 concentrations and GMT in FVU were around 0.22% and 0.28% of those in serum. Similarly, employing the Nluc method, the median EC50 concentrations and GMT in FVU were approximately 0.34% and 0.19% of those found in serum.

3.5 HPV16-specific neutralizing antibody titers correlate with HPV16-IgG in FVU and serum samples

Spearman rank tests were used to analyze the correlation among various assays and sample types for the entire group and separately for vaccinated and unvaccinated women (Figure 3). For serum, the highest correlation was found between EGFP HPV16 EC50 nAb and DELFIA HPV16-IgG concentrations ($r_s = 0.94$). For FVU, the highest significant Spearman rank correlation coefficient was found between Nluc HPV16 EC50 and DELFIA HPV16-IgG concentrations ($r_s = 0.73$).

To explore the feasibility of establishing a DELFIA IU/ml concentration cutoff for the PBNA assay, we conducted ROC analyses. These analyses, using the empirical rule (ER) and concordance probability method (CZ), revealed optimal cutoff titers of 0.047 IU/ml for EGFP, with a sensitivity of 0.8 and specificity of 0.9, and 0.0168 IU/ml for Nluc, with a sensitivity of 0.84 and specificity of 0.87 (Supplementary Figure S3) [280]. We visually represented the correlations between different PBNA assays and DELFIA assays for both

FVU and serum samples (Figure 6) and provide an overview figure of all results (Supplementary Figure S4).

An excellent correlation (r_s = 0.91) was observed for HPV16 DELFIA concentrations between FVU and serum. For EGFP and Nluc HPV16 EC50 nAb concentrations, the correlations between FVU and serum were 0.44 and 0.73, respectively. In the vaccinated cohort, similar trends as the total cohort were observed (Figure 3). Notably, in this subgroup, the correlation coefficients between FVU and serum samples for EGFP (r_s = 0.61) and Nluc (r_s = 0.68) HPV16 EC50 nAb concentrations showed less variability.

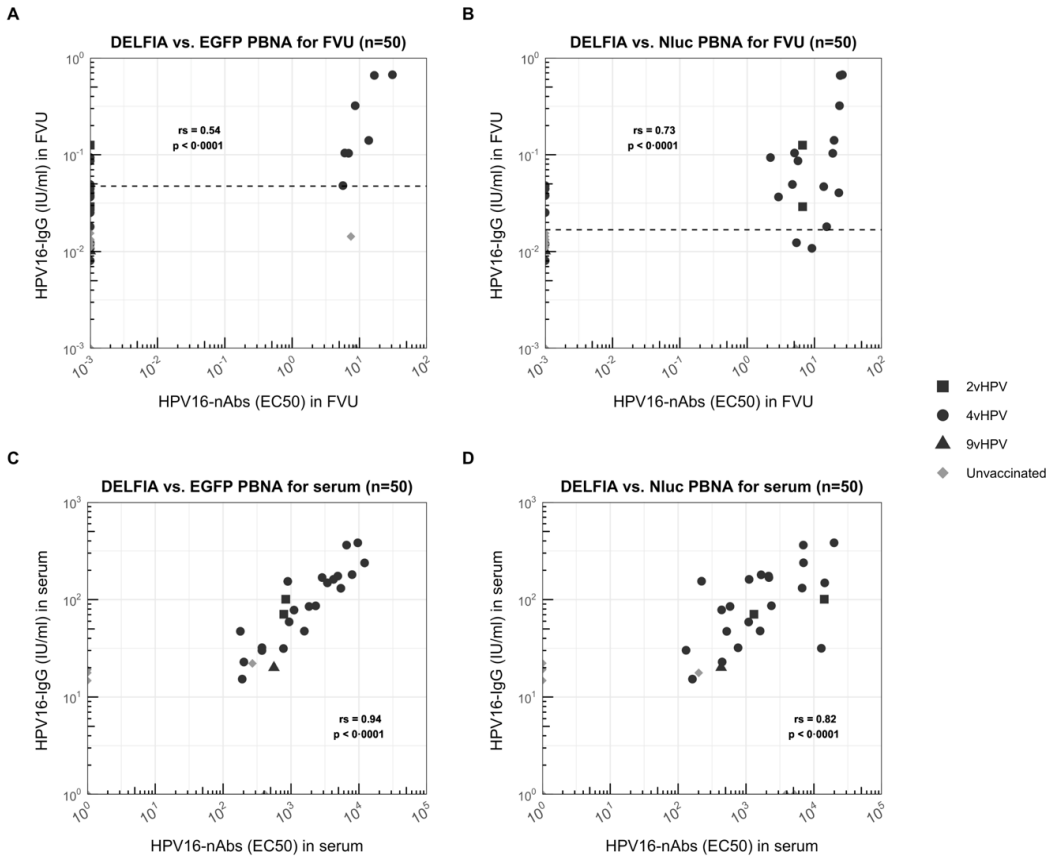

Figure 6: *Correlation curves for the HPV16-IgG and HPV16-nAb concentrations quantified using the two different PBNA assays. (A, B) Correlation curves between FVU samples. The ROC analyses determined the IU/ml optimal cut point, with highest sensitivity and specificity from where nAbs in FVU are detectable using the PBNA with EGFP or Nluc method. This cutoff is presented as a dotted line. (C, D) Correlation curves between serum samples. Spearman rank correlation coefficients are presented in the figures. The color of the dots is dependent on the vaccination status and shapes are dependent on the vaccine type used.*

4 Discussion

The humoral immune response following HPV vaccination or infection is important, and antibody analysis is vital in understanding the protection mechanism [75,79]. Traditionally, antibody quantification has relied on blood samples, requiring minimally invasive procedures that may not fully capture local immune responses. FVU sampling, however, presents a noninvasive alternative that holds promise, particularly in the context of the female genital tract [33]. With FVU, the initial part of the urine stream is collected, acting as a rinsing liquid to capture the accumulated female genital tract secretions around the labia minora [19]. FVU also offers opportunities in settings where cultural preferences or concerns about discomfort make vaginal swabs less desirable or for sampling younger women who have not yet become sexually active [33,281]. Recent studies confirmed the presence of HPV-specific vaccine- or infection-induced antibodies in this sample type [49–51]. This study provides first evidence that antibodies non-invasively obtained from this local source have neutralizing abilities. Two orthogonal PBNA readout methods were used and compared, demonstrating good correlations between HPV16-nAb concentrations in FVU and serum, as well as with HPV16-specific IgG levels in both sample types. This breakthrough not only highlights the potential of FVU as a valuable and accessible sample for immunological studies but also has implications for diagnostics and therapeutic advancement [150,151].

As described previously and shown in this study, HPV16-specific IgG concentrations in FVU are at least three logs lower than those detected in serum samples [49–51]. Therefore, highly sensitive immunoassays become crucial. For HPV16-IgG, we used our in-house developed DELFIA assay, able to detect concentrations until 0.00803 IU/ml and of which in-house experiments showed strong correlations with established HPV-specific immunoassays like M9ELISA, M4ELISA, and GST-L1-MIA [164,166,282]. The DELFIA assay offers superior sensitivity and a broader dynamic range than traditional ELISA due to the prolonged luminescence of lanthanides [217]. In this study, we were able to demonstrate excellent correlations between HPV16-specific IgG concentration in FVU and serum (r_s = 0.91) using this HPV16 DELFIA assay. Since the golden standard for nAb detection is still a PBNA, we amended the well-described PBNA protocol from Pastrana et al. to adequately detect low concentrations of nAbs [261]. Additionally, we introduced a novel EGFP method, utilizing automated microscopy, and compared it with an Nluc-based method. The EGFP method calculated infection ratios irrespective of the variations in cell count across wells [275]. Given the observed differences in cell count among plates (%CV > 26.9) and sample

dilutions (%CV > 6.7), correcting for cell count is crucial when quantifying neutralization, particularly for low antibody titers in FVU.

Both PBNA protocols confirmed the presence of HPV-specific neutralizing antibodies in both FVU and serum, with significant correlations established between the sample types. However, using the current protocols, the Nluc method provided better results for FVU samples. Using the Nluc-based PBNA, 72% of FVU samples from vaccinated women exhibited a detectable nAb titer, compared to 32% for the EGFP method. Additionally, the correlation between the FVU Nluc-based PBNA concentrations and the FVU DELFIA (r_s = 0.73) or the serum Nluc-based PBNA (r_s = 0.73) were higher than for the EGFP method ($r_s \geq 0.54$). For both assays, the GMT of only the nAb positive FVU samples was similar. For serum samples, both PBNA readouts provided comparable results, but the EGFP method exhibited a better correlation with the HPV16-IgG assay, indicating slightly improved specificity. The EGFP method demonstrated significantly lower %CV across different plates, emphasizing enhanced reproducibility, likely attributed to the incorporation of cell count correction – a distinct and valuable advantage.

In the unvaccinated cohort, only four serum samples showed detectable naturally induced HPV16-specific antibodies. However, a higher number of FVU samples (n = 6) tested positive for HPV16-IgG before vaccination. Variations in memory B cell isotypes might explain these differences in antibody detection between sample types [283]. Memory B cell activation, crucial for producing high-quality antibodies, occurs not only after vaccination but also to some extent after natural infection [238,284]. The isotypes of memory B cells can vary between individuals, and the locally present spectrum of B cells overlaps with, but is distinct from, systemic B-cells [237]. This makes it particularly intriguing to investigate local immune responses, detectable in first-void urine. Furthermore, our study highlights the limited neutralizing capacity of these naturally induced antibodies, aligning with existing literature. Notably, among the six HPV16-IgG positive FVU samples from unvaccinated volunteers, only one or two exhibited neutralizing antibodies, depending on the PBNA method.

While achieving the detection and quantification of HPV16-specific neutralizing antibodies in FVU is significant, opportunities for further optimization persist. Various steps in the PBNA protocol were optimized, including diverse antibody enrichment and isolation methods. Given the substantial impurities in FVU, sample purification was essential to prevent contamination of the cells in the PBNA. Methods such as protein G magnetic bead

purification and melon gel purification yielded purified samples but resulted in nonspecific quantification of nAbs due to interference with HPV PsV infectivity. Desalting and buffer exchange failed to resolve the issue. Ultimately, overcoming aspecific interference with the infection was achieved through a method combining Amicon filtration with a medium-speed centrifugation step. We attempted further optimization by comparing various concentrate collection volumes following Amicon filtration. However, capturing the concentrate in volumes lower than 500 µl or utilizing a reversible Amicon® Ultra-2 device led to aspecific inhibition of infection, mirroring challenges encountered in other purification methods. In addition to the optimization of the enrichment protocol, various dilution series and PsV-sample ratios were explored. This evaluation led to the identification of a dilution series where the lowest concentrations were reliably detectable, as used in this study. Exploring an alternative to the current EC50 calculation method, requiring fewer dilutions of a certain sample, might be another avenue for quantifying lower nAb levels. While our current capabilities allow for detection and quantification of HPV16-specific neutralizing antibodies in FVU, further optimizations of purification and protocol methodologies are crucial to refine our understanding and ensure accurate detection at even lower concentrations.

While the units differ between DELFIA and PBNA assays, we compared the ratio of nAbs for both sample types by dividing the GMT of nAbs by the GMT of IgG. For FVU, this ratio was for 90 for the EGFP method and 99 for Nluc, and for serum, this was 26 for EGFP and 42 for Nluc. Additionally, the ratio of FVU/serum was higher for the HPV16-nAbs measured using the PBNAs (≥ 0.22) compared to the HPV16-IgG using the DELFIA (0.07). This indicates that the fraction of HPV16-specific antibodies that are neutralizing is higher in FVU compared to serum, again showing the added value of using this sample to investigate the specific local humoral immune response.

The primary limitation of this study is the relatively small sample size due to the exploratory nature of the study. Although the findings on feasibility and methodology provide valuable insights and directions for future research, there is a need for larger studies, especially including larger cohorts of samples with measurable local nAbs. The complexity of FVU samples and low antibody concentrations necessitates ongoing protocol refinement. Additionally, the lack of identical units between the DELFIA and the PBNA-derived concentrations introduces a potential source of variability in the interpretation of results. Furthermore, results are only established for HPV16-specific

antibodies, and the neutralizing capacity of antibodies against other HPV types needs to be investigated.

This study reveals that HPV16-specific antibodies in noninvasive FVU samples maintain their neutralizing capacity and can be detected up to ten years after vaccination. Two PBNA methods, based on fluorescence (EGFP) and luminescence (Nluc) provided results supporting that FVU is a robust sample for immunological studies. The Nluc method shows a higher sensitivity for FVU samples, but the EGFP method showed better specificity for serum and overall higher reproducibility for detecting neutralizing antibodies. While acknowledging current achievements, future studies are essential for fully leveraging FVU's potential in advancing our understanding of HPV-specific immune responses. The findings of this study highlight the importance of future research related to this topic, including larger cohort studies.

5 Supplementary materials

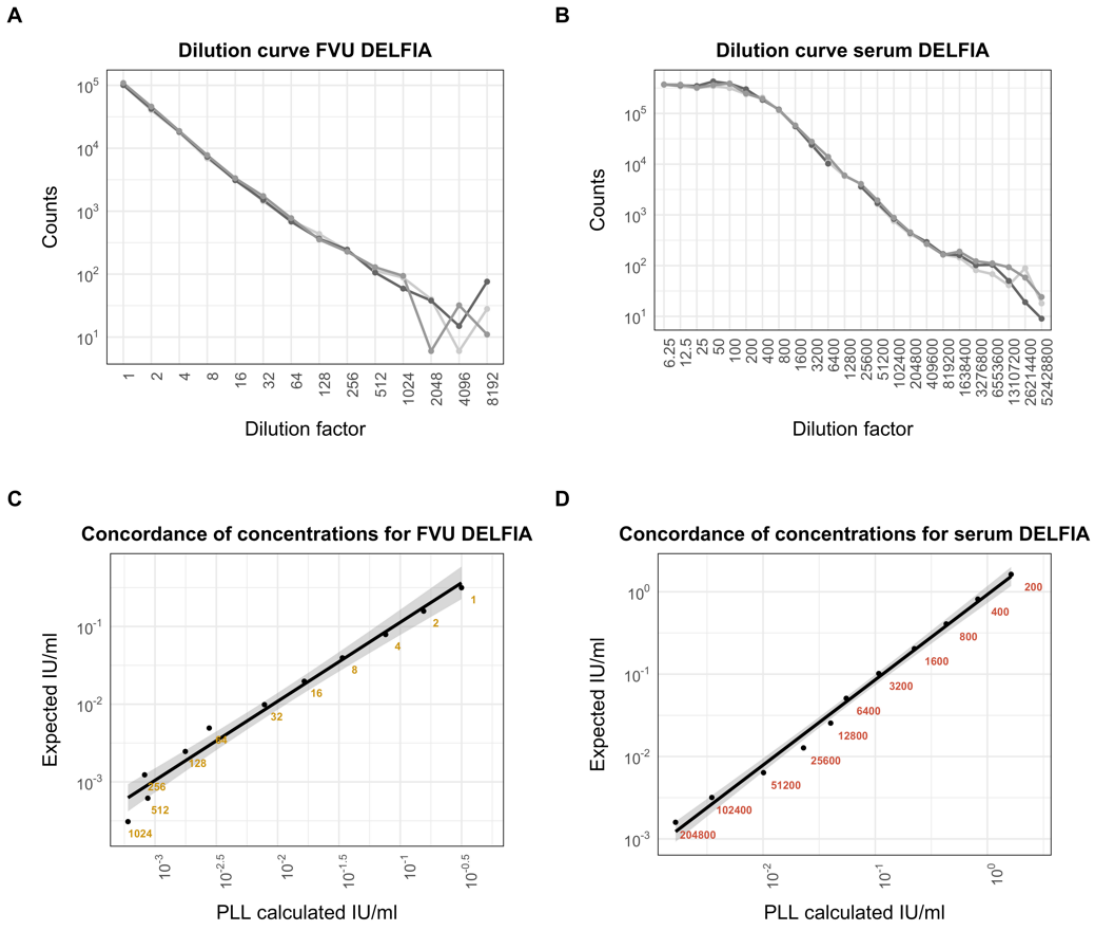

***Figure S1**: DELFIA dilution curves for (A) FVU and (B) serum samples. The concordance between the PLL calculated IU/mL and expected IU/mL concentration (based on known start concentration and dilutions) are presented for (C) FVU and (D) serum samples.*

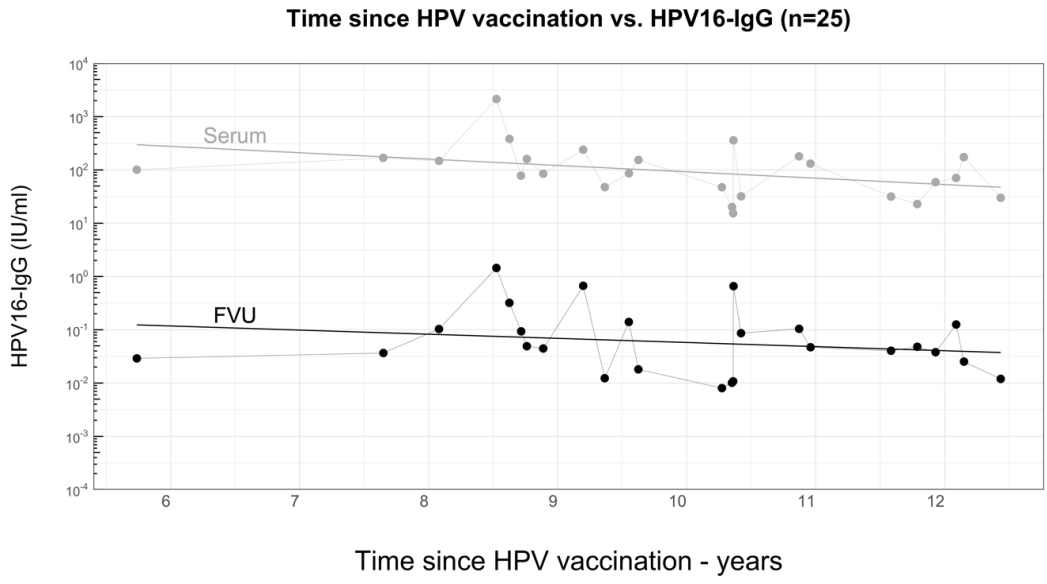

Figure S2: *HPV16-IgG (IU/ml) concentrations over the time since receiving HPV vaccination (years) for all 25 included vaccinated female volunteers. Results for serum are presented in gray (top), and results for FVU are presented in black (bottom). The plot includes regression lines to depict trends over time.*

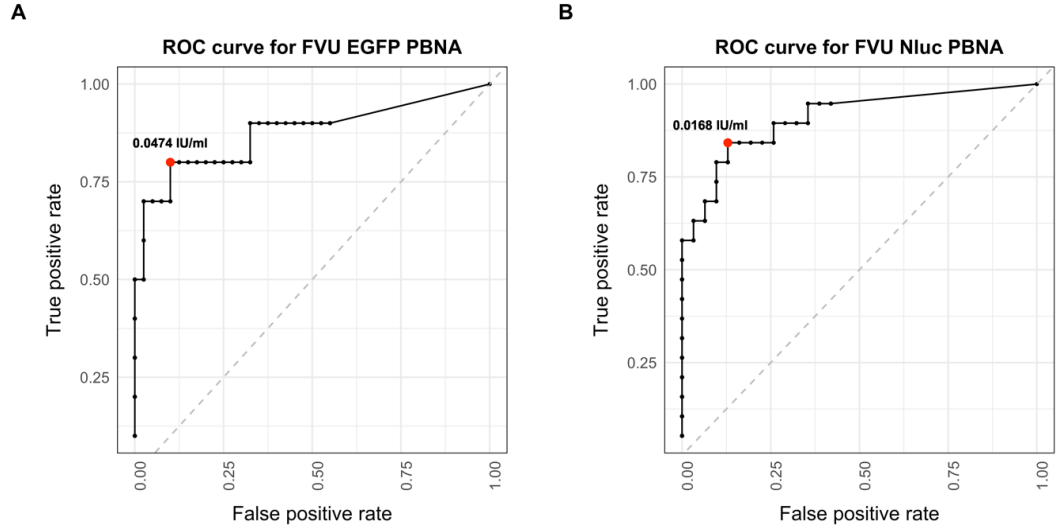

Figure S3: *ROC analysis to determine the lower limit of IU/ml concentration from where nAbs in FVU are detectable using the EGFP or Nluc method. The optimal cut point, with highest sensitivity and specificity, is shown by a red dot.*

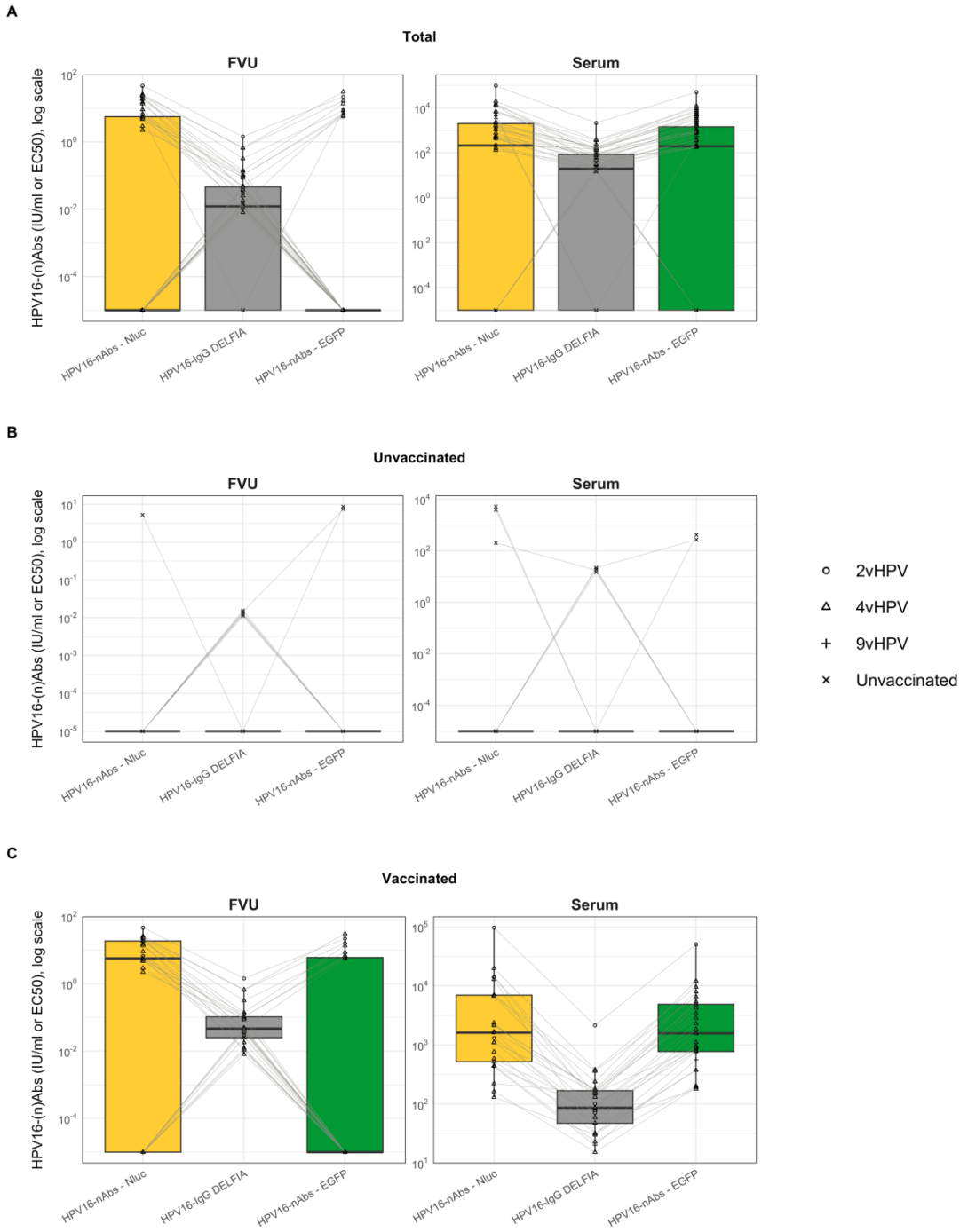

Figure S2: *HPV16 IgG and nAb concentrations for all measurement methods and both sample types.*

CHAPTER

04–B

Concentration strategies for spiked and naturally present biomarkers in non-invasively collected first-void urine.

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Published in European Journal of Medical Research, February 2024.

Abstract

Background

First-void urine (FVU) provides a non-invasive method for collecting a wide range of biomarkers found in genital tract secretions. To optimize biomarker collection in FVU, this study investigated the impact of naturally present and supplemented precipitating agents: uromodulin (UMOD) and polyethylene glycol (PEG), on the concentration of human papillomavirus (HPV) pseudovirions (PsV), cell-free DNA (cfDNA), and cellular genomic DNA (gDNA) through centrifugation.

Methods

FVU samples from ten healthy female volunteers, along with a control sample, were spiked with seal herpesvirus 1 (PhHV-1) DNA, HPV16 plasmid DNA, and HPV16 PsV with an enhanced green fluorescent protein (EGFP) reporter. The samples were subjected to various concentration protocols involving PEG precipitation, low-speed centrifugation (5 min at 1000×*g*), and medium-speed centrifugation (1 h at 3000×*g*). Subsequently, quantitative PCR (qPCR) was used to assess cellular and cell-free glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA, cell-free PhHV-1 and HPV16 DNA, and PsV (EGFP) DNA. In addition, UMOD levels were measured.

Results

The findings revealed that PEG significantly increased the concentration of cfDNA and gDNA in the pellet after centrifugation, with the most pronounced effect observed for cfDNA. Moreover, low-speed centrifugation without PEG effectively depleted cellular gDNA while preserving cfDNA in the supernatants. Pseudovirions were consistently pelleted, even with low-speed centrifugation, and a positive but not significant effect of PEG on PsV (EGFP) DNA yield in the pellet was observed. Additionally, a significant correlation was observed between UMOD and GAPDH, HPV16, and PsV (EGFP) DNA quantities in the pellet. Furthermore, large variations among the FVU samples were observed.

Conclusions

With this study, we provide novel insights into how various biomarker precipitation protocols, including both the properties of FVU and the use of PEG as a precipitating agent, influence the concentration of cfDNA, cellular gDNA, and pseudovirions.

1 Introduction

Secretions originating from the female genital tract (FGT), which include cervical mucus, proteins, pathogens, and other biomarkers, contain essential biological information [285,286]. These secretions can be captured by the first stream of urine, known as first-void urine (FVU) [19,171]. FVU is a non-invasive and convenient sample that can be collected by individuals from home, making it a practical and accessible option for population-level studies [35,46,158].

For human papillomavirus (HPV) based studies, FVU sampling has demonstrated added value as it allows for the detection of virological (HPV DNA), diagnostic (methylation markers), and immunological (HPV-type specific antibodies) endpoints [38,48–51,287]. Given that cervical cancer, primarily caused by HPV, remains the fourth most common cancer in women worldwide, large epidemiological trials are warranted, and the impact and effect of vaccinating adults needs to be elucidated [151,288]. Using FVU as a source of local biomarkers at the site of infection simplifies studies in this context. In addition to DNA and antibodies, intact HPV virions are hypothesized to be present in FVU, enabling the investigation of antibody–virion interactions [151,289]. Moreover, FVU sampling holds potential beyond HPV-related research, containing valuable information on sexually transmitted infections (STIs) and serving as a biomarker source for cancer-related research [17,26,27,66,290,291].

