Value of circulating tumor cell DNA in HPV-associated anal carcinoma for monitoring treatment effects and detection of disease relapses after treatment with definitive chemoradiation

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VALUE OF CIRCULATING TUMOR CELL DNA IN HPV-ASSOCIATED ANAL CARCINOMA FOR MONITORING TREATMENT EFFECTS AND DETECTION OF DISEASE RELAPSES AFTER TREATMENT WITH DEFINITIVE CHEMORADIATION

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LIST OF ABBREVIATIONS

HPV – Human papillomavirus

DNA – Deoxyribonucleic acid

EV – Epidermodysplasia verruciformis

UV light – Ultraviolet light

AJCC – American Joint Association Committee on Cancer

PD-1 – Programmed cell death protein 1

PD-L1 – Programmed cell death 1 – ligand 1

TGFß1 – Transforming growth factor beta

STD – Sexually transmitted disease

HR-HPV – high-risk HPV

LR-HPV – low-risk HPV

p53 – Tumor protein p53

pRB – Retinoblastoma protein

E6 and E7 – Oncogenic proteins of HPV16

URR – Upstream Regulatory Region

E1 and E2 – Replication proteins

L1 and L2 – Capsid proteins

mRNA – Messenger ribonucleic acid

MSM – Men who have sex with men

HIV – Human immunodeficiency virus

AIDS – Acquired Immunodeficiency Syndrome

WHO – World Health Organization

AIN – Anal intraepithelial neoplasia

CT – Computed tomography

MRI – Magnetic resonance imaging

PET – Positron emission tomography

BT – Brachytherapy

CRT – Chemoradiation

Gy – Gray

EBRT – External beam radiation therapy

SCCA – Squamous cell carcinoma

PAP-smear – Cytological smear after Papanicolaou of the cervix

CIN – Cervical intraepithelial neoplasia

FIGO – International Federation of Gynecology and Obstetrics

ACIS – Adenocarcinoma in situ of cervix

LSIL – Low-grade squamous epithelial lesion

HSIL – High-grade intraepithelial lesion

LEEP – Loop electrosurgical excision procedure

IMRT – Intensity-modulated radiotherapy

BSA – Body surface area

TIL – Tumor infiltrating lymphocyte

FDA – Food and Drug Administration

LB – Liquid biopsy

CTC – Circulating tumor cell

ctDNA – Circulating-tumor DNA

cfDNA – Cell-free DNA

ddPCR – Digital droplet polymerase chain reaction

NGS – Next-generation sequencing

qPCR – Quantitative PCR

Cq – Quantitation cycle

MRD – Minimal residual disease

OS – Overall survival

DFS – Disease-free survival

PFS – Progression-free survival

CR – Complete remission

SUV – Standardized uptake curves

5-FU – 5-Fluoruracil

CI – Confidence interval

IQR – Interquartile range

SD – Standard deviation

LN – Lymph nodes

mg/m^2 – Milligrams per square meter

ml/min – Milliliters per minute

mm^3 – Cubic millimeter

1 INTRODUCTION

1.1 Human Papillomavirus

The human papillomavirus (HPV) is a double-stranded, circular, non-enveloped DNA virus with icosahedral capsid belonging to the papillomavirus family (1). HPV infection is the most prevalent sexually transmitted infection, greatly impacting individuals' social lives. They can be classified as non-genital (cutaneous), mucosal, anogenital or Epidermodysplasia verruciformis (EV). The route of transmission is primarily through skin-to-skin or skin-to-mucosa contact. The virus enters through epithelial disruptions, infects keratinocytes in the basal layers of stratified epithelia and potentially integrates into DNA that is a key event of HPV-induced carcinogenesis. Most HPV infections are latent, and the majority of clinical lesions manifest as warts rather than a malignancy (2). Treatment options in particular advanced lesions are very limited up to now; therefore, the HPV vaccination campaign has been strongly promoted in recent years.

There are more than 180 subtypes of HPV, which can be classified into high-risk (HR-HPV) and low-risk (LR-HPV) groups. HPV16 and HPV18 belong to the high-risk subtypes, which cause high-grade intraepithelial lesions that can potentially progress to malignancies. HPV is estimated to be the cause of 96% of cervical, 93% of anal cancer, 64% vaginal cancers, 51% vulvar cancers, and 45-90% oropharyngeal cancers (3). However, HPV alone is not sufficient to cause cancer; it necessitates triggers such as smoking, folate deficiency, exposure to UV light, immunosuppression, and pregnancy (2).