FVU captures proteins, DNA, metabolites, viral particles, bacteria, immune cells and (debris of) exfoliating cells [16,51,292,293]. However, targeting the secretions of interest can be challenging as certain biomarkers are present in only small amounts. Therefore, concentrating the sample is essential for identifying all the information of interest [41], and various methods, including centrifugation/precipitation, have been explored for this purpose [18,63].

Urine contains a variety of proteins that may influence the detection and concentration of biomarkers of interest, with uromodulin (UMOD), also called Tamm–Horsfall protein (THP), being a notable example [294,295]. UMOD is produced primarily by the kidneys, plays a role in kidney function, and is considered the most abundant protein in urine [296]. The UMOD in urine self-assembles into large, linear polymers, known as uromodulin filaments, which may interact with other proteins and biomarkers in urine. This makes it interesting to study the effect of UMOD on biomarker precipitation [297]. Another potential

precipitating agent that is not present in urine is polyethylene glycol (PEG), which induces the formation of protein aggregates by altering the solution conditions, leading to changes in protein solubility and promoting protein precipitation [298–300]. However, the effect of PEG and UMOD on the precipitation of (DNA) biomarkers in FVU has not yet been investigated.

In addition to the complexity introduced by sample variations, different types of DNA in FVU may react, bind, and concentrate in various ways. While cellular genomic DNA (gDNA) or large proteins and cellular debris present in FVU may be more prone to precipitate and interact with internal or external precipitating agents, cell-free DNA (cfDNA), which is smaller in size, may remain unaffected. Furthermore, DNA captured in viral particles is larger in size than cell-free DNA (cfDNA) and might also affect precipitation. Thus, to evaluate the effect of specific parameters on the precipitation and concentration of biomarkers, it is essential to investigate different types of DNA.

In this study, we evaluated the effects of different concentration protocols on the precipitation of pseudovirion (PsV) encapsulated DNA (enhanced green fluorescent protein, EGFP), spiked cfDNA (seal herpesvirus 1 (PhHV-1) and HPV16 plasmid), and a combination of human cfDNA and gDNA (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Furthermore, the effects of precipitating agents UMOD and PEG on the concentration protocols were evaluated. Consequently, the results of this study provide essential information on the optimal precipitation protocols for the detection and concentration of different DNA biomarkers and PsV from FVU.

2 Materials and methods

2.1 Sample collection

We collected FVU samples from ten healthy female volunteers at the Centre for the Evaluation of Vaccination, University of Antwerp, Belgium. Each female volunteer collected one first-void urine sample using a 20 ml Colli-pee first-void urine collection device (Novosanis, Belgium) prefilled with 1/3 urine conservation medium (UCM), resulting in the collection of 13.67 ml FVU directly preserved in 6.33 ml of UCM. The samples were divided into 1-ml aliquots and immediately stored at – 80 °C before further analysis. We obtained informed consent from all volunteers, and the data and samples were

coded to ensure participant privacy. This study was approved by the Institutional Review Board of UZA/University of Antwerp (B300201734129).

2.2 Sample processing

Aliquots of 1 ml FVU and control samples, consisting of 0.33 ml UCM and 0.67 ml dPBS, were used. For each ID and the control samples, 6 aliquots were spiked with (I) 0.74 ng/µl HPV16 PsV, (II) 16.7 µl of a 1:1000 dilution of PhHV-1 DNA, and (III) 5×10^6 copies/ml HPV16 plasmid DNA. HPV16 PsV were produced based on the protocol of Buck et al. with enhanced green fluorescent protein (EGFP) serving as a reporter plasmid [236]. A PhHV-1 stock was created by extracting DNA from 200 µl MEM culture medium from a PhHV-1 infected Crandell Rees Feline kidney cell line using NucliSENS® EasyMag® (bioMérieux, off-board lysis protocol) and elution in 100 µl. Commercially available plasmids containing the HPV16 genome were used (Clonit, Milan, Italy). One 1 ml aliquot of each ID and the control sample was not spiked with DNA. In total, six aliquots of each sample were used for sample concentration based on the different protocols described below (Figure 1), and one aliquot (spiked) was used for baseline analysis.

2.2.1 Low-speed centrifugation

Two aliquots of each sample were centrifuged for 5 min at 1000×*g* at 4 °C (LSC). After centrifugation, the supernatant was removed, whereof 50 µl was collected for direct DNA extraction and the remaining volume of supernatant, approximately 900 µl, was used for PEG precipitation. The pellet was resuspended in dPBS to reach a final volume of 50 µl and was stored for direct DNA extraction.

2.2.2 PEG precipitation

2.2.2.1 PEG stock preparation

We prepared a 50 ml stock of 20% (w/v) PEG6000 solution by adding 10 g of PEG6000 and 0.5 M NaCl to 50 ml dH_2O. A 50 ml stock of 16% (w/v) PEG20000 was prepared by adding 8 g of PEG20000 and 0.5 M NaCl to 50 ml dH_2O. Both stocks were stored for a maximum of 1 week at 4 °C until use.

2.2.2.2 Medium-speed centrifugation

A 1-ml aliquot and one aliquot of 900 µl supernatant after low-speed centrifugation (2.2.1) were diluted with an equal amount of 20% (w/v) PEG6000 solution to obtain a final concentration of 10% (w/v) PEG6000. The same procedure was used for the 16% (w/v) PEG20000 solution, which was added in equal amounts to a 1-ml aliquot and one aliquot of 900 µl supernatant after low-speed centrifugation (2.2.1) to obtain a final concentration of 8% PEG20000. The samples were incubated with PEG at 4 °C for 16 h. Additionally, one 1-ml aliquot with spiked DNA (Spiked–No PEG, Sp–NP) and one without (Unspiked–No PEG, Usp–NP), were incubated at 4 °C for 16 h without the addition of PEG. After incubation, all 6 aliquots were centrifuged at 3000×*g* for 1 h at 4 °C. The supernatant was collected, and 50 µl was used for DNA extraction. The pellet was resuspended in dPBS to reach a final volume of 500 µl.

2.3 DNA extraction and qPCR

In this experiment, various purifications (both supernatant and pellet) and baseline aliquots were subjected to quantitative PCR (qPCR) to detect specific biomarker DNA [GAPDH, PhHV-1, HPV16, and PsV (EGFP)]. For all the samples and purifications, 50 µl was added to 2 ml of NucliSENS Lysis Buffer (bioMérieux Benelux, Schaarbeek, Belgium). DNA extraction was performed using NucliSENS® EasyMag® (bioMérieux) with an off-board lysis generic protocol and elution in 55 µl. The LightCycler480 Real-Time PCR instrument was used (Roche Diagnostics, Machelen, Belgium) following the protocol described by Vorsters et al. (2014) [169] . Briefly, for GAPDH and HPV16, a 20-µl portion of the PCR mixture containing 1×LightCycler® 480 Probes Master (Roche Applied Science, Belgium), 0.5 µM of each primer, 0.1 µM of the probe, and 5 µl of DNA solution was loaded into the LightCycler. For PsV (EGFP), qPCR was performed on 20 µl of a mixture containing 1×LightCycler® 480 Probes Master (Roche Applied Science, Belgium), 0.25 µM forward primer, 0.25 µM reverse primer, 0.2 µM probe, and 5 µl DNA solution. For PhHV-1, qPCR was performed on 20 µl of a mixture containing 1×LightCycler® 480 Probes Master (Roche Applied Science, Belgium), 0.05 µM forward primer, 0.2 µM reverse primer, 0.1 µM probe, and 5 µl sample. The GAPDH, PhHV-1, and HPV16 plasmid primers and probes used have been published previously [186,190,301]. For EGFP, the following primers were used; EGFP-F: CACTACCTGAGCACCCAGTC, EGFP-R: CACGAACTCCAGCAGGACCATG, and EGFP-TM: F-CGCTTCTCGTTGGGGTCTT

TGCT–Q. The lengths of the amplification products were 156 bp for GAPDH, 89 bp for PhHV-1, 81 bp for HPV16, and 58 bp for EGFP. The thermal cycling protocol consisted of the following steps: initial activation of DNA polymerase at 95 °C for 10 min; 45 cycles of denaturation at 95 °C for 10 s; and annealing at 60 °C for 15 s. To ensure reproducibility, both positive and negative controls were included in each run. DNA concentrations for each parameter were calculated based on the Cq values.

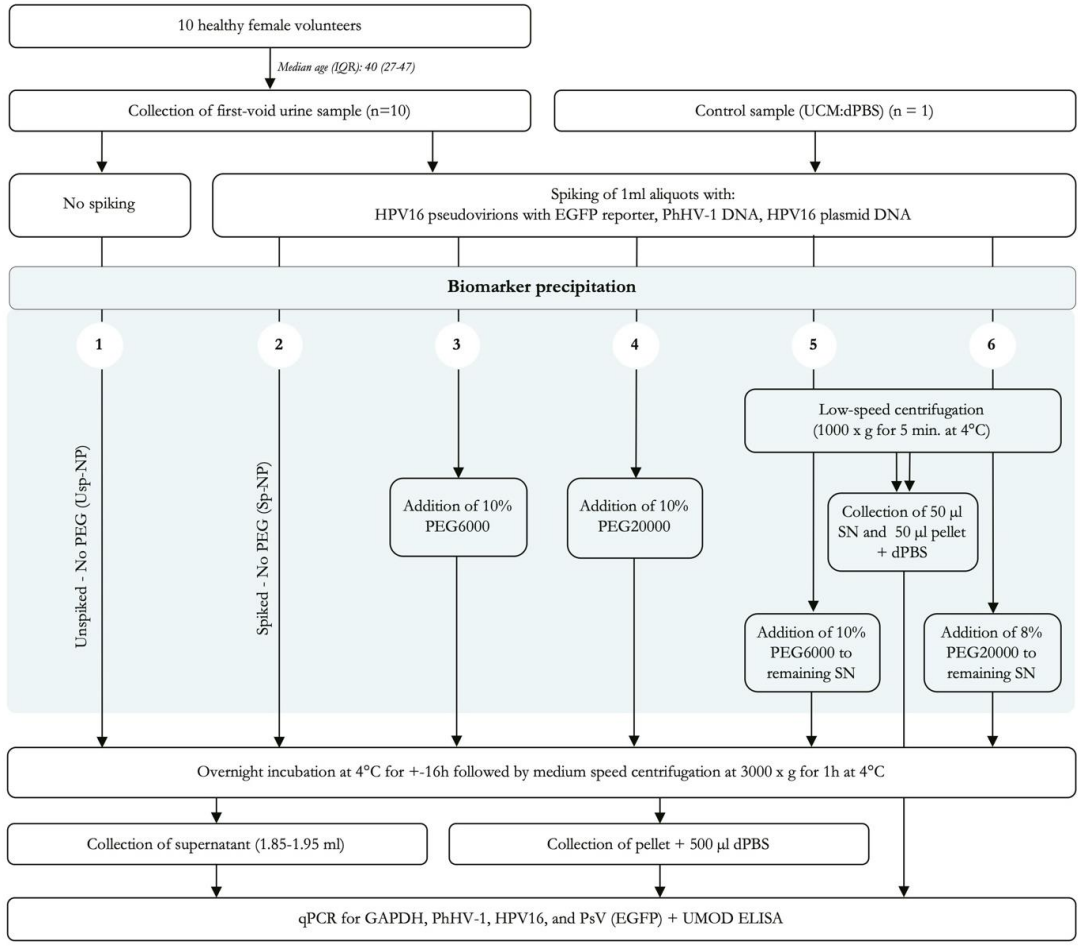


Figure 1: *Schematic overview. First-void urine (FVU) samples were collected by 10 female volunteers. Aliquots of 1 ml of the FVU the control sample were spiked using HPV16 PsV, PhHV-1 DNA, and HPV16 plasmid DNA. Also, one aliquot was not spiked. All samples underwent biomarker precipitation using six different protocols; (1) precipitation of unspiked samples without PEG (Usp-NP), (2) precipitation of spiked samples without PEG (Sp-NP), (3) precipitation using 10% (w/v) PEG6000, (4) precipitation using 8% (w/v) PEG20000, (5) low-speed centrifugation followed by precipitation of the supernatant using 10% (w/v) PEG6000, and (6) low-speed centrifugation followed by precipitation of the supernatant using 8% (w/v) PEG20000. Supernatant and pellet were collected for all purifications and qPCR was performed for GAPDH, PhHV-1, HPV16, and PsV (EGFP). In addition, ELISA for UMOD was performed.*

For PsV (EGFP) and PhHV-1, the results are reported as arbitrary copies/ml as the concentration of the used standards were unknown. For GAPDH, results were reported as ng/ml, and for HPV16 as copies/ml. The following standard curves were used: EGFP: $y = -0.2997 * x + 14.496$, PhHV-1: $y = -0.301 * x + 13.614$, GAPDH: $y = -3.657 * x + 41.60$, HPV16: $y = -0.2997 * x + 12.816$, with x = log(concentration) and y = Cq. All standard curves had an R^2 close to one (>0.98). Results are converged to quantity (ng, copies, or arbitrary copies) of DNA for further analysis, taking the volumes and dilutions into account.

2.4 UMOD ELISA

UMOD protein concentrations were measured using a Uromodulin Huma ELISA kit (BioVendor, Czech Republic) according to the manufacturer's instructions. Before analysis, baseline samples were centrifuged at 3820×*g* for 10 min at 20 °C using an Amicon Ultra-4 50K filter device (Merck Millipore). 1×dPBS (Gibco) was added to the concentrate retained on the filter to reach a final volume of 0.5 ml. All the other arms/fractions were not further processed. The dilutions to be tested were optimized for each sample type, and we selected 1:2000 for Amicon-filtered baseline samples, 1:1000 for supernatant and pellet samples. The absorbance at 450 nm and 630 nm was measured using the Victor Nivo multimode plate reader (Revvity, Belgium). The reference signal at 630 nm was subtracted from the signal at 450 nm for each well, and this measurement was used to calculate the concentration using four-parameter logistic (4PL) curve fitting. Results are calculated as ng/ml UMOD and converged to UMOD quantities (ng) in that specific arm and volume.

2.5 Statistical analysis

All the statistical analyses were performed using R statistical software version 4.2.2. All the data did not meet the normality assumption (Shapiro-Wilk test), and therefore the non-parametric Wilcoxon signed-rank test was applied to check for significant differences between arms and fractions. Statistical significance was determined as p-adjusted < 0.05, and the Holm–Bonferroni method was used for adjusting the p-values. To explore potential correlations between UMOD and DNA amount in certain fractions, Spearman's rank correlations were calculated.

3 Results

For this study, FVU samples from ten female volunteers and a control sample were spiked with (I) seal herpesvirus 1 (PhHV-1) DNA, which is a nonhuman viral control [41,186]; (II) HPV16 plasmid DNA, which consists of small, circular pieces of double-stranded DNA; and (III) HPV16 pseudovirions consisting of EGFP reporter DNA to compare different concentration protocols. In addition to the spiked DNA, human cell-free and genomic cellular GAPDH present in FVU was used as an additional parameter. Since the control sample did not contain human DNA, this sample was excluded for GAPDH analysis.

3.1 Effect of PEG on concentrating DNA

For each supernatant, pellet and baseline sample, DNA extraction was performed, and the concentration was determined or calculated using standard curves. To evaluate the effect of PEG and centrifugation on the concentration of specific DNA biomarkers, we calculated the amount of biomarker DNA in that specific part/sample, taking the volumes and dilutions into account. Based on pilot experiments, we compared two different PEG conditions on the pelleting of several biomarkers and compared this to the yields without PEG addition. Furthermore, we evaluated the effect of a low-speed centrifugation step on the concentration of DNA. The amount of DNA for these arms and fractions is presented as boxplots (Figure 2), and median (IQR) quantities are summarized (Table 1). For the Sp-NP arm, PhHV-1 and HPV16 plasmid DNA was most abundant in the supernatant, and PsV (EGFP) and GAPDH were most abundant in the pellet. Both PEG conditions had a positive effect on the yield of GAPDH, PhHV-1, and HPV16 DNA in the pellet after centrifuging for 1h at 3000×g, as the amount of pelleted DNA was significantly higher after PEG addition ($p \leq 0.04$).

Table 1: *Overview of DNA and UMOD quantities (median, IQR) in different fractions of various arms.*

	GAPDH (ng)	PhHV-1 (arbitrary copies)	HPV16 (DNA copies)	PsV (EGFP) (arbitrary copies)	UMOD (ng)
No PEG					
Baseline	311 (76 - 807)	1709 (1380 - 2120)	1330 (1110 - 1635)	189500 (125000 - 404500)	7.62 (4.71 - 10.84)
Low-speed centrifugation					
Pellet	193 (41 - 388)	109 (52 - 638)	78 (58 - 255)	74000 (48138 - 107125)	
Supernatant	23 (12 - 55)	1131 (961 - 1473)	979 (861 - 1223)	50730 (32728 - 130388)	
Unspiked–No PEG					
Pellet	193 (51 - 756)	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)	
Supernatant	14 (12 - 42)	0 (0 - 0)	0 (0 - 0)	0 (0 - 0)	
Spiked–No PEG					
Pellet	314 (80 - 786)	277 (66 - 984)	162 (98 - 1068)	177500 (142250 - 350000)	13.62 (8.19 - 17.98)
Supernatant	11 (7 - 35)	768 (674 - 1169)	766 (408 - 1102)	20093 (11329 - 29403)	2.01 (1.80 - 2.64)
PEG					
PEG6000					
Pellet	384 (104 - 1244)	928 (709- 2064)	1415 (133 - 1584)	266750 (211750 - 697125)	12.69 (4.36 - 21.33)
Supernatant	1 (0 - 3)	118 (18 - 294)	47 (10 - 79)	2106 (1331 - 2642)	2.36 (1.49 - 3.35)
PEG20000					
Pellet	486 (154 - 1323)	1188 (617 - 2130)	1290 (1103 - 1468)	197500 (180375 - 224375)	5.43 (3.49 - 19.35)
Supernatant	0 (0-3)	346 (0 - 768)	65 (17 - 86)	2447 (1149 - 5572)	2.94 (1.98 - 4.00)
Low-speed centrifugation + PEG6000					
Pellet	20 (11 - 43)	630 (226 - 943)	643 (549 - 1011)	67500 (51063 - 90750)	0.3 (0.19 - 0.36)
Supernatant	2 (0 - 3)	224 (129 - 522)	116 (59 - 185)	2485 (841 - 4870)	1.80 (1.30 - 2.09)
Low-speed centrifugation + PEG20000					
Pellet	15 (12 - 42)	620 (386 - 775)	615 (410 - 771)	67100 (21750 - 106500)	0.23 (0.16 - 0.31)
Supernatant	3 (1 - 3)	226 (141 - 366)	211 (115 - 246)	9380 (6387 - 12034)	2.48 (1.21 - 3.14)

No PEG was added to the baseline samples, the low-speed centrifugation (5min centrifugation at 1000×g), and the unspiked and spiked–No PEG arms (1h centrifugation at 3000×g). Two different PEG precipitation protocols were evaluated separately or combined with the low-speed centrifugation step.

For PsV (EGFP), the addition of both PEG6000 and PEG20000 caused an increase in the quantity of DNA in the pellet, but this change was not statistically significant ($p \geq 0.17$). Additionally, when PEG was used, the amount of DNA in the pellet was significantly higher than in supernatant ($p \leq 0.0068$) for all arms except for the PEG20000 arm on PhHV-1 ($p = 0.48$). The difference between the pellet and supernatant for the samples that were centrifuged at low-speed for 5 min at 1000×g before PEG precipitation was significant for GAPDH, HPV16, and PsV (EGFP) ($p \leq 0.028$) but not for PhHV-1 ($p \geq 0.13$) DNA. Overall DNA quantities were lower for all the arms including a low-speed centrifugation step (Table 1). Altogether, the difference between the amount of DNA in the pellet and the supernatant was most significant for the PEG6000 precipitation arms ($p \leq 0.0068$), although there was no significant difference in the concentration of DNA in the pellet between PEG6000 and PEG20000 ($p \geq 0.28$).

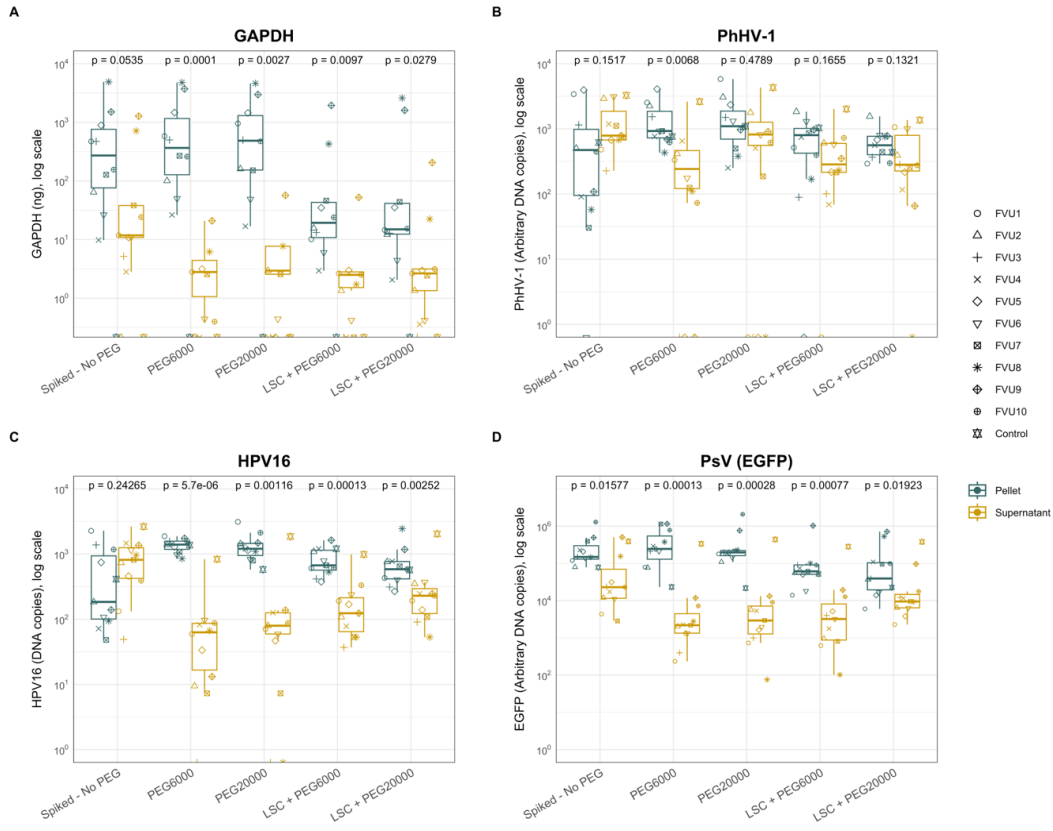


Figure 2: *Boxplots representing the amount of A) GAPDH; B) PhHV-1; C) HPV16; and D) PsV (EGFP) DNA in the respective samples. Results are presented as nanogram (ng), arbitrary DNA copies, and DNA copies. The spiked–No PEG arm did not undergo PEG treatment, whereas the other arms were incubated with 10% (w/v) PEG6000 or 8% (w/v) PEG20000 for approximately 16h at 4 °C. All samples underwent centrifugation at 3000×g at 4 °C for 1h and the low-speed centrifugation (LSC) samples underwent low-speed centrifugation without PEG addition at 1000×g for 5 min before subsequent PEG precipitation. Significant differences between pellet and supernatant for each arm are presented in the figure.*

3.2 Effect of centrifugation protocol on concentrating DNA

We compared the effects of two different centrifugation protocols on the presence of DNA in each fraction (Figure 3). Samples were centrifuged at 1000×*g* for 5 min (low-speed centrifugation) or at 3000×*g* for 1h. Since both centrifugation protocols were performed for both PEG precipitation protocols, two data points per ID were included for these arms. For PhHV-1 and HPV16, DNA was more abundant in the supernatant for both conditions, whereas for GAPDH and EGFP (PsV), the pellet contained the majority of the DNA. For all biomarkers, increasing the centrifugal force and centrifugation time resulted in higher

DNA quantities in the pellet. This increase was significant for GAPDH, HPV16, and EGFP (PsV) ($p \leq 0.0098$) but not for PhHV-1 ($p \geq 0.064$).

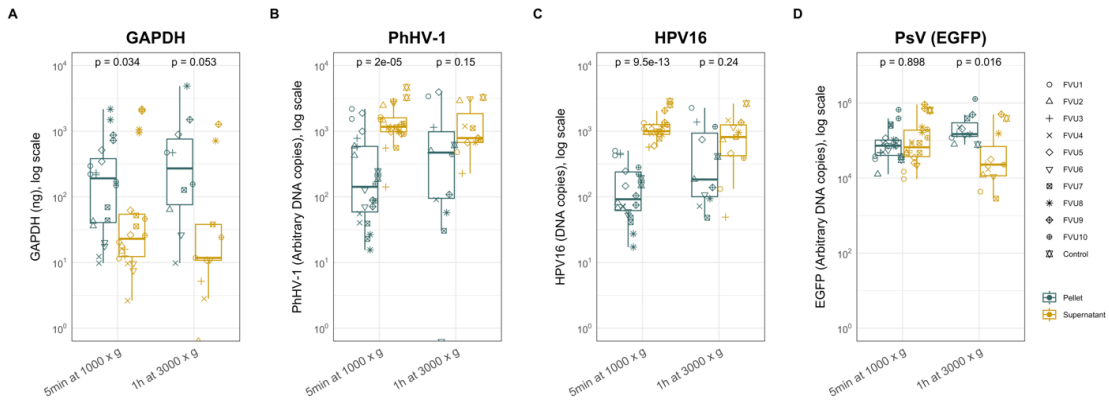


Figure 3: *Boxplots representing the amount of A) GAPDH; B) PhHV-1; C) HPV16; and D) PsV (EGFP) DNA in the respective samples after various centrifugation steps. Results are presented as ng, arbitrary copies, and DNA copies. All 1 ml samples were spiked with the same amount of PhHV-1 DNA, HPV16 plasmid DNA and HPV16 PsV (EGFP). Samples underwent centrifugation at 1000×g at 4 °C for 5 min or at 3000×g at 4 °C for 1h. Centrifugation at 1000×g was performed before both PEG precipitation protocols, resulting in two data points per ID for this arm. Significant differences between pellet and supernatant for centrifugation condition are presented in the figure.*

3.3 The effect of UMOD

UMOD concentrations were measured for specific arms and baseline samples (Table 1). We observed no significant correlations between the respective DNA biomarker quantity and UMOD in the baseline samples (Additional file 1). Additionally, no significant correlations were observed between the quantity of UMOD at baseline and the amount of pelleted DNA for any of the biomarkers and all arms ($p \geq 0.08$). However, combining all acquired pellet data on DNA and UMOD quantities, we did observe a significant correlation between the amount of GAPDH ($r_s = 0.54$, $p = 0.0001$) and PsV (EGFP) ($r_s = 0.62$, $p < 0.0001$) DNA and the amount of UMOD in the pellet (Figure 4). This was not observed for PhHV-1 or HPV16 DNA ($p \geq 0.2$). Analyzing only the pellet data of the arms with PEG precipitation, thus excluding the Sp-NP arm, we found significant correlations between the levels of GAPDH ($r_s = 0.59$, $p = 0.0002$), HPV16 ($r_s = 0.51$, $p = 0.002$), and PsV (EGFP) ($r_s = 0.66$, $p < 0.0001$) and the amount of UMOD in the pellet but not for PhHV-1 DNA ($p = 0.07$) (Figure 4). The median (IQR) amount of UMOD was higher in pellet than in the supernatant for the Sp-NP arm, or for the two PEG arms without low-

speed centrifugation (Table 1). However, for the arms with low-speed centrifugation, the remaining UMOD in the sample was higher in the supernatant than in the pellet. The amount of UMOD in the baseline sample significantly correlated with the amount of pelleted UMOD for the PEG arms without LSC ($r_s \geq 0.82$, $p \leq 0.006$) but not in the Sp-NP or LSC + PEG arms ($r_s \leq 0.50$, $p \geq 0.14$).