The oncogenic potential of HPV is quite complex and not yet fully explored. It is rather an interaction of different factors, such as E6 and E7 oncogenes which inhibit p53 and pRB respectively. Through the integration of viral DNA into the host genome, the virus is able to control regulation of native genes, interference with normal cellular growth and inhibition of apoptosis (4).

1.1.1 Epidemiology

On a worldwide scale, 4.5% of all cancer cases can be attributed to the presence of HPV and the infection can affect both sexes (5). Almost all sexually active women and men will be infected at least once in their lifetime, usually without developing any symptoms or pathologies (5). Nonetheless, the epidemiology of HPV varies between male and female populations. Globally, among females, 26.8% of cases affect the genital organs (with the highest rate found in Sub-Saharan Africa), and 14% affect the anus. In contrast, among males, 45.2% of cases involve the genital organs, while 16% affect the anus (6). The global burden of HPV infection

and the consequences of the resulting diseases are increasing every year. Approximately 625,600 women and 69,400 men are diagnosed with HPV-related cancers annually. In 2020, cervical cancer ranked as the fourth most prevalent cancer and the fourth leading cause of cancer-related deaths among women, accounting for an estimated 604,127 new cases and 341,831 fatalities worldwide (7).

The age of infected patients varies depending on the geographical location. While adolescent girls and women under 25 years of age exhibited the highest infection rates, there was a resurgence of HPV infection among adults aged 45 and older in the East and West Africa and regions of Central and Southern America (5).

Cervical cancer comprises 96% of HPV-associated cancers among women. In developing countries, the incidence is almost twice as high as in more developed countries. However, regardless of the level of development, Eastern Europe exhibits a relatively high prevalence rate of 21.4%, while North Africa and Western Asia display lower rates of 9.2% and 2.2%, respectively (5). The second most HPV-associated malignancy is anal cancer. 93% of anal cancers are thought to be caused by HPV, followed by 64% of vaginal cancers, 51% of vulvar cancer, 36% of penile cancers and 63% of oropharyngeal carcinomas (8). HPV-related mortality and the variety of different HPV-associated malignancies are attributed to oncogenic HPV types, which can result in dysplasia and cancer.

Currently, there are over 200 defined HPV types, which can be categorized into two primary groups: high-risk (HR) and low-risk (LR), depending on their potential to cause cancer (9). The main HPV types and their associated diseases are listed below in Table 1.

Table 1. Main HPV types and their associated diseases

	Low Risk	**High Risk**
HPV Types	1,6,10,11,32,42,44	16,18,31,33,35,39,45,51,52,56,58,59, 66, 68
Associated Disease	Anogenital warts Cutaneous warts Recurrent respiratory papillomatosis Heck's disease	Intraepithelial neoplasia Invasive carcinoma: HNSCC, Cervical cancer, Anogenital cancers, Non-melanoma skin cancer

SOURCE: Medda A, Duca D, Chiocca S. Human Papillomavirus and Cellular Pathways: Hits and Targets. Pathogens. 2021;10:262.

The most common LR-HPV-associated diseases are anogenital warts, cutaneous warts and recurrent papillomatosis. HPV-6 and HPV-11 belong both to the low-risk group which are responsible for approximately 90% of these warts but have no association with cancer. In the high-risk group, HPV-16 and HPV-18 are the most common types and together are responsible for approximately 70% of HPV-associated cancers worldwide (10).

1.1.2 Pathogenesis

The HPV-genome, which consists of circular double-stranded DNA spanning approximately 7–8 kbp, is segmented into 3 regions: the Upstream Regulatory Region (URR), which contains the viral promoter, enhancer, and replication origin site; the early gene region (E), which consists of 6 open reading frames: E6, E7, E1, E2, E4, and E5; and the late gene region (L), which encodes the major coat proteins L1 and L2 (11).

All papillomaviruses encode the four highly conserved core proteins: replication proteins E1 and E2, along with capsid proteins L1 and L2. Additionally, the oncogenic Alpha Papillomaviruses E6 and E7 proteins stand out because they are transcribed from polycistronic, alternatively spliced mRNA. It is widely recognized that HPV-related cancers result from the sustained expression of E6 and E7 oncoproteins. which are responsible for regulating cellular proliferation and differentiation and therefore to maintain the cellular environment (1). They facilitate the entry of basal layer epithelial cells into the cell cycle, thereby promoting viral replication. By binding to and deactivating the Rb protein (pRb), a critical tumor suppressor and regulator of the G1 checkpoint, E7 prompt cells to progress into the S phase of the cell cycle, inducing both cell proliferation and viral DNA amplification in differentiated cells. In a normal cell cycle the function of p53 is to act as a tumor suppressor protein, regulating the cell cycle and preventing the formation of tumors by inducing growth arrest, DNA repair, or apoptosis in cells with damaged DNA. The E6 protein of Alpha Papillomaviruses disrupts the function of p53, thus impeding both growth arrest and apoptosis. HR-HPV E7 and E6 play therefore a central role in the HPV cell cycle by degrading pRb and p53 respectively. When these essential tumor suppressor proteins are absent, the activation of DNA damage checkpoints is compromised, permitting unregulated DNA replication, ultimately resulting in genomic instability and resistance to apoptosis (12).

1.2 HPV-associated neoplasms

Around 16% of all cancers worldwide are caused by infection; in developed countries this proportion is around 7%, half of which is caused by HPV. Almost all cervical and anal and the vast majority of anogenital carcinomas are caused by HPV infection and therefore demonstrate the most common HPV-associated neoplasms (1).

1.2.1 Anal Cancer

Anal carcinoma is a rather rare tumor which accounts for less than 5% of all malignant neoplasms of the gastrointestinal tract with an increasing incidence. In 2020, approximately 2,310 individuals were diagnosed in Germany with the disease, with 1,500 of them being women (13). Predominantly squamous cell carcinomas (90%), followed in frequency by adenocarcinomas (10%), rarely malignant melanomas, anaplastic carcinomas and others.

Depending on the location, a distinction is made between anal canal carcinoma (tumor between the anorectal linea and the anocutaneous linea) and anal margin carcinoma (tumor distal to the linea anocutanea or up to a radius of 5 cm distal to the linea anocutanea) (14). The gender distribution in Germany differs according to the location of the tumor: anal canal carcinoma affects more women than men and anal margin carcinoma affects more men than women. The incidence peaks around the age of 60 and the risk groups include HIV-infected people, in particular men who have sex with men (MSM) (15). The pathophysiological progression of anal cancer is thought to be intricately associated with a multifaceted inflammatory response triggered by infections like Human Papillomavirus types 16 and 18 (16).

1.2.1.1 Etiology

The etiology of anal carcinoma is multifactorial, with HPV infection, particularly type 16, being a significant risk factor. There is a clear correlation between HPV infection and development of anal cancer. In addition to HPV, Chlamydia trachomatis and AIDS have been implicated in anal cancer. Patients infected with the human immunodeficiency virus (HIV) face a 40-fold higher risk of anal cancer compared to the general population (17). The progression of this inflammatory process signals the onset of anal intraepithelial neoplasia (AIN), or squamous cell carcinoma in situ (Bowen's disease), representing a precancerous state (17). AIN is classified into grades I to III according to abnormalities in squamous layer differentiation and maturation, mitotic activity, nuclear membrane changes, and the extent of these irregularities (18). In approximately 10% to 11% of cases, AIN progresses to invasive squamous cell

carcinoma. Tumors typically spread through local extension but also possess the capability to metastasize. There are various HPV16-associated risk factors such as sexual activity, receptive anal intercourse, previous sexually transmitted diseases, and a high number of sexual partners (19). In addition to infection with HPV16, factors like immunosuppression, older age, female gender, and tobacco smoking can contribute to the development of anal carcinoma (20). As with other cancers, the cause of developing anal carcinoma is usually a combination of genetic, environmental, and lifestyle factors.