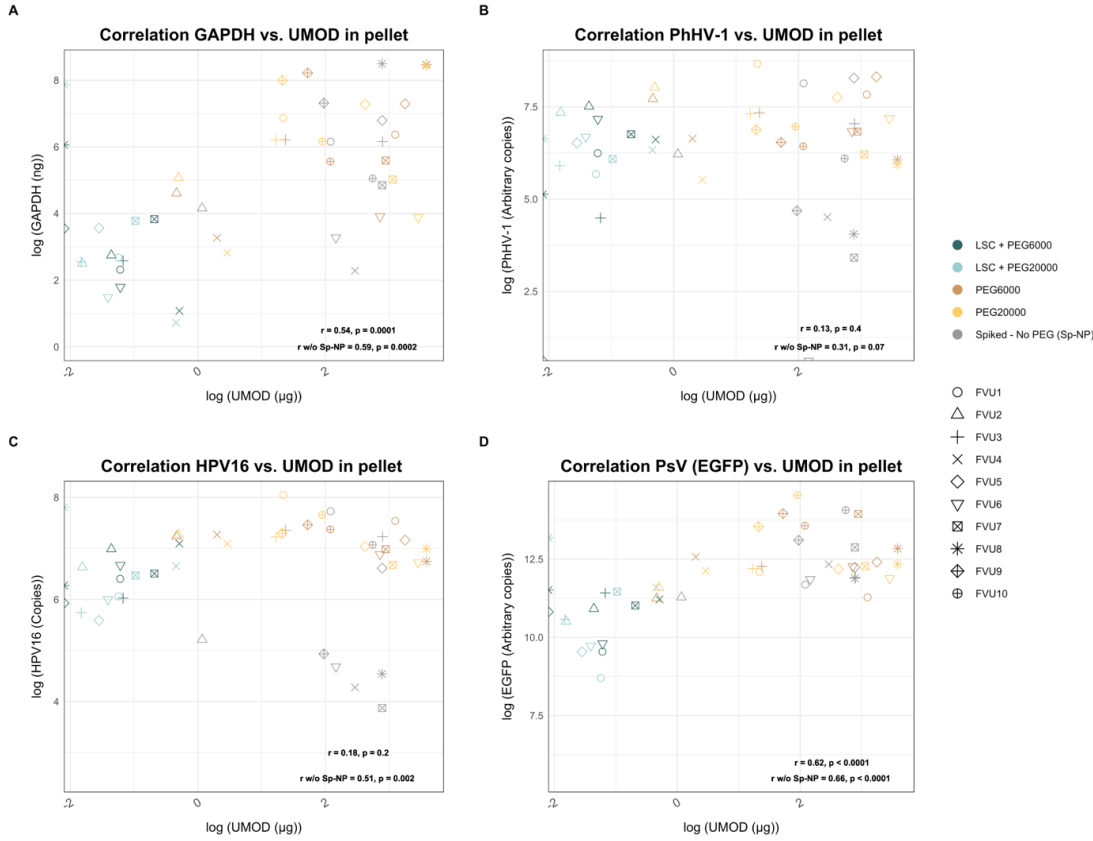


Figure 4: *Correlation plots between the amount of A) GAPDH; B) PhHV-1; C) HPV16; and D) PsV (EGFP) DNA and UMOD in the pellet. Spearman rank correlation coefficients are presented in the figure with, and without (w/o) inclusion of the Sp-NP arm data.*

4 Discussion

Non-invasive sampling can be the solution to reach a larger population for screening, epidemiological trials, and vaccine studies, among others. An example of a non-invasive sample of the urogenital tract is FVU. Secretions of the uterine, cervical, and vaginal epithelium accumulate between the labia minora, are washed away during urination, and are concentrated in the first urine void [25]. In the past decades, studies have proven that FVU has vast advantages for HPV-related research, screening, and vaccine follow-up in women [7,35,38,51,157,302]. Additionally, this non-invasive sample type could play a crucial role in cervical cancer treatment strategies involving immune checkpoint inhibitors, which has also gained interest in the past few years [303,304]. Different timings of collection, sample volumes, and storage buffers for FVU have been evaluated, and good agreement has been observed with cervical and vaginal samples for virological endpoints and with serum for immunological endpoints [24,41,44,46,49,50,169]. In addition to HPV-related research, FVU samples could be used as a sample for other sexually transmitted infections (STIs) or as a biomarker source for cancer-related research [17,26,27,66,290,291].

In this study, we aimed to investigate the effect of an external (PEG) and internal (UMOD) FVU precipitating agent on the concentration of spiked viral cfDNA, human cfDNA and gDNA, and pseudovirion DNA. While one of the advantages of FVU sampling is the concentration of biomarkers of interest related to FGT secretions, other FGT impurities are also concentrated in the sample. These impurities might influence further downstream processing of the sample for future detection of possible biomarkers. As PEG is a known precipitating agent, we evaluated the effect of adding PEG to a FVU sample on the precipitation of cfDNA, gDNA and pseudovirion DNA. It is generally known that cfDNA remains in the supernatant while cellular gDNA precipitates [18,63]. Earlier studies have shown that both transrenal and locally shed DNA are present in FVU [27,66]. In HPV-related research for cervical cancer screening, the most important biomarker source is cellular gDNA originating from exfoliated cells of the cervix [305].

Looking at the results of our study, we observed a median 23% increase in precipitated gDNA (GAPDH) when the samples were incubated with PEG6000 before centrifugation and a 55% increase for PEG20000. Additionally, we found a correlation between the amount of UMOD in the pellet and the amount of GAPDH DNA in the pellet for the PEG arms. This correlation increased when the samples without PEG addition (Sp-NP) were

excluded from analysis, suggesting that there might be some synergy between the external precipitating agent PEG and the UMOD present in the urine. Since we did not observe a correlation between UMOD in the baseline samples and the precipitation of biomarkers, we hypothesize that the interaction between PEG and the precipitation of biomarkers is influenced not by the amount but by the structure and polymerization of UMOD [306,307].

The effect of precipitating agents on cfDNA is of great interest to investigate circulating tumor DNA (ctDNA), which is the tumor fraction of cfDNA. Therefore, we also evaluated various precipitation protocols on spiked PhHV-1 and HPV16 plasmid DNA. As cfDNA mostly remains in the supernatant, the influence of a precipitating agent on the location of the DNA is of interest. Here, we also checked whether depleting cellular DNA by a low-speed centrifugation step influenced the results. For both cfDNA markers, PEG addition indeed had a significant effect on precipitation, with median increases of 235% and 329% in pelleted PhHV-1 DNA, and of 773% and 696% in HPV16 plasmid DNA when the samples were incubated with PEG6000 and PEG20000 before centrifugation, respectively. These results clearly show the potential of the precipitating agent PEG on the concentration of cfDNA in FVU and therefore can be of great interest for novel research towards ctDNA in non-invasive urine samples. By adding a low-speed centrifugation step, we observed that 89% of the GAPDH and 41% of the PsV (EGFP) DNA was pelleted, while only 9% of the PhHV-1 and 7% of the HPV16 DNA were pelleted. This clearly shows the depletion of pseudovirion and cellular DNA after low-speed centrifugation. Performing PEG precipitation after low-speed centrifugation caused pelleting of 73% to 85% of the cfDNA but the amount of cfDNA in the pellet was lower (approximately 50%) when no low-speed centrifugation was performed before PEG precipitation. If a clean cfDNA sample is needed, adding this low-speed centrifugation step can be advantageous, although this step can reduce the cfDNA yield. For cell-free plasmid DNA (HPV16), we observed a correlation between UMOD and the pelleted DNA when PEG was added to the samples. This was, however, not observed for the PhHV-1 DNA. Although we did observe a significant correlation between pelleted PhHV-1 and HPV16 DNA for the Sp-NP and PEG6000 arms ($r_s \geq 0.82$, $p \leq 0.006$), there were differences in precipitation between the PhHV-1 and HPV16 plasmid DNA. These differences may be attributed to variations in DNA fragment size, where the presence of UMOD and PEG enhances DNA precipitation starting from a specific fragment size while having minor effects on smaller DNA fractions.

Another interesting biomarker that was investigated in this study was the DNA of HPV PsV, which was quantified by detecting the DNA of the included reporter (EGFP). It is

hypothesized that HPV virions are present in FVU samples although this is expected at low concentrations [151]. To be able to adequately detect virions and eventually use them to investigate infections, they need to be concentrated. In this study, we mimicked the presence of virus particles in FVU by spiking samples with HPV16 PsV. Our results clearly showed that pseudovirions pelleted during centrifugation. During low-speed centrifugation, 59% of the PsV (EGFP) DNA was present in the pellet, and when the sample was centrifuged for 1h at a faster speed, 90% of the PsV (EGFP) DNA was present in the pellet. We observed a slight increase in the amount of DNA in the pellet when PEG was added as a precipitating agent (99%); however, this change was not significant. A low-speed centrifugation step, to deplete cellular debris and sample impurities is, in this case, not advantageous as 59% of the pseudovirions will be lost. The amount of UMOD in the pellet also correlated significantly with the amount of PsV (EGFP) DNA in the pellet for both the precipitation arms with and without PEG.

An additional and important observation in this study was the difference in the effect of protocols among FVU samples. Each FVU sample has a different composition which also affects the concentration of biomarkers. When examining the samples individually, there was a large variation in the presence of spiked DNA in the pellet or supernatant. Although we spiked all the samples with the same amount of PhHV-1 DNA, HPV16 plasmid DNA, or HPV16 PsV, we did not observe the same trend in concentration for each sample. Therefore, it is essential to always include a minimal amount of samples while optimizing protocols. The UCM:dPBS control supports the fact that the urine sample composition affects the precipitation of biomarkers. For cfDNA, the control sample results closely aligned with the median values observed in the pellet arms. However, when examining PsV (EGFP), urine composition had a clear positive impact on precipitation as the quantities were the lowest for this control sample.

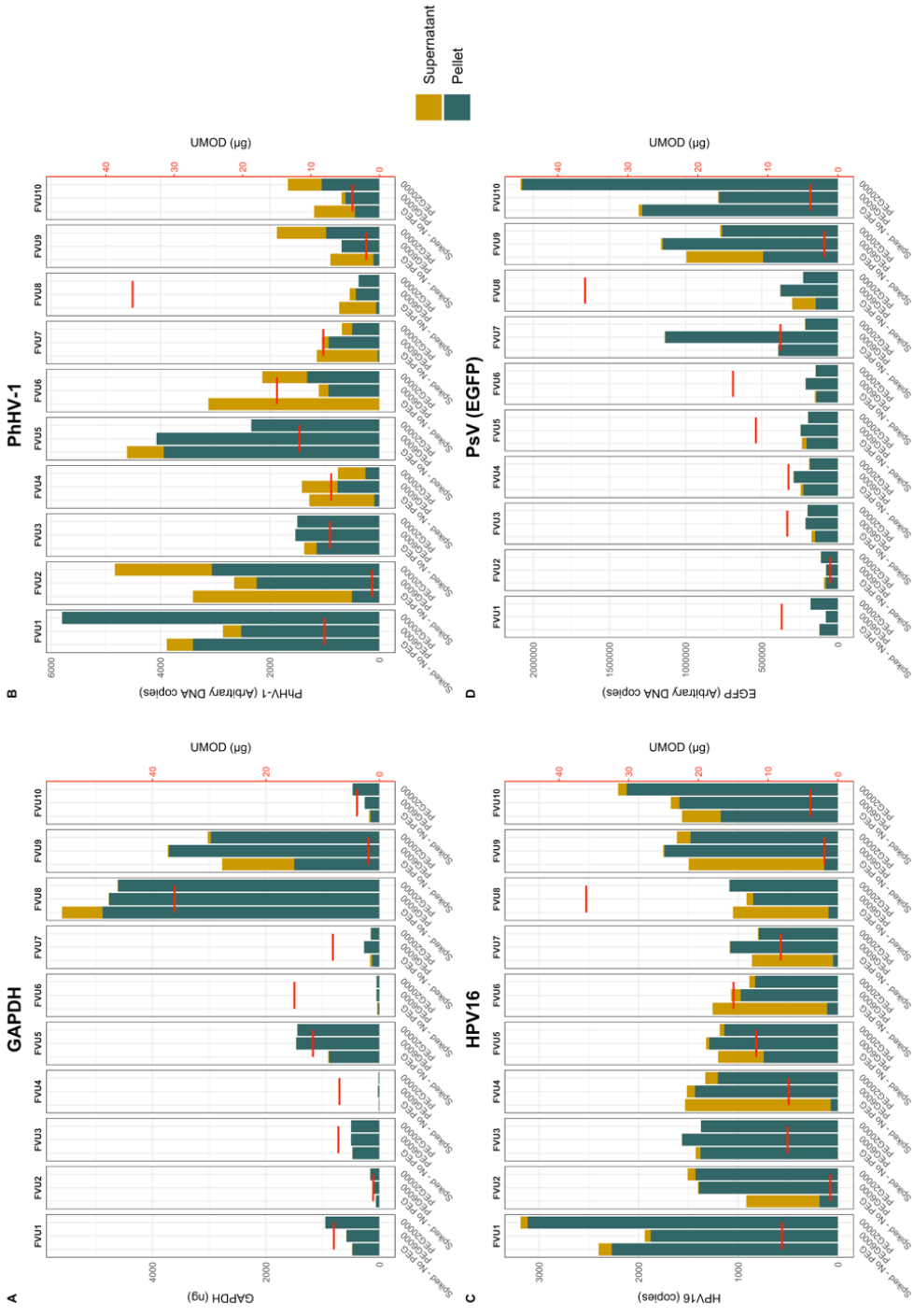
This study has certain limitations. The first limitation is the limited sample size of this study. However, the results clearly show heterogeneity among the different FVU samples while also showing significant effects of the various protocols, ensuring that the sample size is sufficient to support the interpretation of our results. Another limitation is the absence of true standard curves for PhHV-1 and PsV (EGFP) DNA. This limitation also had minimal effect on the data, as the goal of this study was to assess the effect of several protocols and precipitating agents on the presence of DNA. Additionally, spiking the FVU samples with PsV mimics the presence of HPV wild-type virions but is not identical since virions are not only freely present as particles but also encapsulated within cells. Furthermore,

extrapolation to urine collected in a different way or without buffer needs to be handled carefully as the storage and collection of the sample might influence the results. The buffer used aids in adjusting the urine sample to a near-neutral pH (between 6.04 and 6.95), which is required for PEG to precipitate and contains salt, known to enhance PEG precipitation [308]. Evaluating unbuffered urine samples may lead to different results.

5 Conclusion

In summary, this study presents compelling data regarding the effect of precipitation agents and protocols on the pelleting of cfDNA, cellular gDNA, and pseudovirions in non-invasively collected FVU. The results showed that PEG has a clear positive effect on the concentration of DNA in the pellet and that the effect was largest for cfDNA. A low-speed centrifugation step might also be helpful for depleting FVU of gDNA, pseudovirions, and other impurities when cfDNA is of interest. Additionally, the results showed that there might be an interaction between UMOD present in FVU and the added precipitating agent PEG, but the amount of UMOD in the baseline sample did not influence the concentration of biomarkers. The results of this study will help researchers choose and optimize protocols for biomarker DNA precipitation.

6 Supplementary materials



Additional file 1: *Histogram representing the amount of A) GAPDH; B) PhHV-1; C) HPV16; and D) PsV (EGFP) DNA in specific arms and fractions on the left y-axis and the amount of UMOD in the baseline sample (red line).*

CHAPTER

05

General discussion and future research

GENERAL DISCUSSION

In 2020, the World Health Organization (WHO) unveiled a global strategy for cervical cancer elimination [143]. Despite a notable decline in cervical cancer prevalence, it continues to rank as the fourth most common cancer in females globally, with approximately 604,000 new cases and 342,000 deaths reported in 2020. The three pillars to reach cervical cancer elimination are enhancing vaccination coverage (pillar 1) and improving access to screening (pillar 2) and treatment (pillar 3). Modeling predictions estimated that reaching the set targets would reduce the cervical cancer incidence rate by over 90% in 78 low- and lower-middle-income countries, preventing more than 70 million cases over the next century. Additionally, these interventions are anticipated to contribute to a reduction in cervical cancer mortality, with a 10% decrease by 2030 and a 39% reduction by 2045 [309].

PILLAR 1: HPV VACCINATION

The first pillar of the WHO cervical cancer eradication program is achieving 90% coverage of girls fully vaccinated with a human papillomavirus (HPV) vaccine by the age of 15 [143]. Vaccination serves as a crucial preventive measure against HPV infection, the primary cause of almost all cervical cancers [118]. Despite the widespread implementation of vaccination programs globally, the current coverage remains low [113,138]. Enhancing coverage requires ensuring a sufficient supply of affordable vaccines, delivered through cost-effective platforms. Tracking vaccination coverage and effectiveness is vital for assessing progress toward the set targets and evaluating the real-world impact of primary prevention efforts. This thesis contributes to the ability to track vaccine impact data.

In countries where vaccination coverage is not reliably recorded, obtaining a blood sample is necessary to measure the HPV-specific antibody response and determine an individual's vaccination status, particularly in the context of surveillance or vaccine studies [289,310]. Using first-void urine for monitoring HPV-specific antibody responses eliminates the need for invasive blood sampling by a trained medical professional [33]. Beyond assessing vaccine coverage, this sample allows us to evaluate the impact of vaccination by measuring both virological (HPV DNA) and immunological (antibodies) endpoints. Our focus in these studies is not on the urine itself, which acts as a washing fluid, but on the female genital tract secretions washed away with the initial stream of urine [19]. While it was already recognized that cervicovaginal secretions contain HPV-specific antibodies, the hypothesis

that these are also present in first-void urine lacked evidence [31]. In 2019, our research team provided the first data on the ability to accurately detect these HPV-specific antibodies in first-void urine [49,50]. Despite promising results and correlations between first-void urine and serum levels, certain challenges remained to be addressed. Within this thesis, we successfully overcame certain obstacles, laying the foundation for using this sample for the specified objectives.

One primary aim of this thesis was to assess the stability of antibodies derived from first-void urine. While data on the stability and preservation of HPV DNA in first-void urine samples had already been provided [169], uncertainties persisted regarding HPV-specific antibodies. **Chapter 03-A** addresses this concern by confirming the stability of HPV-specific antibodies in first-void urine. Moreover, this study verifies the compatibility of antibody storage with the required DNA preservative, which is crucial for preventing DNA degradation. This alignment is crucial to reach the ultimate goal of using a single first-void urine sample for the follow-up of both immunological and virological endpoints in vaccination and infection studies. This information is particularly crucial for implementing this sample type for screening purposes in low- and middle-income countries where sample storage poses greater challenges.

Moreover, we addressed an additional challenge related to the lower concentration of HPV-specific antibodies in first-void urine compared to serum. In the semi-quantitative GST-L1-MIA assay, the ratio of HPV16-specific antibodies in first-void urine to serum was 0.39% (MFI), while in the quantitative M4ELISA, it was 0.03% (IU/mL) [49,50]. A key objective of the thesis was to develop an HPV-specific immunoassay with enhanced sensitivity capable of detecting the low antibody concentrations present in first-void urine. The ultimate aim is to establish an assay that not only evaluates the antibody response induced by three or two doses of an HPV vaccine but also after a single dose or even following natural infection, which would result in even lower concentrations of antibodies in first-void urine. The added value of such single dose regiments is to be seen in the context of vaccine shortages and the robustness of existing vaccines, which encourage researchers to explore the possibility of administering just one dose of an HPV vaccine [311]. Administering only one dose also simplifies the implementation of vaccination and reduces costs. Findings from the first trials investigating single-dose HPV vaccination, the India IARC trial and the Costa Rica HPV Vaccine Trial, demonstrated one-dose vaccine efficacy of respectively 96% and 82.1% against prevalent HPV 16/18 infection at 10 or 11.3 years post-vaccination [116,312]. Ongoing randomized controlled trials in Tanzania and Kenya (KEN

SHE Study) further investigate the efficacy of one-dose HPV vaccination, with preliminary findings indicating significant protection against HPV16/18 infections [313]. Despite the high effectiveness of a single HPV vaccine dose, HPV-specific antibody concentrations approximately two years after vaccination are less than 30% of the concentrations measured after 2 or 3 doses. Nevertheless, even after just one vaccine dose, the HPV-specific antibody titers remain approximately 10 times higher than those observed after natural infection. This, together with the three-log difference between first-void urine and serum titers, emphasizes the need for a highly sensitive immunoassay to adequately detect antibodies in first-void urine.

In our laboratory, we made the decision to develop an HPV immunoassay using time-resolved fluorescence. In our search for highly sensitive immunoassays or detection systems, we quickly came across the DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) technology, which relies on lanthanides to induce time-resolved fluorescence [222,223]. Lanthanides generate long-lived fluorescent signals, introducing the possibility of a delay between excitation and emission measurements. This feature theoretically aligns with reduced background interference, leading to increased sensitivity. Furthermore, the durable stability of lanthanide fluorescent signals contributes to a robust and reproducible assay [216,217,314].

Essential components of our assay are the antigens, HPV-pseudovirions or virus-like particles. Due to the limited availability of these antigens, we initiated in-house production of pseudovirions. Combining the expertise within our university (Laboratory of Cell Biology and Histology, prof. De Vos and Laboratory of Microbiology, Parasitology and Hygiene, prof. Delputte) and the knowledge gained during a valuable two-week traineeship at the Centers for Disease Control and Prevention (CDC) in Atlanta, we successfully established the capability to produce high quality HPV-pseudovirions internally. This achievement marked a significant advancement for our work, allowing us to measure antibody responses in-house and unlocking new opportunities for further development of assays.

Results of the preliminary HPV16 DELFIA show excellent correlations with previously used immunoassays, M4/9ELISA and GST-L1-MIA, for both serum and first-void urine samples. Additionally, good correlations between serum and first-void urine concentrations were reaffirmed (**Chapter 03-B**). It is important to note, however, that these results do not indicate increased sensitivity compared to the M9ELISA [164]. Future

optimization of the DELFIA assay is required to boost the assay's sensitivity. Additionally, optimization of the M9ELISA for first-void urine is another option for future research (discussed in future perspectives).

Before we can implement first-void urine as the 'golden sample' for monitoring both HPV infection and vaccination, it is essential to demonstrate its utility in monitoring the humoral immune response post-9vHPV vaccination (**Chapter 03-C**) [51]. We set-up a sub-study integrated into the HPV V503-004 study, a phase III randomized controlled trial where volunteers were vaccinated with a 9vHPV vaccine at 0, 1, and 6 months. In addition to serum samples, a cohort of 58 females provided first-void urine samples before vaccination, seven months, and approximately three years after the initial dose. The M9ELISA, validated for serum but not for first-void urine, was used in this study. Results provided promising data, showing that first-void urine can be used in HPV vaccine trials to monitor HPV infection (see pillar 2) and immune responses. This suggests the potential replacement of clinician-taken cervical or self-collected vaginal samples and blood samples with a non-invasive, self-collected urine sample [51]. Moving forward, next steps include further refining the assay to increase sensitivity, as discussed earlier, and validating this method in more extensive cohorts (discussed in future perspectives).

While the humoral immune response, characterized by pathogen-specific antibodies, plays a crucial role in preventing HPV infection and related diseases, the cellular immune response also has its value. Assessing helper T cell ($CD4^+$ T cells) and memory B cell responses can offer valuable insights into vaccine effectiveness [315]. Furthermore, therapeutic vaccines are primarily designed to stimulate a cellular immune response specifically directed at HPV E6 and E7. This response is intended to eliminate HPV-infected cells through a cytotoxic T cell response, marked by the secretion of multifunctional cytokines [135,249]. Surprisingly, no studies have been published on the presence of these cellular immune responses in cervicovaginal secretions (CVS) captured in first-void urine. The ability to detect these specific responses at the site of infection could provide insightful information on protective responses against HPV, potential tissue damage and help in the development of targeted and personalized treatment strategies. A new PhD student in our team will delve into this topic in the next few years.

PILLAR 2: SCREENING

The second pillar aims to achieve 70% cervical cancer screening coverage for females, using a high-performance test at both the ages of 35 and 45 [143]. This screening is crucial in preventing the progression of pre-cancerous conditions to cancer [115,251,316]. Despite its importance, a significant number of females either do not participate or remain unreached by existing screening programs [5,185]. Screening is still mostly based on cytology, where a clinician needs to collect a cervical sample. This approach presents challenges in terms of accessibility, discomfort during sample collection, and consequent hesitancy to visit healthcare facilities [317,318]. Recognizing that cervical cancer is caused by an HPV infection, the focus for cervical cancer screening has shifted toward primary HPV DNA testing in the last decade [88,94,115,319]. HPV DNA testing is typically performed on clinician taken cervical samples but can also be performed on self-samples, including vaginal swabs or first-void urine samples [6,184,320,321].

In this thesis, we focused on the use of first-void urine as a sample for monitoring immunological endpoints (antibodies) but also for monitoring HPV-related virological endpoints (HPV DNA). First-void urine is a non-invasive self-sample that can be collected at the participant's home and does not cause any trauma at the collection site. The acceptability and useability of this sample type for HPV screening have been confirmed by various studies [19,35,188,198,290]. Using this sample type as an alternative screening strategy, could lead to higher coverage and might be an alternative to reaching the un(der)-screened population, aligning with the objective outlined in the second pillar of the WHO guidelines [171].

While previous studies established that the initial stream of urine is rich in secretions, and particularly rich in HPV DNA, the most effective volume for collection had not been defined [24,25]. Currently, the device mostly used for first-void urine collection captures a total volume of 20 mL, consisting of two parts first-void urine and one part DNA preservative [169]. To streamline the high-throughput use of this sample, it is beneficial to harmonize the sample volume with established screening methodologies, ensuring optimal compatibility, and to optimize the sample volume for efficient transportation and storage. Consequently, we assessed various, smaller first-void urine collection volumes, specifically, 4 mL and 10 mL, and observed nearly no impact on HPV DNA yield (**Chapter 02**) [41]. The implementation of a 10 mL collection device, potentially directly compatible with HPV DNA testing equipment used in Belgium (CMA and AML), further

simplifies transport (e.g., via postal mail) and high throughput testing for screening programs.

Following screening, females with clinically significant lesions or a positive HPV DNA test require follow-up. Since cytology is impossible on self-samples, host DNA methylation has been identified as a promising triage option, presenting a possibility of adopting a one-sample approach for both cervical cancer screening and triage [7,48,100,322]. Presently, the CASUS study, in collaboration with Novosanis (a subsidiary of OraSure Technologies, Inc.), Self-Screen B.V., and Amsterdam University Medical Centers (UMC), is actively developing a dual screening and triage strategy. This approach incorporates information from our evaluation of various collection volumes and DNA extraction methods [41]. In this ongoing study, a total of 332 females, all referred to colposcopy due to a (probable) high-risk HPV infection and/or abnormal cervical lesion, used the 10 mL urine collection device. The study involves HPV DNA testing followed by DNA methylation testing, using a marker panel linked to cervical cancer incidence and compatible with first-void urine. If the results on first-void urine show promise, this integrated approach could represent a significant step toward cervical cancer eradication.