1.2.1.2 Symptoms

In about 20% of cases, anal carcinomas are incidental findings during other anal inspections because initial stages are often asymptomatic (21). Sometimes there are palpable, non-painful skin changes. In cases of chronic, slow-healing conditions in the anal region, the possibility of anal carcinoma should be carefully considered as a potential diagnosis (22). The most common symptoms are bleedings, which is the main symptom, feeling of pressure, pain, pruritus ani and fecal incontinence (23). Clinically, hemorrhoids or tears in the anal canal could present in the same way and must therefore always be considered as differential diagnosis.

1.2.1.3 Diagnostics and Staging

Medical history and physical examination are crucial for setting the diagnosis. Because of the stigma surrounding this diagnosis, approximately 19% of patients typically delay seeking medical care, often presenting at a later stage of the disease (17). A thorough medical history, including past medical records, sexual activity history, and any previous venereal diseases, is crucial. Equally important is a comprehensive physical examination, which should encompass a digital rectal examination, palpation of inguinal lymph nodes, and gynecologic evaluation in females, including screening for cervical cancer (18). In the case of conspicuous or questionable findings, imaging procedures, such as CT, MRI or PET scan can be consulted for further diagnostics (18). Biopsy is the definitive diagnostic test for anal carcinoma. There are various methods for obtaining tissue, including punch biopsy, endoscopic biopsy, or surgical excision (24). After the diagnosis of anal cancer is confirmed, additional tests may be conducted to determine the stage of cancer, aiding in finding a treatment strategy. CT, MRI, and PET scan can evaluate the tumor stage, depth of invasion, lymph node status and the presence of metastases (25). In addition to that, laboratory tests can be done to assess the overall health status of the patient, such as liver and kidney function. Screening tests for HPV, gonorrhea,

chlamydia, and HIV in patients at risk can help in planning the therapy, monitoring its development and have different prognostic outcomes and survival rates (26).

The predominant staging system for anal cancer is the American Joint Committee on Cancer (AJCC) TNM system. It is based on the extent of primary tumor (T), the spread to nearby lymph nodes (N), the spread to distant sites (M) and consists of stage I to IV (27). The TNM classification of anal cancer according to AJCC 2017 is listed in Table 2 below (28).

Table 2. TNM Classification for Anal Cancer

Primary Tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	High-grade squamous intraepithelial lesion
T1	Tumor ≤ 2 cm in greatest dimension
T2	Tumor > 2 cm but ≤ 5 cm in greatest dimension
T3	Tumor > 5 cm in greatest dimension
T4	Tumor of any size invades adjacent organ(s)
Regional Lymph Nodes (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in inguinal, mesorectal, internal iliac, or external iliac nodes
	N1a - Metastasis in inguinal, mesorectal, or internal iliac lymph nodes
	N1b - Metastasis in external iliac lymph nodes
	N1c - Metastasis in external iliac with any N1a nodes
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

SOURCE: Anal Cancer Staging: TNM Classification for Anal Cancer [Internet]. 2021 [cited 2024 Feb 20]. Available from: https://emedicine.medscape.com/article/2003634-overview?form=fpf

According to the TNM classification, four different stages of anal carcinoma can be classified which is shown in Table 3 (29).

Table 3. Anal cancer staging according to AJCC 2017

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
IIA	T2	N0	M0
IIB	T3	N0	M0
IIIA	T1, T2	N1	M0
IIIB	T4	N0	M0
IIIC	T3, T4	N1	M0
IV	Any T	Any N	M1

SOURCE: Ärzteblatt DÄG Redaktion Deutsches. Deutsches Ärzteblatt [Internet]. Anal Cancer. 2021 [cited 2024 Feb 20]. Available from: https://www.aerzteblatt.de/int/archive/article?id=218468

Once the diagnosis and staging are established, a multidisciplinary tumor-board consultation including gastroenterology, radiation oncology, oncology, radiology, surgery and other professionals will collaborate to develop a personalized treatment plan tailored to the individual patient's needs.

1.2.1.4 Therapy of Anal Carcinoma

The main objective of treatment is to attain cure while maintaining local and regional control, preserving anal function, and optimizing the patient's quality of life (30). Gold standard therapeutic approach is a combination of mitomycin C and 5-fluorouracil based CRT (31).