Alternative strategies to achieve the screening goal include the exploration of vaginal self-sampling, a method currently also being thoroughly investigated [321]. However, first-void urine presents distinct advantages over vaginal self-sampling due to its non-invasive nature, standardized collection method, combined with the convenience that individuals urinate multiple times daily. Moreover, the use of first-void urine sampling helps avoid potential cultural and ethical concerns that, in certain cases, act as barriers within cervical screening programs.

PILLAR 3: TREATMENT

The third pillar aims to treat 90% of females with precancer and 90% of females with invasive cancer [143]. To enhance cervical cancer treatment, efforts are directed towards expanding capacities in surgical oncology, radiotherapy, and chemotherapy. Additionally, improvements in symptom management, supportive care, and health coverage are crucial aspects. Furthermore, a cautious treatment approach is essential, exclusively targeting lesions with a high likelihood of progressing to cancer. This targeted approach is essential to prevent over-treatment, which could result in adverse outcomes such as preterm births, hemorrhage, and infection [323–325].

Females treated for high-grade disease face an elevated susceptibility to recurrent conditions, with approximately 10% receiving a diagnosis of cancer or a high-grade lesion within a 2-year timeframe. In this subgroup, the risk of developing HPV-related cancer is 2-6 times higher compared to the general population [326,327]. Due to this heightened risk, females post-treatment require increased follow-up to ensure treatment success [328,329]. Current follow-up protocols often use invasive and clinic-based cervical samples for cytology or cytology/high-risk HPV co-testing. Existing cytology-based protocols often result in unnecessary diagnostic procedures, encouraging the reconsideration of using HPV DNA testing for post-treatment follow-up. It has been demonstrated that HPV testing detects residual or recurrent high-grade CIN more quickly and with higher sensitivity than follow-up cytology [330,331].

First-void urine sampling is confirmed as an accurate method for monitoring HPV DNA, offering a highly acceptable approach to monitoring treated patients. Using this non-invasive sampling method that can be performed at home, there is potential to enhance symptom management by reducing follow-up duration, associated concerns, and overall health costs [13,44,169]. Due to its non-invasive nature, the sample can be collected sooner after treatment, as it does not disrupt the still sensitive treatment site. This way, earlier HPV DNA testing is allowed, and preliminary outcome data can be collected before clinician consultation, enabling intensified monitoring activities and a more individualized follow-up schedule. Over the past several years, our study team has been conducting the 'follow-up after treatment (FAT)' study, exploring the potential of self-collected first-void urine and vaginal samples from females treated for high-grade cervical lesions. We expect the release of the study results in the near future.

ADDED VALUE OF HPV VACCINATION

The current HPV vaccines, while not therapeutic, may influence the transmission of the virus and potentially hinder its spread towards other sites (autoinoculation), including the transformation zone, which is highly susceptible to malignant progression [150,151]. When a female has an epithelial lesion, HPV may infect cells in that lesion, and in some cases, the infection progresses to invasive cervical cancer. Surgical excision or ablation, common treatments for high-grade lesions, may create new lesions in other cervical sites, allowing for potential new infections or additional infection sites. Even during screening, a cervical sample can disrupt the epithelial cell layer and increase the likelihood of new infections.

Consequently, there has been a significant interest in vaccinating individuals previously infected with HPV, given their sustained high-risk status [153].

As vaccination programs primarily target young individuals, biological proof regarding the hypothesis that vaccine-induced antibodies interact with newly produced virions and neutralize these new infections, might lead to adaptations of the vaccination programs. This evidence could potentially reduce the current hesitancy in vaccinating females post-sexual debut and, in the future, potentially lead to fewer precancerous lesions and cancer.

HPV vaccination after infection has added value not only for HPV-related cancers but also for other HPV-related diseases. Specifically in the case of respiratory papillomatosis, where HPV6 or 11 cause papillomas in the respiratory tract, HPV vaccination has provided a prolonged intersurgical interval and complete remission [332].

First-void urine is an ideal sample to investigate the hypothesis stated above, as genital tract secretions and intact/functional HPV-specific antibodies at the infection site are captured, along with potentially intact HPV virions [151]. While it has been demonstrated that HPV-specific antibodies are present in first-void urine, the neutralizing capacity of these local antibodies has remained unexplored [31,49–51]. To measure neutralizing antibody concentrations, researchers often use a pseudovirion-based neutralization assay (PBNA). This cell-based assay involves incubating samples with pseudovirions corresponding to the specific HPV type of interest, and subsequently determining the levels of neutralizing antibodies present in the added sample. For the detection of HPV DNA and HPV-specific antibodies, samples usually undergo an enrichment step (such as centrifugation or Amicon filtration) or are used without preprocessing. Given the various impurities found in first-void urine, introducing an enriched sample could lead to contamination of cells, hindering the accurate measurement of neutralization. Therefore, it is crucial to eliminate most contaminants, impurities, and components that might interfere with cell growth and infection. Within this thesis, we identified an effective method for enriching antibodies and removing contaminants from first-void urine. This method was subsequently employed in two PBNA assays (**Chapter 04-A**). We were able to provide first proof of the neutralizing potential of HPV16-specific antibodies in first-void urine. Although further evaluation of larger cohorts and additional optimization of assay protocols are necessary, these results already support the hypothesis that these antibodies maintain their neutralizing capacity, potentially interfering with autoinoculation and transmission to sexual partners.

It is crucial to recognize that wild-type HPV virions will exhibit different behaviors compared to the used HPV pseudovirions in the PBNA. While pseudovirions contain reporter DNA, producing only the reporter protein when a cell is infected, wild type HPV virus replication is tightly linked to the differentiation program of host epithelial cells [333,334]. Additionally, HPV virus particles present in first-void urine might be packaged into shed cells and clumped together. This contrasts with the purified and more controlled nature of pseudovirions used in laboratory settings. Therefore, the ability to isolate intact virions from urine samples is essential to prove the presence of real-life HPV antibody-virion interaction. This approach is crucial to evaluate if antibody-pseudovirion interactions within a PBNA accurately reflect the dynamic interactions occurring after an actual HPV infection.

We are currently in the process of developing a virion isolation protocol specifically tailored for first-void urine samples. Here, HPV DNA positive samples will be utilized to extract virions, which, for vaccinated females, may still be bound to the neutralizing antibodies present at the site of infection. Subsequently, these virions will be used as infecting agents within a cell-based infection model. This setup allows for a comparison of infection rates between wild-type HPV virions obtained from vaccinated and unvaccinated volunteers. It is anticipated that the infection rate among vaccinated female volunteers will be low due to the presence of antibodies bound to the virus particles, preventing the virus from effectively infecting cells. These protocols are presently in the developmental phase (discussed in future perspectives). A major challenge here is the low abundance of viral particles in a first-void urine sample. It is therefore required to implement a concentration step before purification. This concentration step needs to generate a high concentration of virions without causing disruption of the antibody-virion interaction.

To develop the protocol, first-void urine samples spiked with pseudovirions were employed as a model. While this does not precisely replicate a real-life infection, it effectively simulates the presence of HPV virions in first-void urine. This approach serves as a useful model to assess different protocols without relying on HPV16 DNA-positive first-void urine samples. Within this thesis, we examined the impact of naturally present and supplemented precipitating agents on the concentration of HPV16 pseudovirions during the centrifugation process (**Chapter 04-B**). The evaluated protocols consistently resulted in concentration of pseudovirions, and the addition of the precipitating agent positively influenced the quantity of pseudovirions reporter-related DNA in the pellet.

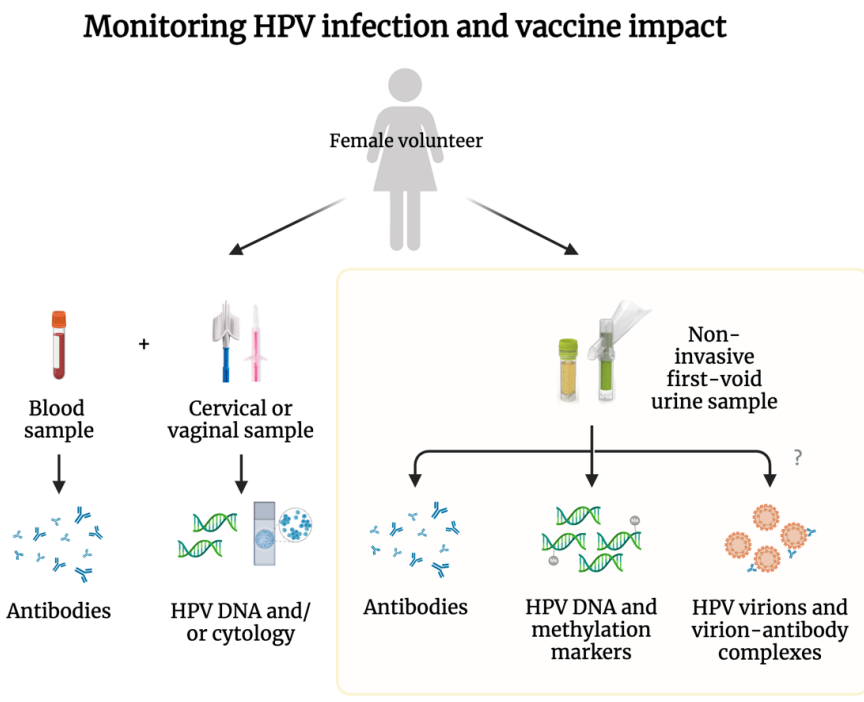


***Figure 1**: Potential of first-void urine self-sampling. First-void urine sampling can be used for detection of antibodies, HPV DNA, and potentially for HPV virions and other biomarkers. This non-invasive liquid biopsy can be used instead of the combination of a blood sample for serology and a clinician-taken cervical smear for detection of HPV infection and other biomarkers. Adapted from Téblick at al. (2022)* [335].

EXTRA APPLICATIONS FOR FIRST-VOID URINE SAMPLING

Urine is a rich source of essential biological information, including proteins, DNA, RNA, extracellular vesicles, metabolites, and more. [178,293,336–338]. It is increasingly recognized as a promising biofluid for studying sexually transmitted infections (STIs), urogenital tract diseases, and serving as a potential liquid biopsy for various cancers. Its ability for repeated collection allows for serial sampling and facilitates multi-omics analysis, emphasizing its potential for large-scale applications [339,340].

First-void urine sampling enables the collection of a concentrated amount of larger molecular weight biomarkers such as proteins and cellular DNA, primarily originating from exfoliated cells of the urogenital tract [24,169]. Previous research already demonstrated the potential of detecting infections such as *Chlamydia trachomatis*, cytomegalovirus, HIV, hepatitis A, B, and C, and gonorrhea in urine [55–57,59,290,341]. Given that these are infections

of the urogenital tract, capturing the first urine void for monitoring is hypothesized to yield even better results than the currently used full void or midstream urine. Additionally, research has proven that urine has potential as a biofluid to monitor immune responses against these infections, making it a potential source of immunological markers [57,212,342].

In addition, first-void urine also captures RNA and smaller cell-free (cf)DNA derived from the glomerular filtration of blood cfDNA [336,343,344]. However, as these biomarkers are likely not concentrated by collecting the first urine void, the primary advantage of collecting first-void urine compared to any other urine fraction in this context might be the standardized collection method. Recent studies have demonstrated that free nucleic acids and circulating tumor (ct)DNA can cross the renal barrier and are excreted in urine, presenting trans-renal cfDNA as potential diagnostic markers for various cancers and inflammation, both urogenital and non-urogenital in origin [66,345]. A recent systematic review delved into current literature-based data on urine sampling as a liquid biopsy for non-invasive cancer research, revealing its versatility in investigating numerous cancer types [346]. These include cancers originating from various organs, such as urothelial cancer, prostate 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. Again, it is essential to note that capturing the first urine void only leads to the concentration of genital tract secretions and not of transrenal biomarkers [27]. Furthermore, the variations in urogenital tract secretions between males and females must be acknowledged [20].

FUTURE RESEARCH

This thesis makes a substantial contribution to advancing the use of first-void urine as a versatile and non-invasively collected sample for monitoring both HPV infection and vaccine impact. While we have successfully evaluated the enrichment and quantification of HPV-specific antibodies in first-void urine and demonstrated its suitability in HPV vaccine trials, there is still additional data that needs to be generated for the complete integration of this sample type into practical applications.

The concentration of HPV-specific antibodies in first-void urine has proven to be about three logs lower than in serum [51]. While we can already adequately detect antibody concentrations in first-void urine samples at peak levels after full HPV vaccination and additionally at 3.5 years after vaccination (**Chapter 03-C**) or even longer (**Chapter 04-A**), the antibody positivity in first-void urine remains lower than in serum samples. Further optimization and development of immunoassays to enhance sensitivity are therefore necessary.

The HPV immunoassay introduced in **Chapter 03-B** will be further developed within the ongoing ERC project, URISAMP, and a complementary IOF-SBO project. To enhance the sensitivity and specificity of the assay, we plan to create monoclonal antibodies (mAbs) with high type specificity. Currently, mAbs targeting HPV vaccine types are not commercially available, and although they are produced to interact exclusively with a specific HPV type, some cross-neutralization occurs [274,347–349]. The production of mAbs involves mice as experimental animals. In this project, we will initially deimmunize the mice with all other HPV types included in the 9vHPV vaccine before immunizing them with the specific HPV type of interest. This process ensures that the mouse is devoid of the non-specific response, generating a highly specific response to the type of interest. Additionally, we will explore the neutralizing potential of the produced mAbs and select those that are highly neutralizing. If successful, these mAbs will be employed to create a 3x3 competitive multiplex DELFIA assay. Since there are four available lanthanides, of which three have high performance, we can assess the immune response to three HPV types in a specific well. Through the implementation of a competitiveness assay, wherein the mAbs bind to the specific sites on the PsV targeted by neutralizing antibodies, we can exclusively measure the HPV-specific antibodies that compete with the neutralizing mAbs. This approach allows for the specific detection of neutralizing antibodies only. Given that

the currently used assays to detect neutralizing antibodies are pseudovirion-based neutralizing assays, which are challenging to perform at high throughput and require cell culture, the new proposed assay would present a significant advantage in the field of HPV research.

Building upon the promising results obtained using the VLP-based multiplex ELISA at the CDC (M9ELISA), we aim to further enhance this assay for first-void urine samples in-house. The assay will be set up using in-house-developed HPV pseudovirions for all nonavalent HPV types. Given the multispot nature of the assay plates, we might be interested in expanding their application to assess the natural immune response following HPV infection. Pseudovirions of HPV types currently of significant interest, such as HPV39, 51, and 59, can be produced, coated onto the multispot plates, and evaluated using first-void urine samples [350]. All assays will be created to be compatible with serum as well. Additionally, it might be interesting to incorporate other STI-related antigens into this assay to assess other specific antibody responses. Notably, *Chlamydia trachomatis* is of interest, and the evaluation of antibody responses to human immunodeficiency virus (HIV) will be explored as well.

Because of the low antibody levels in first-void urine, distinguishing between antibody responses post-natural infection and vaccination can be challenging. To effectively monitor HPV vaccination statuses in countries with incomplete vaccination records, it is crucial to differentiate between females who have received an HPV vaccine and those who likely had an HPV infection in the past. The lower avidity and potency of antibodies generated by past infections emphasize the need for discrimination since these probably do not provide enough protection against disease [240]. In addressing this, we aim to develop an avidity assay incorporating a chaotropic agent, such as Guanidinium chloride, into the M9ELISA assay or the 3x3 competitive multiplex DELFIA assay platforms to assess the avidity index of antibodies. This assay will play a vital role in distinguishing between vaccine-induced and infection-induced antibody responses, particularly in situations where vaccination status is unclear due to responses against specific HPV types not included in the respective vaccine(s).

As the vaccine trial incorporating first-void urine sampling involved a limited cohort of 58 females, it is essential to validate the findings in a more extensive participant pool. Rather than initiating a new vaccine trial, we plan to use first-void urine to assess the real-world impact of HPV vaccination. This involves examining the HPV vaccine-induced immune

response and HPV infection prevalence in individuals vaccinated during the GAVI pilot projects. The insights derived from this localized data will provide valuable information for optimizing the HPV vaccination program.

As anticipated, first-void urine sampling has revealed substantial inter- and intra-individual variation. Multiple factors, including contraceptives, the menstrual cycle, hormones, and vaginal washes, influence the secretions within the sample [204,351]. Therefore, establishing a standardized protocol for detecting HPV-specific antibodies in first-void urine is crucial. As part of the URISAMP project, we aim to develop such a protocol through the implementation of the URINORM study. This study involves identifying normalization parameters and collecting samples from diverse cohorts, including young girls premenarchal, young females using ovulation-influencing and non-ovulation-influencing contraceptives, ovulatory young females, post-menopausal females, and males. The results from this study are expected to yield an effective normalization algorithm. Moreover, the inclusion of a male cohort in our study can provide the first data regarding the statement that first-void urine sampling for HPV and antibody detection is exclusively applicable to females.

While we have presented initial evidence of the neutralizing capabilities of antibodies in first-void urine at the infection site, further investigation is crucial. In the above-mentioned URISAMP project, we will delve deeper into the interaction between HPV-specific antibodies and virus particles using first-void urine sampling. The virion isolation model will undergo additional refinement based on our spiking model and subsequently be validated using samples from vaccinated and unvaccinated female volunteers with an HPV infection of a type included in the 2vHPV, 4vHPV, or 9vHPV vaccine.

Because of the high HPV vaccination coverage in Flanders, acquiring samples with HPV DNA from these types is challenging. Our group is currently exploring several options to set-up a trial to collect the required samples. The antibody-virion complexes derived from these samples will then be incorporated into a cell-based infection model. Maintaining the virion-antibody interaction will be the most challenging part of the laboratory process. Additionally, the PBNA protocols will undergo further optimization and validation on larger sample sets.

Being able to comprehensively evaluate all aspects of an HPV infection and the associated immune response, first-void urine might be the ideal sample to help define a correlate of

protection. Given the high efficacy of the HPV vaccine, there are limited breakthrough infections reported, which are required for defining such a correlate. Increasing the sampling frequency and reaching a larger population of females in need of screening could enhance the registration of breakthrough infections, contributing to a robust definition of a correlate of protection. Moreover, using a sample rich in biological information originating from the infection site may facilitate the establishment of a specific threshold for the required protection at that particular site.

CHAPTER

06

Summary

SUMMARY

Effectively monitoring infection, disease, and immune response relies on the availability of appropriate samples. Biomarker sampling plays a crucial role in assessing an individual's health status, facilitating timely diagnosis, and enabling a comprehensive evaluation of the immune response, including pathogen-host interactions. Self-sampling these biomarkers allows for collection at home, increasing accessibility and reducing health care visits. Recent attention has focused on sampling the initial stream of urine, known as first-void urine, particularly for females. Here, urogenital tract secretions accumulate between the labia minora and are non-invasively collected by the first urine flow. This results in a sample rich in biological information.

In human papillomavirus (HPV) research, the leading cause of cervical cancer, first-void urine has shown promise in assessing virological, diagnostic, and immunological endpoints, presenting a valuable single-sample option for disease and vaccine monitoring. This thesis contributed to the optimization of the use of first-void urine as a non-invasive sample for monitoring HPV infection and vaccination, specifically focusing on the detection of HPV DNA and HPV type-specific antibodies.

The objectives of **Chapter 02-A** were to evaluate the impact of three different collection volumes and various DNA extraction methods on the detection of HPV DNA and other biomarkers. Using samples from 25 female volunteers diagnosed with high-risk HPV, we found limited effects of collection volumes, but the DNA extraction method significantly influenced the recovery of HPV DNA and other biomarkers. This chapter contributed to selecting an optimal combination of first-void urine collection and processing methods. Currently, this combination is being assessed in the CASUS project, which aims to evaluate the use of first-void urine as a diagnostic approach for HPV detection and triage.

While substantial evidence supports the feasibility of using first-void urine for detecting virological endpoints, this thesis mainly focused on addressing knowledge gaps related to immunological endpoints in first-void urine. Here, our first objective was to assess the stability, enrichment, and quantification of total and HPV16-specific immunoglobulins (IgG) in concentrated first-void urine samples. Next, we aimed to develop an in-house, highly sensitive assay for detecting HPV16-specific antibodies. Additionally, we

investigated the potential of using first-void urine to monitor the humoral immune response after HPV vaccination.

In **Chapter 03-A**, our findings provided evidence that HPV-specific antibodies in first-void urine are stable for at least 14 days at room temperature. Additionally, we confirmed the compatibility of IgG storage with a preservative required to avoid DNA degradation, an essential step toward using a single first-void urine sample for both immunological and virological assessments. Moreover, we identified IgG enrichment methods that fit with all evaluated total human IgG detection methods and HPV16-IgG detection for first-void urine. However, certain enrichment methods yielded non-specific results for particular assays, highlighting the necessity for researchers to exercise caution and verify compatibility with specific assays when selecting sample processing protocols.

In **Chapter 03-B**, we developed an in-house HPV16-specific immunoassay (DELFIA), based on time-resolved fluorescence, and compared it to widely used immunoassays. Using paired first-void urine and serum samples from two cohorts (225 paired samples in total), we compared the performance of the DELFIA assay to assays from the German Cancer Research Center (DKFZ) and the Centers for Disease Control and Prevention (CDC). Our DELFIA assay demonstrated similar performances, and we observed excellent correlations between HPV16-specific antibodies in first-void urine and serum. The ability to evaluate HPV-specific antibody responses in-house marks a significant scientific achievement. We will further develop this assay as a competitive multiplex assay for antibody detection against nonavalent HPV vaccine (9vHPV) types.

In **Chapter 03-C,** we were the first to demonstrate that first-void urine is a suitable tool for monitoring HPV vaccination with the 9vHPV in females and detecting HPV DNA simultaneously. We set up a longitudinal cohort study including 58 females in a Gardasil9 Phase III randomized controlled trial that collected paired first-void urine and serum samples before vaccination and at two time points after full vaccination. Significant correlations between first-void urine and serum samples were observed at all timepoints, and 95%–100% or 84%–100% of first-void urine samples had detectable antibody titers, depending on the timepoints, and varying by HPV vaccine type.

While current prophylactic HPV vaccines lack therapeutic effects, induced neutralizing antibodies could potentially block the spread of newly produced virions. In this thesis, first-void urine was used for pilot experiments to investigate this hypothesis. Although the assays

require further optimization, in **Chapter 04-A,** we demonstrated that HPV-specific antibodies in first-void urine, originating from the genital tract, have neutralizing capacity. We achieved this by setting up and optimizing two pseudovirion-based neutralization assay protocols compatible with first-void urine samples, with neutralizing antibody concentrations correlating with serum levels.

Lastly, in **Chapter 04-B,** we provided crucial insights into optimal precipitation protocols for detecting and concentrating HPV pseudovirions in first-void urine, potentially extending to naturally occurring HPV virions. Moreover, we identified a positive effect of a precipitating agent on the concentration of cell-free DNA and genomic DNA in first-void urine. This broadens the utility of first-void urine for research on sexually transmitted infections and as a biomarker source for cancer-related studies.

Despite advancements in screening and primary prevention, cervical cancer persists as the fourth most common cancer among females worldwide. Recognizing the urgent need for action, the WHO has initiated a global strategy for cervical cancer elimination. The strategy harnesses the proven success of current HPV vaccines for cancer prevention, the outstanding efficacy of HPV and cervical screening methods, and the effective treatment options when detected early. By scaling up these three key components, we can pave the way toward a future where cervical cancer is no longer a significant threat to women's health worldwide.

This thesis significantly contributes to the implementation of first-void urine as a non-invasively collected multi-purpose sample for monitoring HPV infection and vaccine impact. First-void urine self-sampling supports broader public health initiatives, providing a feasible option for conducting large-scale screening programs, epidemiological studies, and vaccine trials.

CHAPTER

07

Samenvatting

SAMENVATTING

Voor het efficient opvolgen van infectie, ziekte en immuunrespons zijn er geschikte biologische stalen nodig. Het collecteren van biomerkers in biologische stalen speelt een cruciale rol bij het opvolgen van de gezondheidsstatus van een individu. Hierdoor kunnen we zorgen voor een tijdige diagnose en het opvolgen van de immuunrespons, inclusief pathogeen-gastheer interacties. Door deze biomerker stalen zelf af te nemen kunnen ze van thuis uit verzameld worden, wat de toegankelijkheid vergroot en het aantal nodige bezoeken aan gezondheidsfaciliteiten vermindert. Recent werd de aandacht gevestigd op het collecteren van het eerste deel van de urine, bekend als eerste fractie urine. Dit is voornamelijk interessant bij vrouwen, aangezien de secreties van de urinewegen zich verzamelen tussen de kleine schaamlippen en op een niet-invasieve manier verzameld worden in de eerste fractie van de urine. Dit zorgt voor een staal dat rijk is aan biologische informatie.

In onderzoek naar humaan papillomavirus (HPV), de belangrijkste veroorzaker van baarmoederhalskanker, heeft eerste fractie urine aangetoond veelbelovend te zijn voor het opvolgen van virologische, diagnostische en immunologische eindpunten. Hierdoor is het een interessante optie om als één enkel staal te gebruiken voor opvolging van ziekten en vaccinatie. Deze thesis heeft bijgedragen aan het optimaliseren van het gebruik van de eerste fractie urine als een niet-invasief staal voor het monitoren van zowel HPV-infectie als vaccinatie. Hierbij lag de nadruk op de detectie van HPV-DNA en specifieke antilichamen tegen HPV-types.

De doelstellingen van **hoofdstuk 02-A** waren het evalueren van de invloed van drie verschillende collectie volumes en diverse DNA-extractiemethoden op de detectie van HPV-DNA en andere biomerkers. Gebruik makend van stalen van 25 vrouwelijke vrijwilligers, bij wie een hoog-risico HPV infectie was vastgesteld, vonden we beperkte effecten van collectie volumes, terwijl de DNA-extractiemethode een aanzienlijke impact had op de opbrengst van HPV-DNA en andere biomarkers. Dit hoofdstuk heeft bijgedragen aan het kiezen van een optimale combinatie van de eerste fractie urine collectie en verwerkingsmethoden. Deze combinatie wordt momenteel geëvalueerd in het CASUS-project dat evalueert of eerste fractie urine gebruikt kan worden voor het detecteren en triëren van HPV infecties.

Ondanks dat er al veel bewijs is voor de geschiktheid van de eerste fractie urine als staal voor het detecteren van virologische aspecten, lag de focus van deze thesis voornamelijk op het opvullen van ontbrekende kennis met betrekking tot immunologische eindpunten in eerste fractie urine. Binnen dit hoofdstuk was onze eerste doelstelling gericht op het beoordelen van de stabiliteit, opconcentratie en kwantificering van zowel de totale hoeveelheid immunoglobulines (IgG) als HPV16-specifieke IgG in geconcentreerde eerste fractie urine stalen. Daarnaast werd een zeer gevoelige immunoassay ontwikkeld voor het detecteren van HPV16-specifieke antilichamen. Verder onderzochten we het potentieel van het gebruik van eerse fractie urine voor het opvolgen van de humorale immuunrespons na HPV-vaccinatie.

In **hoofdstuk 03-A** bevestigden onze bevindingen dat HPV-specifieke antilichamen in de eerste fractie urine minstens 14 dagen stabiel blijven bij kamertemperatuur. Daarnaast hebben we vastgesteld dat de bewaring van IgG compatibel is met de buffer dat noodzakelijk is om DNA degradatie te vermijden, een cruciale stap voor het gebruik van slechts één urinestaal voor zowel immunologische als virologische doeleinden. Bovendien hebben we methoden voor IgG-opconcentratie geïdentificeerd die compatibel zijn met alle geëvalueerde detectiemethoden voor totaal humaan IgG en de detectie van HPV16-IgG in de eerste fractie urine. Het is echter essentieel om op te merken dat bepaalde opconcentratie methoden aspecifieke resultaten opleverden bij specifieke assays, wat benadrukt dat onderzoekers voorzichtig moeten zijn en de compatibiliteit met specifieke assays moeten verifiëren bij het kiezen van protocollen voor staalverwerking.

In **hoofdstuk 03-B** hebben we een HPV16-specifieke immunoassay (DELFIA) ontwikkeld, gebaseerd op 'time-resolved fluorescence', en deze assay vergeleken met veelvuldig toegepaste immunoassays. Door gebruik te maken van gepaarde eerste fractie urine- en serumstalen uit twee cohorten (in totaal 225 gepaarde stalen) hebben we de resultaten van onze DELFIA-assay vergeleken met assays van het DKFZ en CDC. Onze DELFIA assay vertoonde vergelijkbare prestaties waarbij we opnieuw uitstekende correlaties verkregen tussen HPV16-specifieke antilichamen in de eerste fractie urine en het serum. De mogelijkheid om HPV-specifieke antilichaamresponsen intern te evalueren markeert een belangrijke wetenschappelijke prestatie voor onze onderzoeksgroep. Deze assay zal verder worden ontwikkeld als een competitieve multiplex assay voor het detecteren van antilichamen tegen negenvalente HPV-vaccin (9vHPV)-types.

In **hoofdstuk 03-C** hebben we als eerste aangetoond dat eerste fractie urine een geschikt staal is voor het monitoren van HPV-vaccinatie met het 9vHPV vaccin bij vrouwen, in combinatie met HPV-DNA-detectie. Hiervoor werd een longitudinale cohortstudie opgezet waarbij 58 vrouwen geincludeerd werden die deelnamen aan een fase III gerandomiseerd gecontroleerd onderzoek naar Gardasil9. Gepaarde eerste fractie urine- en serumstalen werden verzameld vóór vaccinatie en op twee tijdstippen na volledige vaccinatie. Op alle tijdstippen werden significante correlaties waargenomen tussen urine- en serumstalen, waarbij 95%–100% of 84%–100% van de eerste fractie urinestalen detecteerbare antilichaamtiters vertoonden, afhankelijk van de tijdstippen en variërend per HPV-vaccintype.

Hoewel de huidige profylactische HPV-vaccins geen therapeutische effecten hebben, zouden geïnduceerde neutraliserende antilichamen mogelijk de verspreiding van nieuw geproduceerde virionen kunnen blokkeren. In deze thesis werden proefexperimenten uitgevoerd met eerste fractie urine om deze hypothese te onderzoeken. Hoewel verdere optimalisatie van de assay nodig is werd in **hoofdstuk 04-A** aangetoond dat HPV-specifieke antilichamen in de eerste fractie urine, afkomstig uit het genitale stelsel, neutraliserend vermogen vertonen. Dit werd bereikt door het ontwikkelen en optimaliseren van twee neutralisatieassays op basis van pseudoviruspartikels, beide compatibel met eerste fractie-urinestalen, waarbij eveneens goede correlaties met neutraliserende antilichaamconcentraties in serum werden aangetoond.

Daarnaast hebben we in **hoofdstuk 04-B** precipitatieprotocollen geëvalueerd voor het detecteren en concentreren van HPV-pseudoviruspartikels in de eerste fractie urinestalen. Deze protocollen zouden mogelijk kunnen worden uitgebreid naar natuurlijk voorkomende HPV-viruspartikels. Bovendien hebben we een positief effect geïdentificeerd van een precipitatiemiddel op de concentratie van cel-vrij DNA en genomisch DNA in eerste fractie urine. Dit vergroot de bruikbaarheid van eerste fractie urine als bron voor onderzoek naar seksueel overdraagbare infecties en kankeronderzoek.

Ondanks de vooruitgang op het gebied van screening en primaire preventie, blijft baarmoederhalskanker wereldwijd de vierde meest voorkomende vorm van kanker onder vrouwen. De WHO erkent dat er nood is aan actie en heeft het initiatief genomen voor een wereldwijde strategie om baarmoederhalskanker te elimineren. Deze strategie bouwt voort op het bewezen succes van de huidige HPV-vaccins voor kankerpreventie, de uitstekende doeltreffendheid van HPV- en baarmoederhalskankerscreeningmethoden, en

de effectieve behandelingsopties wanneer de ziekte in een vroeg stadium wordt ontdekt. Door deze drie cruciale componenten op grotere schaal toe te passen, streven we naar een toekomst waarin baarmoederhalskanker wereldwijd niet langer een aanzienlijke bedreiging vormt voor de gezondheid van vrouwen.

Deze thesis draagt aanzienlijk bij aan de implementatie van de eerste fractie urine als een niet-invasief verzameld staal met meerdere toepassingen voor het monitoren van HPV-infectie en de impact van vaccinatie. Zelfafname van eerste fractie urine ondersteunt bredere initiatieven op het gebied van volksgezondheid en biedt een haalbare optie voor het uitvoeren van grootschalige screeningsprogramma's, epidemiologische studies en vaccinonderzoek.

CHAPTER

08

Curriculum vitae

CURRICULUM VITAE

Education

- **Master in Bioscience Engineering: cell- and gene biotechnology**
  2016 – 2018, Ghent University
  Graduated with honors

- **Bachelor in Bioscience Engineering**
  2013-2016, University of Antwerp

- **Certificate Laboratory Animal Science**
  2017, Ghent University

Professional experience

- **PhD researcher at Centre for the Evaluation of Vaccination, Vaccine and Infectious Disease Institute, Faculty of Medicine and Health Science**
  2019-2024, University of Antwerp

- **Course coordinator of the Summer School on Vaccinology**
  Editions of 2021, 2022, 2023, and 2024

- **Research stay abroad at the Centre for Disease Control and Prevention, Atlanta, United States of America**
  Supervised by Prof. Elizabeth Ünger and dr. Gitika Panicker
  Goal: production of HPV pseudovirions, performing a pseudovirions based neutralization assay and a multiplex HPV ELISA
  Financed by FWO travel grant for short stay abroad

- **Master dissertation at the Laboratory for Immunology and Animal Biotechnology**
  Subject: DNA vaccination against *Chlamydia* infections in pigs and chickens
  2017-2018, Ghent University

- **Bachelor dissertation at the Laboratory of Environmental Ecology and Applied Microbiology**
  Subject: Identification and antipathogenic activity of vaginal *Lactobacillus* isolates
  2015-2016, University of Antwerp

Publications in peer-reviewed scientific journals

- **Téblick L,** Van Keer S, De Smet A, Van Damme P, Laeremans M, Rios Cortes A, Beyers K, Vankerckhoven V, Matheeussen V, Mandersloot R, Floore A, Meijer CJLM, Steenbergen RDM, Vorsters A. (2021). Impact of Collection Volume and DNA Extraction Method on the Detection of Biomarkers and HPV DNA in First-Void Urine. Molecules, 26, 1989.

- Pattyn J, Panicker G, Willhauck-Fleckenstein M, Van Keer S, **Téblick L,** Pieters Z, Tjalma W, Matheeussen V, Van Damme P, Waterboer T, Unger E, Vorsters A. (2020). Comparison of a VLP-based and GST-L1-based multiplex immunoassay to detect vaccine-induced HPV-specific antibodies in first-void urine. Journal of Medical Virology, 92(12), 3774-3783.

- Pattyn J, Van Keer S, **Téblick L**, Van Damme P, and Vorsters A. (2019). HPV DNA detection in urine samples of women: 'an efficacious and accurate alternative to cervical samples?'. Expert review of anti-infective therapy, 1-3.

- Pattyn J, Van Keer S, **Téblick L**, Van Damme P, Vorsters A. (2020), Non-invasive Assessment of Vaccine-Induced HPV Antibodies via First-Void Urine. Frontiers in Immunology, 11, 1657.

- Waheed DE, Burdier FR, Eklund C, Baussano I, Mariz FC, **Téblick L**, Mugo N, Watson-Jones D, Stanley M, Baay M, Vorsters A. (2023). An update on one-dose HPV vaccine studies, immunobridging and humoral immune responses – A meeting report. Preventive Medicine Reports, 35, 102368.

- **Téblick L**, Pattyn J, Van Keer S, De Smet A, De Coster I, Tjalma WAA, Rajbhandari I, Panicker G, Ünger ER, Vorsters A (2023). Follow-up of humoral immune response after HPV vaccination using first-void urine: A longitudinal cohort study. Journal of Medical Virology, 95(10).

- **Téblick L**, Lipovac M, Burdier FR, De Smet A, Bell M, van den Borst E, Matheeussen V, and Vorsters A (2024). Concentration strategies for spiked and naturally present biomarkers in non-invasively collected first-void urine. European Journal of Medical Research, 29(1), 131.

- **Téblick L**, Lipovac M, Molenberghs F, Delputte P, De Vos WH, Vorsters A (2024). HPV-specific antibodies in female genital tract secretions captured via first-void urine retain their neutralizing capacity. Human Vaccines & Immunotherapeutics, 20(1).

Manuscripts submitted or ready for submission:

- **Téblick L**, Lipovac M, Bell M, De Smet A, De Meester I, Delputte P, Vorsters A. Stability, enrichment, and quantification of total and HPV16-specific IgG present in first-void urine. Submitted to Scientific Reports.

- Lipovac M*, **Téblick L***, Bell M, Van Caesbroeck A, De Smet A, Van Keer S, Delputte P, De Coster I, Tjalma WAA, Vorsters A. Time-resolved fluorescence (TRF) for total IgG and HPV16-specific antibody detection in first-void urine and serum: A comparative study. Submitted to Journal of Virological Methods.

- Hendrickx JO, Van Keer S, Donders G, Weyers S, Doyen J, Beyers KCL, Rios-Cortes A, Meers N, **Téblick L**, Vorsters A, Vankerckhoven VVJ. Home-based urinary HPV self-sampling for the detection of cervical cancer precursor lesions: attitudes and preferences from Belgian females participating in the CASUS study. Will be submitted in 2024.

International journal publications

Téblick L, Van Keer S, Vorsters A. First-void urine as liquid biopsy for HPV vaccine-induced antibody detection. HPV World, March 2022.

National journal publications

Téblick L, Van Keer S, Vorsters A. Urine als gouden staal voor HPV-opsporing en opvolging van vaccinatie. Medi-Sfeer, Sept 2022.

Conference proceedings (1st author only)

- Determination of the optimal first-void urine collection volume for the detection of viral and host biomarkers, and evaluation of an internal control. **Téblick L**, Van Keer S, Pattyn J, De Smet A, Floore A, Steenbergen RDM, Vorsters A. EUROGIN 2019, Oral presentation.

- Determination of the optimal first-void urine collection volume for the detection of viral and host biomarkers, and evaluation of an internal control. CASUS study. **Téblick L**, Van Keer S, Pattyn J, De Smet A, Floore A, Steenbergen RDM, Vorsters A. International Papillomavirus Conference 2020, Poster presentation.

- Detection of HPV-specific antibodies in first-void urine of women vaccinated with the nonavalent HPV vaccine. **Téblick L**, Pattyn J, Van Keer S, De Smet A, De Smedt P, Tjalma WAA, Panicker G, Ünger ER, Van Damme P,Vorsters A. EUROGIN 2021, Oral presentation.

- Monitoring antibody mediated immune response using first void urine samples. **Téblick L**, Pattyn J, Van Keer S, De Smet A, Vorsters A. International Papillomavirus Concerence 2021, Oral presentation.

- Detection of HPV-specific antibodies in first-void urine of women vaccinated with a nonavalent HPV vaccine. **Téblick L**, Pattyn J, Van Keer S, De Smet A, De Smedt P, Panicker G, Ünger ER, Van Damme P, Tjalma WAA, Vorsters A. International Papillomavirus Concerence 2021, Oral presentation.

- Urine for the monitoring of vaccinated women, **Téblick L**, De Smet A, Van Keer S, Vorsters A. EUROGIN 2022, Invited speaker.

- Follow-up of HPV vaccination using first-void urine as a non-invasive genital tract liquid biopsy. **Téblick L**, Pattyn J, Van Keer S, De Smet A, De Coster I, Vorsters A. International Papillomavirus Conference 2023, Oral presentation.

- Non-Invasive HPV antibody monitoring using first-void urine: implications and applications. **Téblick L**, Lipovac M, Bell M, De Smet A, Van Keer S, Vorsters A. EUROGIN 2024, Invited speaker.

CHAPTER

09

Dankwoord

DANKWOORD

Eindelijk is het dan zo ver, mijn doctoraat is afgerond. Na ongeveer vijf jaar is het gelukt om er een goed gevuld boekje van te maken. Hiervoor zijn natuurlijk erg veel personen te bedanken.

Allereerst wil ik graag prof. Alex Vorsters bedanken om mij de kans te geven het HPVplus team aan te vullen en mee uit te breiden. Bedankt voor het vertrouwen, voor de vele constructieve feedback, goede ideeën, ondersteuning en een deur die altijd open staat. Ook bedankt aan prof. Peter Delputte voor de aanvullende feedback, het aanleveren van uitzonderlijke kennis van virologie en immunologie en eveneens de steun de afgelopen jaren. Severien, prof. Van Keer, jou wil ik ook enorm bedanken voor alles, zowel voor je professionele steun als voor je persoonlijke steun de afgelopen jaren. Dank je wel voor je aanmoediging en voor de vele fijne momenten die we hebben gedeeld, zowel op kantoor als op congres. Verder wil ik ook graag de interne en externe juryleden van mijn doctoraat, prof. Didier Ebo, prof. Peter Van Dam, prof. John Doorbar en dr. Fiona van der Klis, bedanken voor de opbouwende feedback en om jullie kennis met ons te delen.

Het ondertussen al erg uitgebreide HPVplus team zou ik ook graag willen bedanken. Zo'n team van uitzonderlijk getalenteerde, fijne collega's vormt elke dag een bron van inspiratie en motivatie. Annemie, zonder jouw vertrouwen, onze vele gesprekken, al jouw hulp en verhalen, zouden de afgelopen jaren er heel anders hebben uitgezien. Bedankt voor de fundamentele rol die je hebt gespeeld in mijn ontwikkeling de afgelopen vijf jaar. Marijana, bedankt dat je ons laboteam bent komen versterken. Al vanaf het begin had ik er het volste vertrouwen in dat we er een paar zeer capabele handen hadden bijgekregen, en ik ben blij dat ik je nog verder kan zien groeien. Margo, jouw gedrevenheid voor je onderzoek is indrukwekkend, en je enthousiasme draagt elke dag bij aan de positieve sfeer binnen de groep, bedankt hiervoor. Eef, ik bewonder je vastberadenheid, medeleven en vriendelijkheid enorm. Bedankt voor je constante bereidheid om anderen te helpen, wederom een waardevolle toevoeging aan het team. Nayab, ik ben erg blij dat ik jou de afgelopen jaren beter heb leren kennen. Je bent een geweldige, hardwerkende vrouw, en ik kijk ernaar uit om nauwer met je samen te werken. Ricardo, jouw bruisende persoonlijkheid en enthousiasme zijn van onschatbare waarde voor ons team. Ik wens je veel succes toe en ben er zeker van dat je het nog erg ver zal schoppen. Anne, bedankt om van ons HPV team het HPVplus team te maken en met passie en motivatie onze expertise

uit te breiden. Ook Mette, onze tijdelijke collega uit Denemarken, en voormalig teamlid Jade wil ik bedanken voor de fijne tijd die we met jullie hebben gehad. Bedankt, HPVplus team, voor alles, en ik kijk ernaar uit om deel uit te blijven maken van dit fantastische team. Ik kan me geen betere collega's voorstellen.

Eveneens bedankt aan prof. Pierre Van Damme, voor de begeleiding binnen mijn doctoraat en voor de kans om de Summer School on Vaccinology te coördineren samen met een fantastisch team. Van beperkte covid-edities tot volwaardige versies van deze week vol informatie, elke keer heb ik ervan genoten. Het vaste Summer School-team, bestaande uit Kirsten, Esra, Ricardo, Eline, Brenda en Xenia, heeft daar natuurlijk ook aan bijgedragen. Bedankt aan jullie allen, niet alleen voor deze fijne weken, maar ook voor jullie enthousiasme en steun daarbuiten gedurende de afgelopen vijf jaar.

Ook bedankt aan alle overige collega's van het CEV en Vaccinopolis. Of het nu gaat om hulp bij klinische studies, het verzamelen of doneren van stalen (pipietjes), of voor de fijne gesprekken en jullie warme ontvangst. Stuk voor stuk waren/zijn jullie fijne collega's. Eveneens bedankt aan alle deelnemers van de klinische studies.

Verder wil ik prof. Winnok De Vos, Freya en hun collega's binnen het CBH bedanken voor de fijne samenwerking. Zonder jullie kennis was de start van mijn doctoraat nooit zo vlot verlopen. Ik hoop dat we onze samenwerking kunnen voortzetten en dat er nog mooie resultaten uit kunnen komen. Ook bedankt aan andere onderzoekers van de UA en het UZA waarmee we samengewerkt hebben, meer specifiek prof. Ingrid De Meester, prof. Veerle Matheeusssen en prof. Wiebren Tjalma.

Furthermore, I would like to extend my gratitude to prof. Elizabeth Ünger, Gitika, and Ira from the Centers for Disease Control and Prevention in Atlanta. Thank you for guiding me during my two-week internship and for your invaluable input and collaborations. I could not have felt more welcome, and I hope to have the opportunity to see you all again soon.

Ook het CASUS team met o.a. prof. Renske Steenbergen, prof. Chris Meijer, Arno, Vanessa en Koen wil ik graag bedanken voor de hulp en feedback tijdens het begin van mijn doctoraat. Ik wens jullie nog erg veel succes met het afronden van deze mooie studie en ben er zeker van dat er nog toekomstige samenwerkingen met ons team zullen volgen. Ook Alejandra zou ik graag bedanken voor het design van mijn thesiscover.

Naast alle fantastische collega's zijn er natuurlijk nog mensen achter de schermen die een belangrijke rol gespeeld hebben. Hierbij zou ik graag mijn zus (en collega) Eva willen bedanken voor de constante goede zorgen op en naast het werk. Ik heb met bewondering mogen toekijken hoe je de afgelopen jaren gegroeid bent als persoon en ik kijk ernaar uit om je te blijven zien groeien. Ook mijn ouders, die altijd voor me klaarstaan en onvoorwaardelijk in me geloven, verdienen oneindig veel bedankjes. Ik ben jullie ontzettend dankbaar voor alle kansen die jullie mij hebben gegeven en kan me geen warmere en liefdevollere familie voorstellen. Elke dag besef ik hoe gelukkig ik mag zijn met jullie in mijn leven. De rest van mijn familie wil ook bedanken om altijd voor iedereen klaar te staan. Verder verdient ook mijn toekomstige schoonfamilie een dikke merci om mij met open armen te ontvangen, in mij te geloven en mij lief te hebben als jullie eigen familie vanaf dag één.

Verder zijn er natuurlijk ook nog de vrienden en vriendinnen die er al die jaren voor gezorgd hebben dat er afleiding is waar nodig en die altijd klaarstaan als het eens wat moeilijk gaat. Bijzondere dank aan mijn grietjes, Elly, Jolien, Katrien en Sofie, om mij mee door onze studie te loodsen en er onvergetelijke jaren van te maken.

Als laatste zou ik graag mijn verloofde, Jasper, willen bedanken om mijn leven zo veel rijker te maken. Je haalt altijd het beste in mij naar boven en ik kan alleen maar blijven opkijken naar jou. Je bent een prachtig, inspirerend persoon en ik zou hier niet geraakt zijn zonder jou. Mijn liefde voor jou groeit nog elke dag en ik kijk enorm uit naar wat ons nog te wachten staat. Ik zie je graag.

Bedankt voor alles.

Laura

April, 2024

CHAPTER

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References

REFERENCES

1. Aitio A, Anderson D, Blain P, et al. Biomarkers and risk assessment: Concepts and principles. *Environ Heal Criteria*. 1993;(155):3-82.
2. Kang HJ, Jeon SY, Park JS, et al. Identification of clinical biomarkers for pre-analytical quality control of blood samples. *Biopreserv Biobank*. 2013;11(2):94-100. doi:10.1089/bio.2012.0051
3. Strimbu K, Tavel JA. What are biomarkers? *Curr Opin HIV AIDS*. 2010;5(6):463-466. doi:10.1097/COH.0b013e32833ed177
4. Holland NT, Pfleger L, Berger E, Ho A, Bastaki M. Molecular epidemiology biomarkers - Sample collection and processing considerations. *Toxicol Appl Pharmacol*. 2005;206(2):261-268. doi:10.1016/j.taap.2004.10.024
5. Serrano B, Ibáñez R, Robles C, Peremiquel-Trillas P, de Sanjosé S, Bruni L. Worldwide use of HPV self-sampling for cervical cancer screening. *Prev Med (Baltim)*. 2022;154:106900. doi:10.1016/J.YPMED.2021.106900
6. Nishimura H, Yeh PT, Oguntade H, Kennedy CE, Narasimhan M. HPV self-sampling for cervical cancer screening: A systematic review of values and preferences. *BMJ Glob Heal*. 2021;6(5):1-14. doi:10.1136/bmjgh-2020-003743
7. Van Keer S, Pattyn J, Tjalma WAA, et al. First-void urine: A potential biomarker source for triage of high-risk human papillomavirus infected women. *Eur J Obstet Gynecol Reprod Biol*. 2017;216:1-11. doi:10.1016/j.ejogrb.2017.06.036
8. Ducancelle A, Reiser J, Pivert A, Guillou-Guillemette H Le, Le Duc-Banaszuk AS, Lunel-Fabiani F. Home-based urinary HPV DNA testing in women who do not attend cervical cancer screening clinics. *J Infect*. 2015;71(3):377-384. doi:10.1016/j.jinf.2015.05.001
9. Lebeer S, Ahannach S, Gehrmann T, et al. A citizen-science-enabled catalogue of the vaginal microbiome and associated factors. *Nat Microbiol*. 2023;8(11):2183-2195. doi:10.1038/s41564-023-01500-0
10. Nikolaou E, German EL, Howard A, et al. Assessing the use of minimally invasive self-sampling at home for long-term monitoring of the microbiota within UK families. *Sci Rep*. 2023;13(1). doi:10.1038/s41598-023-45574-6
11. German EL, Nabwera HM, Robinson R, et al. Participant perceptions and experiences of a novel community-based respiratory longitudinal sampling method in Liverpool, UK: A mixed methods feasibility study. *PLoS One*. 2023;18(11 November). doi:10.1371/journal.pone.0294133
12. Su X, Xia D, Sun Y, et al. A novel internet sampling for HIV surveillance: feasibility of self-sampling and preparation of DBS for delivery detection of HIV total nucleic acid and complementarity to sentinel surveillance. *BMC Infect Dis*. 2023;23(1). doi:10.1186/s12879-023-08456-w

13. Parker SL, Amboree TL, Bulsara S, et al. Self-Sampling for Human Papillomavirus Testing: Acceptability in a U.S. Safety Net Health System. *Am J Prev Med*. Published online November 2023. doi:10.1016/j.amepre.2023.10.020
14. Burdon DW. Immunoglobulins of normal human urine and urethral secretions. *Immunology*. 1971;21(2):363. Accessed September 14, 2023. /pmc/articles/PMC1408118/?report=abstract
15. Mohandas S, Balan S, Mourya D. Urinary immunoglobulins in viral diagnosis: An overview. *Indian J Med Res*. 2022;155(1):11-21. doi:10.4103/ijmr.IJMR_808_18
16. Decramer S, de Peredo AG, Breuil B, et al. Urine in clinical proteomics. *Mol Cell Proteomics*. 2008;7(10):1850-1862. doi:10.1074/mcp.R800001-MCP200
17. Oshi M, Murthy V, Takahashi H, et al. Urine as a source of liquid biopsy for cancer. *Cancers (Basel)*. 2021;13(11):1-15. doi:10.3390/cancers13112652
18. Zancan M, Franceschini R, Mimmo C, et al. Free DNA in Urine: A New Marker for Bladder Cancer? Preliminary Data. *Int J Biol Markers*. 2005;20(2):134-136. doi:10.1177/172460080502000209
19. Vorsters A, Van Damme P, Clifford G. Urine testing for HPV: Rationale for using first void. *BMJ*. 2014;349(October):6252. doi:10.1136/bmj.g6252
20. Deltourbe L, Lacerda Mariano L, Hreha TN, Hunstad DA, Ingersoll MA. The impact of biological sex on diseases of the urinary tract. *Mucosal Immunol*. 2022;15(5):857-866. doi:10.1038/s41385-022-00549-0
21. Mändar R. Microbiota of male genital tract: Impact on the health of man and his partner. *Pharmacol Res*. 2013;69(1):32-41. doi:10.1016/j.phrs.2012.10.019
22. Christiansen ME, Detti L. Clinically Relevant Female Genital Tract Anomalies. *Clin Obstet Gynecol*. 2017;60(1):18-26. doi:10.1097/GRF.0000000000000258
23. Plummer DC, Walters WA. 6 Female genital tract discharge. *Baillieres Clin Obstet Gynaecol*. 1993;7(1):139-158. doi:10.1016/S0950-3552(05)80150-6
24. Pattyn J, Van Keer S, Biesmans S, et al. Human papillomavirus detection in urine: Effect of a first-void urine collection device and timing of collection. *J Virol Methods*. 2019;264:23-30. doi:10.1016/j.jviromet.2018.11.008
25. Pathak N, Dodds J, Zamora J, Khan K. Accuracy of urinary human papillomavirus testing for presence of cervical HPV: Systematic review and meta-analysis. *BMJ*. 2014;349. doi:10.1136/bmj.g5264
26. Augustus E, van Casteren K, Sorber L, et al. The art of obtaining a high yield of cell-free DNA from urine. *PLoS One*. 2020;15(4). doi:10.1371/journal.pone.0231058
27. Hentschel AE, van den Helder R, van Trommel NE, et al. The origin of tumor DNA in urine of urogenital cancer patients: Local shedding and transrenal excretion. *Cancers (Basel)*. 2021;13(3):1-11. doi:10.3390/cancers13030535
28. Dudley JC, Schroers-Martin J, Lazzareschi D V, et al. Detection and surveillance of bladder cancer using urine tumor DNA Acquisition of data: Analysis and interpretation of data: HHS Public Access. *Cancer Discov*. 2019;9(4):500-509.

doi:10.1158/2159-8290.CD-18-0825

29. Pattyn J, Keer S Van, Téblick L, Damme P Van. Expert Review of Anti-infective Therapy HPV DNA detection in urine samples of women : ' an efficacious and accurate alternative to cervical. *Expert Rev Anti Infect Ther*. 2019;00(00):1-3. doi:10.1080/14787210.2019.1668776
30. Scherpenisse M, Mollers M, Schepp RM, et al. Detection of systemic and mucosal HPV-specific IgG and IgA antibodies in adolescent girls one and two years after HPV vaccination. *Hum Vaccines Immunother*. 2013;9(2):314-321. doi:10.4161/hv.22693
31. Pattyn J, Van Keer S, Tjalma W, Matheeussen V, Van Damme P, Vorsters A. Infection and vaccine-induced HPV-specific antibodies in cervicovaginal secretions. A review of the literature. *Papillomavirus Res*. 2019;8:100185. doi:10.1016/j.pvr.2019.100185
32. Terada K, Niizuma T, Kataoka N, Niitani Y. Testing for rubella-specific IgG antibody in urine. *Pediatr Infect Dis J*. 2000;19(2):104-108. doi:10.1097/00006454-200002000-00003
33. Pattyn J, Van Keer S, Téblick L, Van Damme P, Vorsters A. Non-invasive Assessment of Vaccine-Induced HPV Antibodies via First-Void Urine. *Front Immunol*. 2020;11. doi:10.3389/fimmu.2020.01657
34. De Baetselier I, Smet H, Abdellati S, et al. Evaluation of the "Colli-Pee", a first-void urine collection device for self-sampling at home for the detection of sexually transmitted infections, versus a routine clinic-based urine collection in a one-to-one comparison study design: Efficacy and accept. *BMJ Open*. 2019;9(4). doi:10.1136/bmjopen-2018-028145
35. De Pauw H, Donders G, Weyers S, et al. Cervical cancer screening using HPV tests on self-samples: attitudes and preferences of women participating in the VALHUDES study. *Arch Public Heal*. 2021;79(1). doi:10.1186/s13690-021-00667-4
36. Racey CS, Withrow DR, Gesink D. Self-collected HPV testing improves participation in cervical cancer screening: a systematic review and meta-analysis. *Can J Public Health*. 2013;104(2):e159-66. Accessed September 10, 2019. http://www.ncbi.nlm.nih.gov/pubmed/23618210
37. Arbyn M, Smith SB, Temin S, Sultana F, Castle P. Detecting cervical precancer and reaching underscreened women by using HPV testing on self samples: updated meta-analyses. *BMJ*. 2018;363:4823. doi:10.1136/BMJ.K4823
38. Cem Ertik F, Kampers J, Hülse F, et al. CoCoss-Trial: Concurrent Comparison of Self-Sampling Devices for HPV-Detection. *Public Health*. 2021;18:10388. doi:10.3390/ijerph181910388
39. Combita AL, Gheit T, Gonzalez P, et al. Comparison between Urine and Cervical Samples for HPV DNA detection and typing in young women in Colombia. *Cancer Prev Res*. 2016;9(9):766-771. doi:10.1158/1940-6207.CAPR-16-0038

40. Bober P, Firment P, Sabo J. Diagnostic Test Accuracy of First-Void Urine Human Papillomaviruses for Presence Cervical HPV in Women: Systematic Review and Meta-Analysis. *Int J Environ Res Public Health*. 2021;18(24):13314. doi:10.3390/IJERPH182413314
41. Téblick L, Van Keer S, De Smet A, et al. Impact of Collection Volume and DNA Extraction Method on the Detection of Biomarkers and HPV DNA in First-Void Urine. *Molecules*. 2021;26(7):1989. doi:10.3390/molecules26071989
42. Van Keer S, Tjalma WAA, Pattyn J, et al. Human papillomavirus genotype and viral load agreement between paired first-void urine and clinician-collected cervical samples. *Eur J Clin Microbiol Infect Dis*. 2018;37(5):859-869. doi:10.1007/s10096-017-3179-1
43. Arbyn M, Peeters E, Benoy I, et al. VALHUDES: A protocol for validation of human papillomavirus assays and collection devices for HPV testing on self-samples and urine samples. *J Clin Virol*. 2018;107:52-56. doi:10.1016/j.jcv.2018.08.006
44. Van Keer S, Latsuzbaia A, Vanden Broeck D, et al. Analytical and clinical performance of extended HPV genotyping with BD Onclarity HPV Assay in home-collected first-void urine: A diagnostic test accuracy study. *J Clin Virol*. 2022;155. doi:10.1016/J.JCV.2022.105271
45. Senkomago V, Des Marais AC, Rahangdale L, Vibat CRT, Erlander MG, Smith JS. Comparison of urine specimen collection times and testing fractions for the detection of high-risk human papillomavirus and high-grade cervical precancer. *J Clin Virol*. 2016;74:26-31. doi:10.1016/j.jcv.2015.11.005
46. Van Keer S, Peeters E, Vanden Broeck D, et al. Clinical and analytical evaluation of the RealTime High Risk HPV assay in Colli-Pee collected first-void urine using the VALHUDES protocol. *Gynecol Oncol*. 2021;162(3):575-583. doi:10.1016/J.YGYNO.2021.06.010
47. Latsuzbaia A, Van Keer S, Vanden Broeck D, et al. Clinical Accuracy of Alinity m HR HPV Assay on Self- versus Clinician-Taken Samples Using the VALHUDES Protocol. *J Mol Diagn*. 2023;25(12):957-966. doi:10.1016/J.JMOLDX.2023.09.008
48. Van Keer S, van Splunter AP, Pattyn J, et al. Triage of human papillomavirus infected women by methylation analysis in first-void urine. *Sci Rep*. 2021;11(1). doi:10.1038/s41598-021-87329-1
49. Van Keer S, Willhauck-Fleckenstein M, Pattyn J, et al. First-void urine as a non-invasive liquid biopsy source to detect vaccine-induced human papillomavirus antibodies originating from cervicovaginal secretions. *J Clin Virol*. 2019;117:11-18. doi:10.1016/j.jcv.2019.05.004
50. Pattyn J, Panicker G, Willhauck-Fleckenstein M, et al. Comparison of a VLP-based and GST-L1-based multiplex immunoassay to detect vaccine-induced HPV-specific antibodies in first-void urine. *J Med Virol*. 2020;92(12):3774-3783.

doi:10.1002/jmv.25841
51. Téblick L, Pattyn J, Van Keer S, et al. Follow-up of humoral immune response after HPV vaccination using first-void urine: A longitudinal cohort study. *J Med Virol*. 2023;95(10). doi:10.1002/jmv.29133
52. Pickett ML, Visotcky A, Brazauskas R, Ledeboer NA, Drendel AL. Can a Clean Catch Urine Sample Be Used to Diagnose Chlamydia and Gonorrhea in Adolescent Females? *J Adolesc Heal*. 2021;69(4):574-578. doi:10.1016/j.jadohealth.2021.02.022
53. Tyker A, Pudwell J, Schneiderman M, Hundal P, Thorne J, Jamieson MA. Prevalence of Chlamydia and Gonorrhea Among Pregnant Adolescents Screened in the Third Trimester Using a Urine PCR Test: A Retrospective Review. *J Obstet Gynaecol Canada*. 2021;43(9):1069-1075. doi:10.1016/j.jogc.2020.12.018
54. Lv Y, Li G, Hu M, et al. Anonymous Linkage between College Students and Human Immunodeficiency Virus (HIV) Facilities: Systematic Evaluation of Urine Self-Collection for HIV Testing Initiative in China. *Clin Infect Dis*. 2021;73(5):E1108-E1115. doi:10.1093/cid/ciaa1816
55. Irwin GR, Allen AM, Bancroft WH, et al. Hepatitis B antigen in saliva, urine, and stool. *Infect Immun*. 1975;11(1):142-145. doi:10.1128/iai.11.1.142-145.1975
56. Lu T, Han Y, Zhang R, Zhang K, Lin G, Li J. Quantitative detection of hepatitis C virus RNA in urine of patients with chronic hepatitis C using a novel real-time PCR assay. *J Med Virol*. 2019;91(1):115-123. doi:10.1002/jmv.25280
57. Joshi MS, Bhalla S, Kalrao VR, Dhongade RK, Chitambar SD. Exploring the concurrent presence of hepatitis A virus genome in serum, stool, saliva, and urine samples of hepatitis A patients. *Diagn Microbiol Infect Dis*. 2014;78(4):379-382. doi:10.1016/j.diagmicrobio.2013.12.013
58. Tan NK, Pope CF, Carrington D. Performance evaluation of fully automated cobas ® 6800 CMV PCR for the detection and quantification of cytomegalovirus DNA in neonatal urine and saliva , and adult urine , saliva , and vaginal secretion. *J Med Virol*. 2023;95(October). doi:10.1002/jmv.29223
59. Espiritu MM, Bailey S, Wachtel E V., Mally P V. Utility of routine urine CMV PCR and total serum IgM testing of small for gestational age infants: A single center review. *J Perinat Med*. 2018;46(1):81-86. doi:10.1515/jpm-2016-0287
60. Ardizzone CM, Albritton HL, Lillis RA, et al. Human genital antibody-mediated inhibition of Chlamydia trachomatis infection and evidence for ompA genotype-specific neutralization. *PLoS One*. 2021;16(10 October):e0258759. doi:10.1371/journal.pone.0258759
61. Connell JA, Parry J V., Mortimer PP, et al. Preliminary report: Accurate assays for anti-HIV in urine. *Lancet*. 1990;335(8702):1366-1369. doi:10.1016/0140-6736(90)91245-6
62. Connell JA, Parry J V. Detection of anti-HIV in saliva and urine at the time of

seroconversion. *Clin Diagn Virol*. 1994;1(5-6):299-311. doi:10.1016/0928-0197(94)90060-4

63. Szarvas T, Kovalszky I, Bedi K, et al. Deletion analysis of tumor and urinary DNA to detect bladder cancer: Urine supernatant versus urine sediment. *Oncol Rep*. 2007;18(2):405-409. doi:10.3892/or.18.2.405
64. Togneri FS, Ward DG, Foster JM, et al. Genomic complexity of urothelial bladder cancer revealed in urinary cfDNA. *Eur J Hum Genet*. 2016;24(8):1167-1174. doi:10.1038/ejhg.2015.281
65. Di Meo A, Bartlett J, Cheng Y, Pasic MD, Yousef GM. Liquid biopsy: A step forward towards precision medicine in urologic malignancies. *Mol Cancer*. 2017;16(1). doi:10.1186/s12943-017-0644-5
66. Dermody SM, Bhambhani C, Swiecicki PL, Brenner JC, Tewari M. Trans-Renal Cell-Free Tumor DNA for Urine-Based Liquid Biopsy of Cancer. *Front Genet*. 2022;13:879108. doi:10.3389/fgene.2022.879108
67. Araldi RP, Assaf SMR, de Carvalho RF, et al. Papillomaviruses: A systematic review. *Genet Mol Biol*. 2017;40(1):1-21. doi:10.1590/1678-4685-GMB-2016-0128
68. Araldi RP, Sant'Ana TA, Módolo DG, et al. The human papillomavirus (HPV)-related cancer biology: An overview. *Biomed Pharmacother*. 2018;106:1537-1556. doi:10.1016/J.BIOPHA.2018.06.149
69. Tommasino M. The human papillomavirus family and its role in carcinogenesis. *Semin Cancer Biol*. 2014;26:13-21. doi:10.1016/j.semcancer.2013.11.002
70. Van Doorslaer K. Evolution of the Papillomaviridae. *Virology*. 2013;445(1-2):11-20. doi:10.1016/j.virol.2013.05.012
71. Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses. *Vaccine*. 2012;30(SUPPL.5):F55-F70. doi:10.1016/j.vaccine.2012.06.083
72. Bouvard V, Baan R, Straif K, et al. A review of human carcinogens--Part B: biological agents. *Lancet Oncol*. 2009;10(4):321-322. doi:10.1016/s1470-2045(09)70096-8
73. Richards KF, Bienkowska-Haba M, Dasgupta J, Chen XS, Sapp M. Multiple Heparan Sulfate Binding Site Engagements Are Required for the Infectious Entry of Human Papillomavirus Type 16. *J Virol*. 2013;87(21):11426-11437. doi:10.1128/jvi.01721-13
74. Culp TD, Budgeon LR, Marinkovich MP, Meneguzzi G, Christensen ND. Keratinocyte-Secreted Laminin 5 Can Function as a Transient Receptor for Human Papillomaviruses by Binding Virions and Transferring Them to Adjacent Cells. *J Virol*. 2006;80(18):8940-8950. doi:10.1128/jvi.00724-06
75. Roden RBS, Stern PL. Opportunities and challenges for human papillomavirus vaccination in cancer. *Nat Rev Cancer*. 2018;18(4):240-254. doi:10.1038/nrc.2018.13

76. Day PM, Gambhira R, Roden RBS, Lowy DR, Schiller JT. Mechanisms of Human Papillomavirus Type 16 Neutralization by L2 Cross-Neutralizing and L1 Type-Specific Antibodies. *J Virol*. 2008;82(9):4638-4646. doi:10.1128/jvi.00143-08
77. DiGiuseppe S, Bienkowska-Haba M, Guion LG, Sapp M. Cruising the cellular highways: How human papillomavirus travels from the surface to the nucleus. *Virus Res*. 2017;231:1-9. doi:10.1016/j.virusres.2016.10.015
78. Woodman CBJ, Collins SI, Young LS. The natural history of cervical HPV infection: Unresolved issues. *Nat Rev Cancer*. 2007;7(1):11-22. doi:10.1038/nrc2050
79. Stanley M. HPV - Immune response to infection and vaccination. *Infect Agent Cancer*. 2010;5(1). doi:10.1186/1750-9378-5-19
80. Yousefi Z, Aria H, Ghaedrahmati F, et al. An Update on Human Papilloma Virus Vaccines: History, Types, Protection, and Efficacy. *Front Immunol*. 2022;12. doi:10.3389/FIMMU.2021.805695
81. Bontkes HJ, De Gruijl TD, Walboomers JMM, et al. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. II. Systemic but not local IgA responses correlate with clearance of HPV-16. *J Gen Virol*. 1999;80(2):409-417. doi:10.1099/0022-1317-80-2-409
82. Stanley M. Immune responses to human papillomavirus. *Vaccine*. 2006;24(SUPPL. 1):S16. doi:10.1016/j.vaccine.2005.09.002
83. Schwarz TF, Leo O. Immune response to human papillomavirus after prophylactic vaccination with AS04-adjuvanted HPV-16/18 vaccine: Improving upon nature. *Gynecol Oncol*. 2008;110(3 SUPPL.1):S1. doi:10.1016/j.ygyno.2008.05.036
84. Sasagawa T, Takagi H, Makinoda S. Immune responses against human papillomavirus (HPV) infection and evasion of host defense in cervical cancer. *J Infect Chemother*. 2012;18(6):807-815. doi:10.1007/s10156-012-0485-5
85. Passmore JAS, Marais DJ, Sampson C, et al. Cervicovaginal, oral, and serum IgG and IgA responses to human papillomavirus type 16 in women with cervical intraepithelial neoplasia. *J Med Virol*. 2007;79(9):1375-1380. doi:10.1002/jmv.20901
86. Rocha-Zavaleta L, Pereira-Suarez AL, Yescas G, Cruz-Mimiaga RM, Garcia-Carranca A, Cruz-Talonia F. Mucosal IgG and IgA responses to human papillomavirus type 16 capsid proteins in HPV16-infected women without visible pathology. *Viral Immunol*. 2003;16(2):159-168. doi:10.1089/088282403322017893
87. Stanley MA, Sudenga SL, Giuliano AR. Alternative dosage schedules with HPV virus-like particle vaccines. *Expert Rev Vaccines*. 2014;13(8):1027-1038. doi:10.1586/14760584.2014.935767
88. Formana D, de Martel C, Lacey CJ, et al. Global burden of human papillomavirus and related diseases. *Vaccine*. 2012;30(SUPPL.5):F12-F23.

doi:10.1016/j.vaccine.2012.07.055

89. Zur Hausen H. Papillomavirus infections - A major cause of human cancers. *Biochim Biophys Acta - Rev Cancer*. 1996;1288(2). doi:10.1016/0304-419X(96)00020-0
90. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424. doi:10.3322/caac.21492
91. Biswas A. Human Papillomavirus (HPV) and Cervical Cancer. *J Indian Med Assoc*. 2000;98(2):53-55. Accessed November 9, 2023. https://www.who.int/en/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer
92. Ghittoni R, Accardi R, Hasan U, Gheit T, Sylla B, Tommasino M. The biological properties of E6 and E7 oncoproteins from human papillomaviruses. *Virus Genes 2009 401*. 2009;40(1):1-13. doi:10.1007/S11262-009-0412-8
93. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. *J Clin Virol*. 2016;76(Suppl 1):S49-S55. doi:10.1016/j.jcv.2015.11.015
94. Bierman R, Beardsley L, Chang CJ, Burk RD. Natural History of Cervicovaginal Papillomavirus Infection in Young Women GYF Ho. *J Low Genit Tract Dis*. 1998;2(4):235. doi:10.1097/00128360-199810000-00020
95. Rodríguez AC, Schiffman M, Herrero R, et al. Rapid clearance of human papillomavirus and implications for clinical focus on persistent infections. *J Natl Cancer Inst*. 2008;100(7):513-517. doi:10.1093/JNCI/DJN044
96. Ball SLR, Winder DM, Vaughan K, et al. Analyses of human papillomavirus genotypes and viral loads in anogenital warts. *J Med Virol*. 2011;83(8):1345-1350. doi:10.1002/JMV.22111
97. Gallay C, Miranda E, Schaefer S, et al. Human papillomavirus (HPV) contamination of gynaecological equipment. *Sex Transm Infect*. 2016;92(1):19-23. doi:10.1136/sextrans-2014-051977
98. Tjalma W. HPV negative cervical cancers and primary HPV screening. *Facts, Views Vis ObGyn*. 2018;10(2):107. Accessed January 20, 2023. /pmc/articles/PMC6516188/
99. Verlaat W, Snoek BC, Heideman DAM, et al. Identification and validation of a 3-gene methylation classifier for hpv-based cervical screening on self-samples. *Clin Cancer Res*. 2018;24(14):3456-3464. doi:10.1158/1078-0432.CCR-17-3615
100. van den Helder R, Steenbergen RDM, van Splunter AP, et al. HPV and DNA Methylation Testing in Urine for Cervical Intraepithelial Neoplasia and Cervical Cancer Detection. *Clin Cancer Res*. 2022;28(10):2061-2068. doi:10.1158/1078-0432.CCR-21-3710
101. Castle PE, Murokora D, Perez C, Alvarez M, Quek SC, Campbell C. Treatment of cervical intraepithelial lesions. *Int J Gynecol Obstet*. 2017;138:20-25.

doi:10.1002/ijgo.12191
102. Liontos M, Kyriazoglou A, Dimitriadis I, Dimopoulos MA, Bamias A. Systemic therapy in cervical cancer: 30 years in review. *Crit Rev Oncol Hematol*. 2019;137:9-17. doi:10.1016/J.CRITREVONC.2019.02.009
103. Hill EK. Updates in Cervical Cancer Treatment. *Clin Obstet Gynecol*. 2020;63(1):3-11. doi:10.1097/GRF.0000000000000507
104. Aitken CA, Siebers AG, Matthijsse SM, et al. Management and treatment of cervical intraepithelial neoplasia in the Netherlands after referral for colposcopy. *Acta Obstet Gynecol Scand*. 2019;98(6):737-746. doi:10.1111/aogs.13547
105. Schiller JT, Davies P. Delivering on the promise: HPV vaccines and cervical cancer. *Nat Rev Microbiol*. 2004;2(4):343-347. doi:10.1038/nrmicro867
106. Schiller JT, Lowy DR. Papillomavirus-Like Particle Vaccines. *JNCI Monogr*. 2012;2000(28):50-54. doi:10.1093/oxfordjournals.jncimonographs.a024258
107. Li M, Zhao C, Zhao Y, Li J, Wei L. Immunogenicity, efficacy, and safety of human papillomavirus vaccine: Data from China. *Front Immunol*. 2023;14. doi:10.3389/FIMMU.2023.1112750
108. Akhatova A, Azizan A, Atageldiyeva K, et al. Prophylactic Human Papillomavirus Vaccination: From the Origin to the Current State. *Vaccines*. 2022;10(11). doi:10.3390/VACCINES10111912
109. Sharma H, Parekh S, Pujari P, et al. Immunogenicity and safety of a new quadrivalent HPV vaccine in girls and boys aged 9–14 years versus an established quadrivalent HPV vaccine in women aged 15–26 years in India: a randomised, active-controlled, multicentre, phase 2/3 trial. *Lancet Oncol*. 2023;0(0). doi:10.1016/S1470-2045(23)00480-1
110. Villa LL, Costa RLR, Petta CA, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: A randomised double-blind placebo-controlled multicentre phase II efficacy trial. *Lancet Oncol*. 2005;6(5):271-278. doi:10.1016/S1470-2045(05)70101-7
111. Harper DM, Franco EL, Wheeler CM, et al. Sustained efficacy up to 4·5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet*. 2006;367(9518):1247-1255. doi:10.1016/S0140-6736(06)68439-0
112. Paavonen J, Jenkins D, Bosch FX, et al. Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. *Lancet*. 2007;369(9580):2161-2170. doi:10.1016/S0140-6736(07)60946-5
113. Harper DM, DeMars LR. HPV vaccines – A review of the first decade. *Gynecol Oncol*. 2017;146(1):196-204. doi:10.1016/J.YGYNO.2017.04.004
114. Lehtinen M, Dillner J. Clinical trials of human papillomavirus vaccines and beyond.

Nat Rev Clin Oncol. 2013;10(7):400-410. doi:10.1038/nrclinonc.2013.84
115. Schiffman M, Doorbar J, Wentzensen N, et al. Carcinogenic human papillomavirus infection. *Nat Rev Dis Prim*. 2016;2. doi:10.1038/nrdp.2016.86
116. Joshi S, Anantharaman D, Muwonge R, et al. Evaluation of immune response to single dose of quadrivalent HPV vaccine at 10-year post-vaccination. *Vaccine*. 2023;41(1):236-245. doi:10.1016/j.vaccine.2022.11.044
117. Basu P, Malvi SG, Joshi S, et al. Vaccine efficacy against persistent human papillomavirus (HPV) 16/18 infection at 10 years after one, two, and three doses of quadrivalent HPV vaccine in girls in India: a multicentre, prospective, cohort study. *Lancet Oncol*. 2021;22(11):1518-1529. doi:10.1016/S1470-2045(21)00453-8
118. Falcaro M, Castañon A, Ndlela B, et al. The effects of the national HPV vaccination programme in England, UK, on cervical cancer and grade 3 cervical intraepithelial neoplasia incidence: a register-based observational study. *Lancet*. 2021;398(10316):2084-2092. doi:10.1016/S0140-6736(21)02178-4
119. Zhou C, Tuong ZK, Frazer IH. Papillomavirus Immune Evasion Strategies Target the Infected Cell and the Local Immune System. *Front Oncol*. 2019;9(August). doi:10.3389/fonc.2019.00682
120. Brown DR, Joura EA, Yen GP, et al. Systematic literature review of cross-protective effect of HPV vaccines based on data from randomized clinical trials and real-world evidence. *Vaccine*. 2021;39(16):2224-2236. doi:10.1016/j.vaccine.2020.11.076
121. Harari A, Chen Z, Rodríguez AC, et al. Cross-protection of the Bivalent Human Papillomavirus (HPV) Vaccine Against Variants of Genetically Related High-Risk HPV Infections. *J Infect Dis*. 2016;213(6):939-947. doi:10.1093/infdis/jiv519
122. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. *Nat Rev Immunol*. 2004;4(1):46-54. doi:10.1038/nri1260
123. Stanley M, Pinto LA, Trimble C. Human Papillomavirus Vaccines – Immune Responses. *Vaccine*. 2012;30:F83-F87. doi:10.1016/j.vaccine.2012.04.106
124. Markowitz LE, Schiller JT. Human Papillomavirus Vaccines. *J Infect Dis*. 2021;224(12 Suppl 2):S367-S378. doi:10.1093/INFDIS/JIAA621
125. Schwarz TF, Spaczynski M, Schneider A, et al. Immunogenicity and tolerability of an HPV-16/18 AS04-adjuvanted prophylactic cervical cancer vaccine in women aged 15-55 years. *Vaccine*. 2009;27(4):581-587. doi:10.1016/j.vaccine.2008.10.088
126. Petrosky E, Bocchini JA, Hariri S, et al. Use of 9-valent human papillomavirus (HPV) vaccine: updated HPV vaccination recommendations of the advisory committee on immunization practices. *MMWR Morb Mortal Wkly Rep*. 2015;64(11):300-304. http://www.ncbi.nlm.nih.gov/pubmed/25811679%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4584883
127. Yadav R, Zhai L, Tumban E. Virus-like Particle-Based L2 Vaccines against HPVs:

Where Are We Today? *Viruses*. 2020;12(1). doi:10.3390/V12010018
128. Seitz H, Canali E, Ribeiro-Müller L, et al. A three component mix of thioredoxin-L2 antigens elicits broadly neutralizing responses against oncogenic human papillomaviruses. *Vaccine*. 2014;32(22):2610-2617. doi:10.1016/j.vaccine.2014.03.033
129. Kalnin K, Chivukula S, Tibbitts T, et al. Incorporation of RG1 epitope concatemers into a self-adjuvanting Flagellin-L2 vaccine broaden durable protection against cutaneous challenge with diverse human papillomavirus genotypes. *Vaccine*. 2017;35(37):4942-4951. doi:10.1016/j.vaccine.2017.07.086
130. Jagu S, Karanam B, Gambhira R, et al. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. *J Natl Cancer Inst*. 2009;101(11):782-792. doi:10.1093/jnci/djp106
131. Pouyanfard S, Spagnoli G, Bulli L, et al. Minor Capsid Protein L2 Polytope Induces Broad Protection against Oncogenic and Mucosal Human Papillomaviruses. *J Virol*. 2018;92(4):1930-1947. doi:10.1128/jvi.01930-17
132. Spagnoli G, Pouyanfard S, Cavazzini D, et al. Broadly neutralizing antiviral responses induced by a single-molecule HPV vaccine based on thermostable thioredoxin-L2 multiepitope nanoparticles. *Sci Rep*. 2017;7(1). doi:10.1038/s41598-017-18177-1
133. Rosales R, Rosales C. Immune therapy for human papillomaviruses-related cancers. *World J Clin Oncol*. 2014;5(5):1002-1019. doi:10.5306/wjco.v5.i5.1002
134. Yang A, Farmer E, Wu TC, Hung CF. Perspectives for therapeutic HPV vaccine development. *J Biomed Sci*. 2016;23(1):1-19. doi:10.1186/s12929-016-0293-9
135. Chabeda A, Yanez RJR, Lamprecht R, Meyers AE, Rybicki EP, Hitzeroth II. Therapeutic vaccines for high-risk HPV-associated diseases. *Papillomavirus Res*. 2018;5:46-58. doi:10.1016/j.pvr.2017.12.006
136. Vici P, Pizzuti L, Mariani L, et al. Targeting immune response with therapeutic vaccines in premalignant lesions and cervical cancer: hope or reality from clinical studies. *Expert Rev Vaccines*. 2016;15(10):1327-1336. doi:10.1080/14760584.2016.1176533
137. Yan F, Cowell LG, Tomkies A, Day AT. Therapeutic Vaccination for HPV-Mediated Cancers. *Curr Otorhinolaryngol Rep*. 2023;11(1):44. doi:10.1007/S40136-023-00443-8
138. Bruni L, Saura-Lázaro A, Montoliu A, et al. HPV vaccination introduction worldwide and WHO and UNICEF estimates of national HPV immunization coverage 2010–2019. *Prev Med (Baltim)*. 2021;144:106399. doi:10.1016/j.ypmed.2020.106399
139. Cameron RL, Kavanagh K, Pan J, et al. Human papillomavirus prevalence and herd immunity after introduction of vaccination program, Scotland, 2009–2013. *Emerg Infect Dis*. 2016;22(1):56-64. doi:10.3201/eid2201.150736
140. Romanowski B, Schwarz TF, Ferguson LM, et al. Immunogenicity and safety of the

HPV-16/18 AS04-adjuvanted vaccine administered as a 2-dose schedule compared with the licensed 3-dose schedule: results from a randomized study. *Hum Vaccin*. 2011;7(12):1374-1386. doi:10.4161/HV.7.12.18322

141. Dobson SRM, McNeil S, Dionne M, et al. Immunogenicity of 2 Doses of HPV Vaccine in Younger Adolescents vs 3 Doses in Young Women: A Randomized Clinical Trial. *JAMA*. 2013;309(17):1793-1802. doi:10.1001/JAMA.2013.1625
142. Tsang SH, Basu P, Bender N, et al. Evaluation of serological assays to monitor antibody responses to single-dose HPV vaccines. *Vaccine*. 2020;38(38):5997-6006. doi:10.1016/j.vaccine.2020.07.017
143. Director-General WHO. Accelerating the elimination of cervical cancer as a public health problem: towards achieving 90-70-90 targets by 2030. 2022;19(September):1-5. doi:10.1016/S1470-2045(18)30836-2.4
144. WHO. Human Papillomavirus (HPV) vaccination coverage. Published 2022. https://immunizationdata.who.int/pages/coverage/hpv.html?CODE=Global&YEAR=
145. Lei J, Ploner A, Elfström KM, et al. HPV Vaccination and the Risk of Invasive Cervical Cancer. *N Engl J Med*. 2020;383(14):1340-1348. doi:10.1056/NEJMoa1917338
146. Man I, Vänskä S, Lehtinen M, Bogaards JA. Human Papillomavirus Genotype Replacement: Still Too Early to Tell? *J Infect Dis*. 2021;224(3):481-491. doi:10.1093/INFDIS/JIAA032
147. Schlecht NF, Diaz A, Nucci-Sack A, et al. Incidence and Types of Human Papillomavirus Infections in Adolescent Girls and Young Women Immunized With the Human Papillomavirus Vaccine. *JAMA Netw Open*. 2021;4(8):e2121893-e2121893. doi:10.1001/JAMANETWORKOPEN.2021.21893
148. Tota JE, Struyf F, Merikukka M, et al. Evaluation of Type Replacement Following HPV16/18 Vaccination: Pooled Analysis of Two Randomized Trials. *J Natl Cancer Inst*. 2017;109(7):1-9. doi:10.1093/jnci/djw300
149. Garçon N, Morel S, Didierlaurent A, Descamps D, Wettendorff M, Van Mechelen M. Development of an AS04-adjuvanted HPV vaccine with the adjuvant system approach. *BioDrugs*. 2011;25(4):217-226. doi:10.2165/11591760-000000000-00000
150. Schiller JT. The Potential Benefits of HPV Vaccination in Previously Infected Women. Published online 2016. doi:10.1016/j.ebiom.2016.06.042
151. Vorsters A, Van Damme P, Bosch FX. HPV vaccination: Are we overlooking additional opportunities to control HPV infection and transmission? *Int J Infect Dis*. 2019;88:110-112. doi:10.1016/j.ijid.2019.09.006
152. Haupt RM, Wheeler CM, Brown DR, et al. Impact of an HPV6/11/16/18 L1 virus-like particle vaccine on progression to cervical intraepithelial neoplasia in seropositive women with HPV16/18 infection. *Int J Cancer*. 2011;129(11):2632-

2642. doi:10.1002/ijc.25940
153. Reuschenbach M, Doorbar J, del Pino M, et al. Prophylactic HPV vaccines in patients with HPV-associated diseases and cancer. *Vaccine*. 2023;41(42):6194-6205. doi:10.1016/j.vaccine.2023.08.047
154. Goldstone SE. Human papillomavirus (HPV) vaccines in adults: Learnings from long-term follow-up of quadrivalent HPV vaccine clinical trials. *Hum Vaccin Immunother*. 2023;19(1). doi:10.1080/21645515.2023.2184760
155. World Health Organization Expert Committee on Biological Standardization. Guidelines to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines. 2006;(962):51-113. http://www.who.int/biologicals/publications/trs/areas/vaccines/human_papillomavirus/HPVg Final BS 2050 .pdf
156. Miller C, Kemp TJ, Pinto LA. Development of a proficiency testing program for HPV serology assays used to evaluate antibody responses in vaccine trials. *J Immunol Methods*. Published online November 2023:113585. doi:10.1016/j.jim.2023.113585
157. Baussano I, Sayinzoga F, Tshomo U, et al. Impact of human papillomavirus vaccination, rwanda and Bhutan. *Emerg Infect Dis*. 2021;27(1):1-9. doi:10.3201/eid2701.191364
158. Franceschi S, Chantal Umulisa M, Tshomo U, et al. Urine testing to monitor the impact of HPV vaccination in Bhutan and Rwanda. *Int J Cancer*. 2016;139(3):518-526. doi:10.1002/ijc.30092
159. Baussano I, Tshomo U, Tenet V, et al. Prevalence of Human Papillomavirus and Estimation of Human Papillomavirus Vaccine Effectiveness in Thimphu, Bhutan, in 2011–2012 and 2018. *Ann Intern Med*. 2020;173(11):888-894. doi:10.7326/m20-2849
160. Sehr P, Rubio I, Seitz H, et al. High-Throughput Pseudovirion-Based Neutralization Assay for Analysis of Natural and Vaccine-Induced Antibodies against Human Papillomaviruses. *PLoS One*. 2013;8(10):75677. doi:10.1371/journal.pone.0075677
161. Brown D, Müller M, Sehr P, et al. Concordance assessment between a multiplexed competitive Luminex immunoassay, a multiplexed IgG Luminex immunoassay, and a pseudovirion-based neutralization assay for detection of human papillomaviruse types 16 and 18. *Vaccine*. 2014;32(44):5880-5887. doi:10.1016/j.vaccine.2014.08.004
162. Kemp TJ, Matsui K, Shelton G, Safaeian M, Pinto LA. A comparative study of two different assay kits for the detection of secreted alkaline phosphatase in HPV antibody neutralization assays. *Hum Vaccines Immunother*. 2015;11(2):337-346. doi:10.4161/21645515.2014.990851
163. Nie J, Huang W, Wu X, Wang Y. Optimization and validation of a high throughput

method for detecting neutralizing antibodies against human papillomavirus (HPV) based on pseudovirons. *J Med Virol*. 2014;86(9):1542-1555. doi:10.1002/jmv.23995

164. Panicker G, Rajbhandari I, Pathak HN, Brady AM, Unger ER. Multiplex immunoassay to measure antibody response to nine HPV vaccine types. *J Immunol Methods*. 2021;498:113136. doi:10.1016/j.jim.2021.113136
165. Waterboer T, Sehr P, Michael KM, et al. Multiplex human papillomavirus serology based on in situ-purified glutathione S-transferase fusion proteins. *Clin Chem*. 2005;51(10):1845-1853. doi:10.1373/clinchem.2005.052381
166. Panicker G, Rajbhandari I, Gurbaxani BM, Querec TD, Unger ER. Development and evaluation of multiplexed immunoassay for detection of antibodies to HPV vaccine types HHS Public Access. *J Immunol Methods*. 2015;417:107-114. doi:10.1016/j.jim.2014.12.013
167. Ferguson M, Wilkinson DE, Heath A, Matejtschuk P. The first international standard for antibodies to HPV 16. *Vaccine*. 2011;29(38):6520-6526. doi:10.1016/j.vaccine.2011.07.007
168. Kemp TJ, Panicker G, Eklund C, et al. Collaborative Study to Evaluate Candidate 1st WHO International Standards. 2022;(September).
169. Vorsters A, Van den Bergh J, Micalessi I, et al. Optimization of HPV DNA detection in urine by improving collection, storage, and extraction. *Eur J Clin Microbiol Infect Dis*. 2014;33(11):2005-2014. doi:10.1007/s10096-014-2147-2
170. Vorsters A, Micalessi I, Bilcke J, Ieven M, Bogers J, Van Damme P. Detection of human papillomavirus DNA in urine. A review of the literature. *Eur J Clin Microbiol Infect Dis*. 2012;31(5):627-640. doi:10.1007/s10096-011-1358-z
171. Pattyn J, Van Keer S, Téblick L, Van Damme P, Vorsters A. HPV DNA detection in urine samples of women: 'an efficacious and accurate alternative to cervical samples?' *Expert Rev Anti Infect Ther*. 2019;17(10):755-757. doi:10.1080/14787210.2019.1668776
172. Ieven M, Tjalma WAA, Van Ostade X, et al. First-void urine: A potential biomarker source for triage of high-risk human papillomavirus infected women. *Eur J Obstet Gynecol Reprod Biol*. 2017;216:1-11. doi:10.1016/j.ejogrb.2017.06.036
173. Luttmer R, De Strooper LMA, Steenbergen RDM, et al. Management of high-risk HPV-positive women for detection of cervical (pre)cancer. *Expert Rev Mol Diagn*. 2016;16(9):961-974. doi:10.1080/14737159.2016.1217157
174. Ebisch RMF, Siebers AG, Bosgraaf RP, Massuger LFAG, Bekkers RLM, Melchers WJG. Triage of high-risk HPV positive women in cervical cancer screening. *Expert Rev Anticancer Ther*. 2016;16(10):1073-1085. doi:10.1080/14737140.2016.1232166
175. Cho HW, Ouh YT, Hong JH, et al. Comparison of urine, self-collected vaginal swab, and cervical swab samples for detecting human papillomavirus (HPV) with

Roche Cobas HPV, Anyplex II HPV, and RealTime HR-S HPV assay. *J Virol Methods*. 2019;269:77-82. doi:10.1016/j.jviromet.2019.04.012

176. Snoek BC, Splunter AP va., Bleeker MCG, et al. Cervical cancer detection by DNA methylation analysis in urine. *Sci Rep*. 2019;9(1). doi:10.1038/s41598-019-39275-2
177. Burroni E, Bonanni P, Sani C, Lastrucci V, Carozzi F, The HPV ScreeVacc Working Group Ann KLALBCDPMCMLSBLIASTTABCA. Human papillomavirus prevalence in paired urine and cervical samples in women invited for cervical cancer screening. *J Med Virol*. 2015;87(3):508-515. doi:10.1002/jmv.24085
178. Cómbita AL, Gheit T, González P, et al. Comparison between Urine and Cervical Samples for HPV DNA Detection and Typing in Young Women in Colombia. *Cancer Prev Res (Phila)*. 2016;9(9):766-771. doi:10.1158/1940-6207.CAPR-16-0038
179. Ducancelle A, Legrand MC, Pivert A, et al. Interest of human papillomavirus DNA quantification and genotyping in paired cervical and urine samples to detect cervical lesions. *Arch Gynecol Obstet*. 2014;290(2):299-308. doi:10.1007/s00404-014-3191-y
180. Vorsters A, Van Keer S, Biesmans S, et al. Long-term follow-up of HPV infection using urine and cervical quantitative HPV DNA testing. *Int J Mol Sci*. 2016;17(5):750. doi:10.3390/ijms17050750
181. Rohner E, McGuire FH, Liu Y, et al. Racial and Ethnic Differences in Acceptability of Urine and Cervico-Vaginal Sample Self-Collection for HPV-Based Cervical Cancer Screening. *J Women's Heal*. Published online March 26, 2020:jwh.2019.8132. doi:10.1089/jwh.2019.8132
182. Sellors JW, Lorincz AT, Mahony JB, et al. Comparison of self-collected vaginal, vulvar and urine samples with physician-collected cervical samples for human papillomavirus testing to detect high-grade squamous intraepithelial lesions. *CMAJ*. 2000;163(5):513-518. Accessed November 30, 2020. /pmc/articles/PMC80457/?report=abstract
183. Tamalet C, Le Retraite L, Leandri FX, Heid P, Sancho Garnier H, Piana L. Vaginal self-sampling is an adequate means of screening HR-HPV types in women not participating in regular cervical cancer screening. *Clin Microbiol Infect*. 2013;19(1). doi:10.1111/1469-0691.12063
184. Verdoodt F, Jentschke M, Hillemanns P, Racey CS, Snijders PJF, Arbyn M. Reaching women who do not participate in the regular cervical cancer screening programme by offering self-sampling kits: A systematic review and meta-analysis of randomised trials. *Eur J Cancer*. 2015;51(16):2375-2385. doi:10.1016/j.ejca.2015.07.006
185. Gakidou E, Nordhagen S, Obermeyer Z. Coverage of Cervical Cancer Screening in 57 Countries: Low Average Levels and Large Inequalities. *PLoS Med*. 2008;5(6):e132. doi:10.1371/journal.pmed.0050132

186. Van Doornum GJJ, Guldemeester J, Osterhaus ADME, Niesters HGM. Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J Clin Microbiol*. 2003;41(2):576-580. doi:10.1128/JCM.41.2.576-580.2003
187. Niesters HGM. Molecular and diagnostic clinical virology in real time. *Clin Microbiol Infect*. 2004;10(1):5-11. doi:10.1111/j.1469-0691.2004.00699.x
188. Leeman A, del Pino M, Molijn A, et al. HPV testing in first-void urine provides sensitivity for CIN2+ detection comparable with a smear taken by a clinician or a brush-based self-sample: cross-sectional data from a triage population. *BJOG An Int J Obstet Gynaecol*. 2017;124(9):1356-1363. doi:10.1111/1471-0528.14682
189. BE 71030031000; Biobank Antwerpen, BBMR-ERIC; No. Access: (25), Last: February, 1 2021. [BIORESOURCE].
190. Payan C, Ducancelle A, Aboubaker MH, et al. Human papillomavirus quantification in urine and cervical samples by using the Mx4000 and LightCycler general real-time PCR systems. *J Clin Microbiol*. 2007;45(3):897-901. doi:10.1128/JCM.02022-06
191. Hesselink AT, Berkhof J, Van Der Salm ML, et al. Clinical validation of the HPV-Risk assay, a novel real-time PCR assay for detection of high-risk human papillomavirus DNA by targeting the E7 region. *J Clin Microbiol*. 2014;52(3):890-896. doi:10.1128/JCM.03195-13
192. Verlaat W, Snijders PJF, Novianti PW, et al. Genome-wide DNA methylation profiling reveals methylation markers associated with 3q gain for detection of cervical precancer and cancer. *Clin Cancer Res*. 2017;23(14):3813-3822. doi:10.1158/1078-0432.CCR-16-2641
193. Snellenberg S, Strooper LMAD, Hesselink AT, et al. Development of a multiplex methylation-specific PCR as candidate triage test for women with an HPV-positive cervical scrape. *BMC Cancer*. 2012;12(1):551. doi:10.1186/1471-2407-12-551
194. Overmeer R, Henken F, Snijders P, et al. Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J Pathol*. 2008;215(4):388-397. doi:10.1002/path.2367
195. Luttmer R, De Strooper LMA, Dijkstra MG, et al. FAM19A4 methylation analysis in self-samples compared with cervical scrapes for detecting cervical (pre)cancer in HPV-positive women. *Br J Cancer*. 2016;115(5):579-587. doi:10.1038/bjc.2016.200
196. Van Den Helder R, Van Trommel NE, Van Splunter AP, Lissenberg-Witte BI, Bleeker MCG, Steenbergen RDM. Methylation analysis in urine fractions for optimal CIN3 and cervical cancer detection. *Papillomavirus Res*. 2020;9:100193. doi:10.1016/j.pvr.2020.100193
197. Bosschieter J, Bach S, Bijnsdorp I V, et al. A protocol for urine collection and storage prior to DNA methylation analysis. *PLoS One*. 2018;13(8). doi:10.1371/journal.pone.0200906
198. Tshomo U, Franceschi S, Tshokey T, et al. Evaluation of the performance of Human

Papillomavirus testing in paired urine and clinician-collected cervical samples among women aged over 30 years in Bhutan. *Virol J*. 2017;14(1):74. doi:10.1186/s12985-017-0744-2

199. Wardak S. Human Papillomavirus (HPV) and cervical cancer. Medycyna doswiadczalna i mikrobiologia. doi:10.12968/indn.2020.2.20
200. Verlaat W, Van Leeuwen RW, Novianti PW, et al. Host-cell DNA methylation patterns during high-risk HPV-induced carcinogenesis reveal a heterogeneous nature of cervical pre-cancer. *Epigenetics*. 2018;13(7):769-778. doi:10.1080/15592294.2018.1507197
201. De Strooper LMA, Berkhof J, Steenbergen RDM, et al. Cervical cancer risk in HPV-positive women after a negative FAM19A4/mir124-2 methylation test: A post hoc analysis in the POBASCAM trial with 14 year follow-up. *Int J Cancer*. 2018;143(6):1541-1548. doi:10.1002/ijc.31539
202. Candiano G, Santucci L, Petretto A, et al. 2D-electrophoresis and the urine proteome map: Where do we stand? *J Proteomics*. 2010;73(5):829-844. doi:10.1016/j.jprot.2009.12.003
203. Ludolf F, Ramos FF, Bagno FF, et al. Detecting anti–SARS-CoV-2 antibodies in urine samples: A noninvasive and sensitive way to assay COVID-19 immune conversion. *Sci Adv*. 2022;8(19):7424. doi:10.1126/sciadv.abn7424
204. Nardelli-Haefliger D, Wirthner D, Schiller JT, et al. Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles. *J Natl Cancer Inst*. 2003;95(15):1128-1137. doi:10.1093/jnci/djg018
205. Franceschi S, Clifford GM, Baussano I. Options for design of real-world impact studies of single-dose vaccine schedules. *Vaccine*. 2018;36(32):4816-4822. doi:10.1016/j.vaccine.2018.02.002
206. Téblick L, Lipovac M, Molenberghs F, et al. HPV-specific antibodies in female genital tract secretions captured via first-void urine retain their neutralizing capacity retain their neutralizing capacity. *Hum Vaccin Immunother*. 2024;20(1). doi:10.1080/21645515.2024.2330168
207. Organization WH. *Human Papillomavirus Laboratory Manual Human Papillomavirus Laboratory Manual First Edition , 2009*. Vol 1.; 2009.
208. Giavarina D. Understanding Bland Altman analysis. *Biochem Medica*. 2015;25(2):141-151. doi:10.11613/BM.2015.015
209. Tesana S, Srisawangwong T, Sithithaworn P, Itoh M, Phumchaiyothin R. The ELISA-based detection of anti-Opisthorchis viverrini IgG and IgG4 in samples of human urine and serum from an endemic area of north–eastern Thailand. *http://dx.doi.org/101179/136485907X229068*. 2013;101(7):585-591. doi:10.1179/136485907X229068
210. Hashida S, Hashinaka K, Ishikawa S, Ishikawa E. More reliable diagnosis of infection

with human immunodeficiency virus type 1 (HIV-1) by detection of antibody IgGs to pol and gag proteins of HIV- 1 and p24 antigen of HIV-1 in urine, saliva, and/or serum with highly sensitive and specific enzyme immunoa. *J Clin Lab Anal*. 1997;11(5):267-286. doi:10.1002/(SICI)1098-2825(1997)11:5<267::AID-JCLA5>3.0.CO;2-4

211. Ruantip S, Eamudomkarn C, Kopolrat KY, Sithithaworn J, Laha T, Sithithaworn P. Analysis of Daily Variation for 3 and for 30 Days of Parasite-Specific IgG in Urine for Diagnosis of Strongyloidiasis by Enzyme-Linked Immunosorbent Assay. *Acta Trop*. 2021;218. doi:10.1016/j.actatropica.2021.105896
212. Zeng ZC, Chang Q, Sun ZW, et al. Detection of cytomegalovirus (CMV) infection in wheezing infants by urine DNA and serum IgG testing. *Med Sci Monit*. 2017;23:1242-1246. doi:10.12659/MSM.898589
213. Li X, Wang X, Wei Z, et al. Detection of IgG protein in human urine based on vertical flow paper microfluidic chip. *Shengwu Gongcheng Xuebao/Chinese J Biotechnol*. 2023;39(1):337-346. doi:10.13345/j.cjb.220246
214. Bratcher PE, Gaggar A. Characterization and prevention of the adsorption of surfactant protein D to polypropylene. *PLoS One*. 2013;8(9). doi:10.1371/journal.pone.0073467
215. Bergmann-Leitner ES, Mease RM, Duncan EH, Khan F, Waitumbi J, Angov E. Evaluation of immunoglobulin purification methods and their impact on quality and yield of antigen-specific antibodies. *Malar J*. 2008;7:129. doi:10.1186/1475-2875-7-129
216. Smith DR, Rossi CA, Kijek TM, Henchal EA, Ludwig G V. Comparison of dissociation-enhanced lanthanide fluorescent immunoassays to enzyme-linked immunosorbent assays for detection of staphylococcal enterotoxin B, Yersinia pestis-specific F1 antigen, and Venezuelan equine encephalitis virus. *Clin Diagn Lab Immunol*. 2001;8(6):1070-1075. doi:10.1128/CDLI.8.6.1070-1075.2001
217. Hagan AK, Zuchner T. Lanthanide-based time-resolved luminescence immunoassays. *Anal Bioanal Chem*. 2011;400(9):2847-2864. doi:10.1007/s00216-011-5047-7
218. Brittain HG, Konteatis Z. Solution chemistry of lanthanide complexes-III. Spectroscopic studies of lanthanide complexes of glycine and polyglycines. *J Inorg Nucl Chem*. 1981;43(7):1719-1723. doi:10.1016/0022-1902(81)80370-3
219. Tanner SP, Choppin GR. Lanthanide and Actinide Complexes of Glycine. Determination of Stability Constants and Thermodynamic Parameters by a Solvent Extraction Method. *Inorg Chem*. 1968;7(10):2046-2048. doi:10.1021/ic50068a017
220. Goderski S, Kanno S, Yoshihara K, et al. Lanthanide Luminescence Enhancement of Core-Shell Magnetite-SiO2Nanoparticles Covered with Chain-Structured Helical Eu/Tb Complexes. *ACS Omega*. 2020;5(51):32930-32938. doi:10.1021/acsomega.0c03746

221. Combita A-L, Touzé A, Bousarghin L, Christensen ND, Coursaget P. Identification of Two Cross-Neutralizing Linear Epitopes within the L1 Major Capsid Protein of Human Papillomaviruses. *J Virol*. 2002;76(13):6480-6486. doi:10.1128/JVI.76.13.6480-6486.2002
222. Daikuzono H, Yamazaki M, Sato Y, Takahashi T, Yamagata K. Development of a DELFIA method to detect oncofetal antigen ROR1-positive exosomes. *Biochem Biophys Res Commun*. 2021;578:170-176. doi:10.1016/J.BBRC.2021.08.054
223. Damiani V, Pizzinato E, Cicalini I, et al. Development of a Method for Detection of SARS-CoV-2 Nucleocapsid Antibodies on Dried Blood Spot by DELFIA Immunoassay. *Diagnostics*. 2023;13(5). doi:10.3390/DIAGNOSTICS13050897/S1
224. Einhorn L, Krapfenbauer K. HTRF: A technology tailored for biomarker determination-novel analytical detection system suitable for detection of specific autoimmune antibodies as biomarkers in nanogram level in different body fluids. *EPMA J*. 2015;6(1). doi:10.1186/s13167-015-0046-y
225. Yuan J, Wang G. Lanthanide complex-based fluorescence label for time-resolved fluorescence bioassay. *J Fluoresc*. 2005;15(4):559-568. doi:10.1007/s10895-005-2829-3
226. Darwish IA. Immunoassay Methods and their Applications in Pharmaceutical Analysis: Basic Methodology and Recent Advances. *Int J Biomed Sci*. 2006;2(3):217-235. Accessed September 6, 2023. /pmc/articles/PMC3614608/
227. Bielefeld-Sevigny M. AlphaLISA immunoassay platform- the "no-Wash" high-throughput alternative to ELISA. *Assay Drug Dev Technol*. 2009;7(1):90-92. doi:10.1089/adt.2009.9996
228. Walboomers JMM, Jacobs M V., Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*. 1999;189(1):12-19. doi:10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F
229. Struyf F, Colau B, Wheeler CM, et al. Post Hoc Analysis of the PATRICIA Randomized Trial of the Efficacy of Human Papillomavirus Type 16 (HPV-16)/HPV-18 AS04-Adjuvanted Vaccine against Incident and Persistent Infection with Nonvaccine Oncogenic HPV Types Using an Alternative Multiplex Type-Specific PCR Assay for HPV DNA. *Clin Vaccine Immunol*. 2015;22(2):235. doi:10.1128/CVI.00457-14
230. Longet S, Schiller JT, Bobst M, Jichlinski P, Nardelli-Haefliger D. A Murine Genital-Challenge Model Is a Sensitive Measure of Protective Antibodies against Human Papillomavirus Infection. *J Virol*. 2011;85(24):13253-13259. doi:10.1128/jvi.06093-11
231. Mestecky J, Moldoveanu Z, Russell MW. Immunologic uniqueness of the genital tract: Challenge for vaccine development. *Am J Reprod Immunol*. 2005;53(5):208-214. doi:10.1111/j.1600-0897.2005.00267.x
232. Zhou JZ, Way SS, Chen K. Immunology of the Uterine and Vaginal Mucosae. *Trends*

Immunol. 2018;39(4):302-314. doi:10.1016/J.IT.2018.01.007
233. Gillison ML, Chaturvedi AK, Lowy DR. HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. *Cancer*. 2008;113(10 SUPPL.):3036-3046. doi:10.1002/cncr.23764
234. Paavonen J, Naud P, Salmerón J, et al. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. *Lancet*. 2009;374(9686):301-314. doi:10.1016/S0140-6736(09)61248-4
235. Villa LL, Costa RLR, Petta CA, et al. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *Br J Cancer*. 2006;95(11):1459-1466. doi:10.1038/sj.bjc.6603469
236. Buck CB, Pastrana DV, Lowy DR SJ. Generation of HPV Pseudovirions Using Transfection and Their Use in Neutralization Assays. *Methods Mol Med*. 2005;119(1):445-462. doi:10.1385/1-59259-982-6:445
237. Gaudet RG, Breden F, Plummer F, Berry JD. Molecular characterization of the cervical and systemic B-cell repertoire. *MAbs*. 2011;3(2):181-191. doi:10.4161/mabs.3.2.14858
238. Scherer EM, Smith RA, Gallego DF, et al. A Single Human Papillomavirus Vaccine Dose Improves B Cell Memory in Previously Infected Subjects. *EBioMedicine*. 2016;10:55-64. doi:10.1016/j.ebiom.2016.06.042
239. Waterboer T, Sehr P, Pawlita M. Suppression of non-specific binding in serological Luminex assays. *J Immunol Methods*. 2006;309(1-2):200-204. doi:10.1016/J.JIM.2005.11.008
240. Beachler DC, Jenkins G, Safaeian M, Kreimer AR, Wentzensen N. Natural Acquired Immunity Against Subsequent Genital Human Papillomavirus Infection: A Systematic Review and Meta-analysis. *J Infect Dis*. 2016;213(9):1444-1454. doi:10.1093/infdis/jiv753
241. Rosillon D, Baril L, Del Rosario-Raymundo MR, et al. Risk of newly detected infections and cervical abnormalities in adult women seropositive or seronegative for naturally acquired HPV-16/18 antibodies. *Cancer Med*. 2019;8(10):4938-4953. doi:10.1002/cam4.1879
242. R H-L, L H, L L-M, et al. Performance of an affordable urine self-sampling method for human papillomavirus detection in Mexican women. *PLoS One*. 2021;16(7). doi:10.1371/JOURNAL.PONE.0254946
243. Moreira ED, Block SL, Ferris D, et al. Safety profile of the 9-valent HPV vaccine: A combined analysis of 7 phase III clinical trials. *Pediatrics*. 2016;138(2). doi:10.1542/peds.2015-4387
244. Depuydt CE, Beert J, Bosmans E, Salembier G. Human Papillomavirus (HPV) virion

induced cancer and subfertility, two sides of the same coin. *Facts, views Vis ObGyn*. 2016;8(4):211-222. Accessed May 15, 2019. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5303699/pdf/FVVinObGyn-8-211.pdf

245. Grabowska K, Wang X, Jacobsson A, Dillner J. Evaluation of cost-precision rations of different strategies for ELISA measurement of serum antibody levels. *J Immunol Methods*. 2002;271(1-2):1-15. doi:10.1016/S0022-1759(02)00334-4
246. Panwar K, Godi A, Cocuzza CE, et al. Binding antibody levels to vaccine (HPV6/11/16/18) and non-vaccine (HPV31/33/45/52/58) HPV antigens up to 7 years following immunization with either Cervarix® or Gardasil® vaccine. *Vaccine*. 2022;40(9):1198-1202. doi:10.1016/j.vaccine.2022.01.041
247. Porras C, Sampson JN, Herrero R, et al. Rationale and design of a double-blind randomized non-inferiority clinical trial to evaluate one or two doses of vaccine against human papillomavirus including an epidemiologic survey to estimate vaccine efficacy: The Costa Rica ESCUDDO trial. *Vaccine*. 2022;40(1):76-88. doi:10.1016/j.vaccine.2021.11.041
248. Hoes J, Pasmans H, Schurink-van 't Klooster TM, et al. Review of long-term immunogenicity following HPV vaccination: Gaps in current knowledge. *Hum Vaccines Immunother*. 2022;18(1). doi:10.1080/21645515.2021.1908059
249. Mo Y, Ma J, Zhang H, et al. Prophylactic and Therapeutic HPV Vaccines: Current Scenario and Perspectives. *Front Cell Infect Microbiol*. 2022;12:909223. doi:10.3389/FCIMB.2022.909223
250. Abraham S, Juel HB, Bang P, et al. Safety and immunogenicity of the chlamydia vaccine candidate CTH522 adjuvanted with CAF01 liposomes or aluminium hydroxide: a first-in-human, randomised, double-blind, placebo-controlled, phase 1 trial. *Lancet Infect Dis*. 2019;19(10):1091-1100. doi:10.1016/s1473-3099(19)30279-8
251. Burd EM. Human papillomavirus and cervical cancer. *Clin Microbiol Rev*. 2003;16(1):1-17. doi:10.1128/CMR.16.1.1-17.2003
252. de Sanjose S, Delany-Moretlwe S. HPV vaccines can be the hallmark of cancer prevention. *Lancet*. Published online June 26, 2019. doi:10.1016/s0140-6736(19)30549-5
253. Pinto LA, Dillner J, Beddows S, Unger ER. Immunogenicity of HPV prophylactic vaccines: Serology assays and their use in HPV vaccine evaluation and development. *Vaccine*. 2018;36(32):4792-4799. doi:10.1016/j.vaccine.2017.11.089
254. Williamson A-L. Recent Developments in Human Papillomavirus (HPV) Vaccinology. *Viruses 2023, Vol 15, Page 1440*. 2023;15(7):1440. doi:10.3390/V15071440
255. Laura A. Koutsky, Kevin A. Ault, Cosette M. Wheeler, Darron R. Brown, Eliav Barr, Frances B. Alvarez, Lisa M. Chiacchierini and KUJ. A controlled trial of a

human papillomavirus type 16 vaccine. *N Engl J Med*. 2002;347(21):1645-1651.
256. Cadman L, Reuter C, Jitlal M, et al. A randomized comparison of different vaginal self-sampling devices and urine for human papillomavirus testing-predictors 5.1. *Cancer Epidemiol Biomarkers Prev*. 2021;30(4):661-668. doi:10.1158/1055-9965.EPI-20-1226
257. Kwak K, Yemelyanova A, Roden RBS. Prevention of cancer by prophylactic human papillomavirus vaccines. *Curr Opin Immunol*. 2011;23(2):244-251. doi:10.1016/j.coi.2010.11.009
258. Bosch FX, Castellsagué X, de Sanjosé S. Prophylactic human papillomavirus vaccines. *Cancer Chemother Rev*. 2008;3(1):23-34. doi:10.1172/JCI28607
259. Dykens JA, Peterson CE, Holt HK, Harper DM. Gender neutral HPV vaccination programs: Reconsidering policies to expand cancer prevention globally. *Front Public Heal*. 2023;11. doi:10.3389/fpubh.2023.1067299
260. Di Donato V, Caruso G, Bogani G, et al. HPV Vaccination after Primary Treatment of HPV-Related Disease across Different Organ Sites: A Multidisciplinary Comprehensive Review and Meta-Analysis. *Vaccines*. 2022;10(2):239. doi:10.3390/vaccines10020239
261. Pastrana D V, Buck CB, Pang YYS, et al. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology*. 2004;321(2):205-216. doi:10.1016/j.virol.2003.12.027
262. Einstein MH, Baron M, Levin MJ, et al. Comparative immunogenicity and safety of human papillomavirus (HPV)-16/18 vaccine and HPV-6/11/16/18 vaccine: Follow-up from months 12-24 in a phase III randomized study of healthy women aged 18-45 y. *Hum Vaccin*. 2011;7(12):1343-1358. doi:10.4161/hv.7.12.18281
263. Einstein MH, Baron M, Levin MJ, et al. Comparison of the immunogenicity and safety of Cervarix™ and Gardasil® human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18-45 years. *Hum Vaccin*. 2009;5(10):705-719. doi:10.4161/hv.5.10.9518
264. Kemp TJ, Hildesheim A, Falk RT, et al. Evaluation of two types of sponges used to collect cervical secretions and assessment of antibody extraction protocols for recovery of neutralizing anti-human papillomavirus type 16 antibodies. *Clin Vaccine Immunol*. 2008;15(1):60-64. doi:10.1128/CVI.00118-07
265. Kemp TJ, García-Piñeres A, Falk RT, et al. Evaluation of systemic and mucosal anti-HPV16 and anti-HPV18 antibody responses from vaccinated women. *Vaccine*. 2008;26(29-30):3608-3616. doi:10.1016/j.vaccine.2008.04.074
266. Téblick L, Lipovac M, Burdier FR, et al. Concentration strategies for spiked and naturally present biomarkers in non-invasively collected first-void urine. *Eur J Med Res*. 2024;29(1):131. doi:10.1186/s40001-024-01719-5
267. Li JZ, Guo ZL, Sato T, et al. Optimized application of the secreted Nano-luciferase reporter system using an affinity purification strategy. *PLoS One*. 2018;13(5):1-15.

doi:10.1371/journal.pone.0196617
268. England CG, Ehlerding EB, Cai W. NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconjug Chem*. 2016;27(5):1175-1187. doi:10.1021/acs.bioconjchem.6b00112
269. Wu X, Zhang C, Feng S, et al. Detection of HPV types and neutralizing antibodies in gansu province, China. *J Med Virol*. 2009;81(4):693-702. doi:10.1002/jmv.21435
270. Nie J, Liu Y, Huang W, Wang Y. Development of a triple-color pseudovirion-based assay to detect neutralizing antibodies against human papillomavirus. *Viruses*. 2016;8(4). doi:10.3390/v8040107
271. Day PM, Pang YYS, Kines RC, Thompson CD, Lowy DR, Schiller JT. A human papillomavirus (HPV) in vitro neutralization assay that recapitulates the in vitro process of infection provides a sensitive measure of HPV L2 infection-inhibiting antibodies. *Clin Vaccine Immunol*. 2012;19(7):1075-1082. doi:10.1128/CVI.00139-12
272. Buck CB, Thompson CD, Roberts JN, Müller M, Lowy DR, Schiller JT. Carrageenan is a potent inhibitor of papillomavirus infection. *PLoS Pathog*. 2006;2(7):0671-0680. doi:10.1371/JOURNAL.PPAT.0020069
273. Buck CB, Thompson CD. Production of Papillomavirus-Based Gene Transfer Vectors. *Curr Protoc Cell Biol*. 2007;37(1):26.1.1-26.1.19. doi:10.1002/0471143030.cb2601s37
274. Christensen ND, Dillner J, Eklund C, et al. Surface conformational and linear epitopes on HPV-16 and HPV-18 L1 virus-like particles as defined by monoclonal antibodies. *Virology*. 1996;223(1):174-184. doi:10.1006/viro.1996.0466
275. Molenberghs F, Verschuuren M, Barbier M, et al. Cells infected with human papilloma pseudovirus display nuclear reorganization and heterogenous infection kinetics. *Cytom Part A*. 2022;101(12):1035-1048. doi:10.1002/cyto.a.24663
276. De Puysseleyr L, De Puysseleyr K, Vanrompay D, De Vos WH. Quantifying the growth of chlamydia suis in cell culture using high-content microscopy. *Microsc Res Tech*. 2017;80(4):350-356. doi:10.1002/jemt.22799
277. De Vos WH, Van Neste L, Dieriks B, Joss GH, Van Oostveldt P. High content image cytometry in the context of subnuclear organization. *Cytom Part A*. 2010;77(1):64-75. doi:10.1002/cyto.a.20807
278. Depuydt CE, Thys S, Beert J, Jonckheere J, Salembier G, Bogers JJ. Linear viral load increase of a single HPV-type in women with multiple HPV infections predicts progression to cervical cancer. *Int J cancer*. 2016;139(9):2021-2032. doi:10.1002/IJC.30238
279. Campbell, M.J., Machin, D., and Walters SJ. *Medical Statistics: A Textbook for Health Sciences.*; 2010.
280. Unal I. Defining an optimal cut-point value in ROC analysis: An alternative

approach. *Comput Math Methods Med*. 2017;2017. doi:10.1155/2017/3762651
281. Franceschi S, Clifford GM, Baussano I. Options for design of real-world impact studies of single-dose vaccine schedules. *Vaccine*. 2018;36(32):4816-4822. doi:10.1016/j.vaccine.2018.02.002
282. Scherpenisse M, Schepp RM, Mollers M, et al. Comparison of different assays to assess human papillomavirus (HPV) type 16- and 18-specific antibodies after HPV infection and vaccination. *Clin Vaccine Immunol*. 2013;20(8):1329-1332. doi:10.1128/CVI.00153-13
283. Oh JE, Iijima N, Song E, et al. Migrant memory B cells secrete luminal antibody in the vagina. *Nature*. 2019;571(7763):122-126. doi:10.1038/s41586-019-1285-1
284. Prabhu PR, Carter JJ, Galloway DA. B Cell Responses upon Human Papillomavirus (HPV) Infection and Vaccination. *Vaccines*. 2022;10(6). doi:10.3390/vaccines10060837
285. Herold BC, Mesquita PM, Madan RP, Keller MJ. Female Genital Tract Secretions and Semen Impact the Development of Microbicides for the Prevention of HIV and Other Sexually Transmitted Infections. *Am J Reprod Immunol*. 2011;65(3):325-333. doi:10.1111/J.1600-0897.2010.00932.X
286. Massé J, Watrin T, Laurent A, Deschamps S, Guerrier D, Pellerin I. The developing female genital tract: From genetics to epigenetics. *Int J Dev Biol*. 2009;53(2-3):411-424. doi:10.1387/ijdb.082680jm
287. Leeman A, del Pino M, Molijn A, et al. HPV testing in first-void urine provides sensitivity for CIN2+ detection comparable with a smear taken by a clinician or a brush-based self-sample: cross-sectional data from a triage population. *BJOG An Int J Obstet Gynaecol*. 2017;124(9):1356-1363. doi:10.1111/1471-0528.14682
288. Cervical cancer. Accessed June 23, 2022. https://www.who.int/news-room/fact-sheets/detail/cervical-cancer
289. Poljak M, Cuschieri K, Alemany L, Vorsters A. Testing for Human Papillomaviruses in Urine, Blood, and Oral Specimens: an Update for the Laboratory. *J Clin Microbiol*. 2023;61(8):e0140322. doi:10.1128/jcm.01403-22
290. De Baetselier I, Smet H, Abdellati S, et al. Evaluation of the "Colli-Pee", a first-void urine collection device for self-sampling at home for the detection of sexually transmitted infections, versus a routine clinic-based urine collection in a one-to-one comparison study design: Efficacy and accept. *BMJ Open*. 2019;9(4). doi:10.1136/bmjopen-2018-028145
291. Grad AI, Vica ML, Matei HV, Grad DL, Coman I, Tataru DA. Polymerase chain reaction as a diagnostic tool for six sexually transmitted infections - preliminary results. *Clujul Med*. 2015;88(1):33-37. doi:10.15386/cjmed-373
292. Harpole M, Davis J, Espina V. Expert Review of Proteomics Current state of the art for enhancing urine biomarker discovery Current state of the art for enhancing urine biomarker discovery. *Expert Rev Proteomics*. 2016;9450(6):609-626.

doi:10.1080/14789450.2016.1190651.Current
293. Bouatra S, Aziat F, Mandal R, et al. The Human Urine Metabolome. *PLoS One*. 2013;8(9). doi:10.1371/journal.pone.0073076
294. Noonin C, Kapincharanon C, Sueksakit K, Kanlaya R, Thongboonkerd V. Application of tandem fast protein liquid chromatography to purify intact native monomeric/aggregated Tamm-Horsfall protein from human urine and systematic comparisons with diatomaceous earth adsorption and salt precipitation: Yield, purity and time-consu. *Anal Methods*. 2021;13(30):3359-3367. doi:10.1039/d1ay00922b
295. Xu X, Barreiro K, Musante L, et al. Management of Tamm–Horsfall Protein for Reliable Urinary Analytics. *Proteomics - Clin Appl*. 2019;13(6). doi:10.1002/prca.201900018
296. Schaeffer C, Devuyst O, Rampoldi L. Uromodulin: Roles in Health and Disease. *Annu Rev Physiol*. 2021;83:477-501. doi:10.1146/annurev-physiol-031620-092817
297. Kipp A, Olinger E. What does uromodulin do? *Clin J Am Soc Nephrol*. 2021;16(1):150-153. doi:10.2215/CJN.06390420
298. Sapula SA, Whittall JJ, Pandopulos AJ, Gerber C, Venter H. An optimized and robust PEG precipitation method for detection of SARS-CoV-2 in wastewater. *Sci Total Environ*. 2021;785:147270. doi:10.1016/j.scitotenv.2021.147270
299. Lin SY, Luo Y, Marshall MM, et al. A new method for improving extraction efficiency and purity of urine and plasma cell-free dna. *Diagnostics*. 2021;11(4):1-9. doi:10.3390/diagnostics11040650
300. Atha DH, Ingham KC. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. *J Biol Chem*. 1981;256(23):12108-12117. doi:10.1016/s0021-9258(18)43240-1
301. Peitsaro P, Johansson B, Syrjänen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol*. 2002;40(3):886-891. doi:10.1128/JCM.40.3.886-891.2002
302. Clifford GM, Vaccarella S, Franceschi S, et al. Comparison of Two Widely Used Human Papillomavirus Detection and Genotyping Methods, GP5+/6+-Based PCR Followed by Reverse Line Blot Hybridization and Multiplex Type-Specific E7-Based PCR. *J Clin Microbiol*. 2016;54(8):2031-2038. doi:10.1128/jcm.00618-16
303. Aghbash PS, Hemmat N, Fathi H, Baghi HB. Monoclonal antibodies in cervical malignancy-related HPV. *Front Oncol*. 2022;12. doi:10.3389/fonc.2022.904790
304. Frazer IH, Chandra J. Immunotherapy for HPV associated cancer. *Papillomavirus Res*. 2019;8:100176. doi:10.1016/J.PVR.2019.100176
305. Hattori M, Nishimura Y, Toyonaga M, Kakinuma H, Matsumoto K, Ohbu M. Cytological significance of abnormal squamous cells in urinary cytology. *Diagn Cytopathol*. 2012;40(9):798-803. doi:10.1002/dc.21645
306. Serafini-Cessi F, Malagolini N, Cavallone D. Tamm-Horsfall glycoprotein: Biology

and clinical relevance. *Am J Kidney Dis*. 2003;42(4):658-676. doi:10.1016/S0272-6386(03)00829-1

307. Stanisich JJ, Zyla DS, Afanasyev P, et al. The cryo-em structure of the human uromodulin filament core reveals a unique assembly mechanism. *Elife*. 2020;9:1-46. doi:10.7554/ELIFE.60265
308. Shulgin IL, Ruckenstein E. Preferential hydration and solubility of proteins in aqueous solutions of polyethylene glycol. *Biophys Chem*. 2006;120(3):188-198. doi:10.1016/j.bpc.2005.11.010
309. World Health Organization. *Stronger Collaboration, Better Health: Global Action Plan for Healthy Lives and Well-Being for All. Strengthening Collaboration among Multilateral Organizations to Accelerate Country Progress on the Health-Related Sustainable Development Goals.*; 2019.
310. Pasmans H, Schurink-van't Klooster TM, Bogaard MJM, et al. Long-term HPV-specific immune response after one versus two and three doses of bivalent HPV vaccination in Dutch girls. *Vaccine*. 2019;37(49):7280-7288. doi:10.1016/j.vaccine.2019.09.066
311. Waheed D e. N, Burdier FR, Eklund C, et al. An update on one-dose HPV vaccine studies, immunobridging and humoral immune responses – A meeting report. In: *Preventive Medicine Reports*. Vol 35. Prev Med Rep; 2023. doi:10.1016/j.pmedr.2023.102368
312. Kreimer AR, Sampson JN, Porras C, et al. Evaluation of durability of a single dose of the bivalent HPV vaccine: The CVT trial. *J Natl Cancer Inst*. 2020;112(10):1038-1046. doi:10.1093/jnci/djaa011
313. Barnabas R V., Brown ER, Onono MA, et al. Efficacy of Single-Dose Human Papillomavirus Vaccination among Young African Women. *NEJM Evid*. 2022;1(5). doi:10.1056/evidoa2100056
314. Malle E, Münscher G, Müller T, Vermeer H, Ibovnik A. Quantification and mapping of antigenic determinants of serum amyloid A (SAA) protein utilizing sequence-specific immunoglobulins and Eu3+ as a specific probe for time-resolved fluorometric immunoassay. *J Immunol Methods*. 1995;182(1):131-144. doi:10.1016/0022-1759(95)00035-9
315. Einstein MH, Levin MJ, Chatterjee A, et al. Comparative humoral and cellular immunogenicity and safety of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine and HPV-6/11/16/18 vaccine in healthy women aged 18-45 years: Follow-up through Month 48 in a Phase III randomized study. *Hum Vaccines Immunother*. 2014;10(12):3455-3465. doi:10.4161/hv.36117
316. Bosch FX, Tsu V, Vorsters A, Van Damme P, Kane AM. Reframing cervical cancer prevention. Expanding the field towards prevention: Of human papillomavirus infections and related diseases. *Vaccine*. 2012;30(SUPPL.5):F1-F11. doi:10.1016/j.vaccine.2012.05.090

317. Shin HY, Lee B, Hwang S-H, et al. Evaluation of satisfaction with three different cervical cancer screening modalities: clinician-collected Pap test vs. HPV test by self-sampling vs. HPV test by urine sampling. *J Gynecol Oncol*. 2019;30(5). doi:10.3802/jgo.2019.30.e76
318. Everett T, Bryant A, Griffin MF, Martin-Hirsch PPL, Forbes CA, Jepson RG. Interventions targeted at women to encourage the uptake of cervical screening. *Cochrane Database Syst Rev*. 2011;2011(5):CD002834. doi:10.1002/14651858.CD002834.pub2
319. Balasubramaniam SD, Balakrishnan V, Oon CE, et al. Key Molecular Events in Cervical Cancer Development. *Medicina (B Aires)*. 2019;55(7):384. doi:10.3390/medicina55070384
320. De Sanjose S, Holme F. What is needed now for successful scale-up of screening? *Papillomavirus Res*. 2019;7:173-175. doi:10.1016/j.pvr.2019.04.011
321. Ertik FC, Kampers J, Hülse F, et al. Cocoss-trial: Concurrent comparison of self-sampling devices for HPV-detection. *Int J Environ Res Public Health*. 2021;18(19):10388. doi:10.3390/ijerph181910388
322. von Knebel Doeberitz M, Prigge ES. Role of DNA methylation in HPV associated lesions. *Papillomavirus Res*. 2019;7:180-183. doi:10.1016/j.pvr.2019.03.005
323. Kyrgiou M, Athanasiou A, Paraskevaidi M, et al. Adverse obstetric outcomes after local treatment for cervical preinvasive and early invasive disease according to cone depth: Systematic review and meta-analysis. *BMJ*. 2016;354. doi:10.1136/bmj.i3633
324. Sroczynski G, Esteban E, Widschwendter A, et al. Reducing overtreatment associated with overdiagnosis in cervical cancer screening—A model-based benefit–harm analysis for Austria. *Int J Cancer*. 2020;147(4):1131-1142. doi:10.1002/ijc.32849
325. Rongthongaram W, Plumworasawat S, Charakorn C, Lertkhachonsuk AA, Satitniramai S. Overtreatment in the see-and-treat approach for high-grade squamous cervical cytology. *Eur J Obstet Gynecol Reprod Biol X*. 2023;19(January):100205. doi:10.1016/j.eurox.2023.100205
326. Strander B, Hällgren J, Sparén P. Effect of ageing on cervical or vaginal cancer in Swedish women previously treated for cervical intraepithelial neoplasia grade 3: Population based cohort study of long term incidence and mortality. *BMJ*. 2014;348. doi:10.1136/bmj.f7361
327. Arbyn M, Sasieni P, Meijer CJLM, Clavel C, Koliopoulos G, Dillner J. Chapter 9: Clinical applications of HPV testing: A summary of meta-analyses. *Vaccine*. 2006;24(SUPPL. 3):S78-S89. doi:10.1016/j.vaccine.2006.05.117
328. Hoffman SR, Le T, Lockhart A, et al. Patterns of persistent HPV infection after treatment for cervical intraepithelial neoplasia (CIN): A systematic review. *Int J Cancer*. 2017;141(1):8-23. doi:10.1002/ijc.30623

329. Arbyn M, Redman CWE, Verdoodt F, et al. Incomplete excision of cervical precancer as a predictor of treatment failure: a systematic review and meta-analysis. *Lancet Oncol*. 2017;18(12):1665-1679. doi:10.1016/S1470-2045(17)30700-3
330. Cuschieri K, Bhatia R, Cruickshank M, Hillemanns P, Arbyn M. HPV testing in the context of post-treatment follow up (test of cure). *J Clin Virol*. 2016;76:S56-S61. doi:10.1016/j.jcv.2015.10.008
331. Kocken M, Uijterwaal MH, De Vries ALM, et al. High-risk human papillomavirus testing versus cytology in predicting post-treatment disease in women treated for high-grade cervical disease: A systematic review and meta-analysis. *Gynecol Oncol*. 2012;125(2):500-507. doi:10.1016/j.ygyno.2012.01.015
332. Ponduri A, Azmy MC, Axler E, et al. The Efficacy of Human Papillomavirus Vaccination as an Adjuvant Therapy in Recurrent Respiratory Papillomatosis. *Laryngoscope*. 2023;133(9):2046-2054. doi:10.1002/lary.30560
333. Biryukov J, Meyers C. Papillomavirus infectious pathways: A comparison of systems. *Viruses*. 2015;7(8):4303-4325. doi:10.3390/v7082823
334. Doorbar J, Egawa N, Heather Griffin CK and IM. Human papillomavirus molecular biology. *Rev Med Virol*. 2016;25:2-23. doi:10.1002/rmv.1822
335. Téblick, Laura; Severien, Van Keer; Vorsters A. First-void urine as liquid biopsy for HPV vaccine-induced antibody detection. *HPVWorld.com*. 2022;(March):191. www.HPVWorld.com
336. Bryzgunova OE, Laktionov PP. Extracellular Nucleic Acids in Urine: Sources, Structure, Diagnostic Potential. *Acta Naturae*. 2015;7(3):48-54. doi:10.32607/20758251-2015-7-3-48-54
337. Hulstaert E, Morlion A, Levanon K, Vandesompele J, Mestdagh P. Candidate RNA biomarkers in biofluids for early diagnosis of ovarian cancer: A systematic review. *Gynecol Oncol*. 2021;160(2):633-642. doi:10.1016/j.ygyno.2020.11.018
338. Miranda KC, Bond DT, McKee M, et al. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. *Kidney Int*. 2010;78(2):191-199. doi:10.1038/ki.2010.106
339. Nikanjam M, Kato S, Kurzrock R. Liquid biopsy: current technology and clinical applications. *J Hematol Oncol*. 2022;15(1):1-14. doi:10.1186/s13045-022-01351-y
340. Ferrara F, Zoupanou S, Primiceri E, Ali Z, Chiriacò MS. Beyond liquid biopsy: Toward non-invasive assays for distanced cancer diagnostics in pandemics. *Biosens Bioelectron*. 2022;196:113698. doi:10.1016/j.bios.2021.113698
341. Le Guern R, Miaux B, Pischedda P, Herwegh S, Courcol R. DNA stability of Chlamydia trachomatis and Neisseria gonorrhoeae in urine. *Diagn Microbiol Infect Dis*. 2016;85(3):313-314. doi:10.1016/j.diagmicrobio.2016.04.005
342. Taye B, Woldeamanuel Y, Kebede E. Diagnostic detection of human immunodeficincy virus type-1 antibodies in urine, Jimma Hospital, south west Ethiopa. *Ethiop Med J*. 2006;44(4):363-368. Accessed October 8, 2020.

https://europepmc.org/article/med/17370436
343. Peng M, Chen C, Hulbert A, Brock M V., Yu F. Non-blood circulating tumor DNA detection in cancer. *Oncotarget*. 2017;8(40):69162-69173. doi:10.18632/oncotarget.19942
344. Hulstaert E, Morlion A, Avila Cobos F, et al. Charting Extracellular Transcriptomes in The Human Biofluid RNA Atlas. *Cell Rep*. 2020;33(13). doi:10.1016/j.celrep.2020.108552
345. Crisafulli G, Mussolin B, Cassingena A, et al. Whole exome sequencing analysis of urine trans-renal tumour DNA in metastatic colorectal cancer patients. *ESMO Open*. 2019;4(6):1-9. doi:10.1136/esmoopen-2019-000572
346. Jordaens S, Zwaenepoel K, Tjalma W, et al. Urine biomarkers in cancer detection: A systematic review of preanalytical parameters and applied methods. *Int J Cancer*. 2023;152(10):2186-2205. doi:10.1002/ijc.34434
347. Xia L, Xian Y, Wang D, et al. A human monoclonal antibody against HPV16 recognizes an immunodominant and neutralizing epitope partially overlapping with that of H16.V5 OPEN. Published online 2015. doi:10.1038/srep19042
348. Day PM, Thompson CD, Buck CB, Pang Y-YS, Lowy DR, Schiller JT. Neutralization of Human Papillomavirus with Monoclonal Antibodies Reveals Different Mechanisms of Inhibition. *J Virol*. 2007;81(16):8784. doi:10.1128/JVI.00552-07
349. Smith JF, Kowalski R, Esser MT, Brown MJ, Bryan JT. Evolution of type-specific immunoassays to evaluate the functional immune response to Gardasil®: A vaccine for human papillomavirus types 16, 18, 6 and 11. *Hum Vaccin*. 2008;4(2):134-142. doi:10.4161/hv.4.2.5261
350. García FR, Norenhag J, Edfeldt G, et al. Prevalence of the human papillomavirus (HPV) types among cervical dysplasia women attending a gynaecological clinic in Sweden. *BJC Reports 2023 11*. 2023;1(1):1-7. doi:10.1038/s44276-023-00012-y
351. Safaeian M, Falk RT, Rodriguez AC, et al. Factors associated with fluctuations in IgA and IgG levels at the cervix during the menstrual cycle. *J Infect Dis*. 2009;199(3):455-463. doi:10.1086/596060

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Thesis submitted for the degree of Doctor of Medical Sciences
Faculty of Medicine and Health Sciences

Promotors: Prof. dr. Alex Vorsters, Prof. dr. Peter Delputte,
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Antwerp, 2024