According to ESMO Clinical Practice Guidelines from 2021, locoregional anal cancer in Stage I (anal margin subtype) is treated with local excision, while Stage II-III (anal margin subtype) and Stage I-III (anal canal subtype lesions) are primarily treated with definitive CRT (32). In case of local relapse or residual tumor after CRT radical surgery is the second step. A systemic treatment approach with carboplatin-paclitaxel (first-line) or cisplatin-5-FU, carboplatin, doxorubicin, taxane, irinotecan ± cetuximab (second-line) is chosen in Stage IV advanced anal cancer (32).

1.2.1.4.1 Surgery

In most cases, surgery is not the first-line treatment for anal carcinoma except for patients with anal margin cancer and a tumor size being ≤ 2cm and no lymph nodes affected (33). A local resection only removes the tumor with a defined safety margin of >1 mm. The aim

of this procedure is to maintain the sphincter tone. In some cases, CRT is needed afterwards if some residual tumor mass remains (33).

1.2.1.4.2 Chemoradiation

CRT is the standard therapy for treatment of anal carcinomas which cannot be removed without harming the anal sphincter. It is a combination of external beam radiation therapy (ERBT) and chemotherapy usually with 5-FU and mitomycin (34). In Stages I to III, 5-FU and mitomycin are given during the first and fifth week and EBRT is given daily on 5 days for 5 to 7 weeks with total doses of 45-56 Gy (32,35). Research has shown that CRT with 5-FU and mitomycin is more effective for local control than radiation alone (35). After complete response to CRT, patients should regularly have a follow-up and in the event of residual tumor, surgery should be performed. This combination of therapy can affect the quality of life in regard to fecal incontinence, sexual dysfunction and urinary problems (36).

1.2.1.4.3 Chemotherapy and Immunotherapy

Patients with distant metastases of anal carcinomas shall receive first-line treatment using carboplatin-paclitaxel (37). Other possible chemotherapeutic agents are cisplatin-5-FU, doxorubicin, taxanes and irinotecan ± cetuximab or a combination (16). Best supportive care is last possible therapeutic option for patients who are not suitable or not fit enough for all other therapy options (38).

Considering the immunogenic potential of HPV in squamous cell anal carcinoma, the enhancement of PD-L1 expression and upregulation of TGFβ via immunotherapy are areas of active investigation in SCCA. The clinical use of immune checkpoint inhibitors, such as PD-1 blocker, are under current investigation (39).

1.3 Liquid Biopsy

Liquid biopsy (LB) is an all-encompassing term used to describe the testing of bodily fluids, that involves the real-time analysis of tumor cells or products, such as cell-free circulating nucleic acids (cfDNA, cfRNA), extracellular vesicles, or proteins, released into the blood or other body fluids by primary or metastatic tumor lesions (40). It is a minimally invasive method for diagnosis, therapy monitoring and early detection of tumor, as well as its heterogenicity, recurrence or its progression. Circulating extracellular nucleic acids (cfDNA) and circulating tumor DNA (ctDNA) can be isolated from the blood. cfDNA refers to DNA that

circulates freely in the bloodstream, potentially originating from various sources including tumors, whereas ctDNA specifically originates from tumor (41). ctDNA constitutes a portion of circulating cfDNA, which comprises short fragments found in plasma and serum due to both lymphocyte release and general cell lysis.

Additionally, circulating tumor cells (CTCs) which are shed from primary or secondary tumor sites into blood, can be detected by LB for 1-2.5 hours before they are eradicated by the immune system (41). Despite their low prevalence, which is estimated at around 1–10 tumor cells per 10^6–10^8 white blood cells, they have the potential to seed distant metastases (42). The cells are characterized by a pronounced plasticity due to their redifferentiation potential and show similar mutations of the genetic material as the original tumor. Tumor cells produce exosomes that can contain a variety of messenger substances including DNA and RNA and can therefore be used to recognize tumor cells. The challenge with utilizing exosomes stems from the abundance of healthy exosomes in comparison to the relatively limited number of altered exosomes (43). While the methodology for ctDNA is well-established and standardized, the approaches for isolating and enriching CTCs, which occur at low concentrations, is more demanding.

The significant advancements in digital droplet polymerase chain reaction (ddPCR) and the diverse enhancements in next-generation sequencing (NGS) technologies are pivotal in the numerous validations of CTC-free DNA as a robust cancer biomarker (44). ddPCR and NGS are methods for the highly sensitive detection of nucleic acids.

LB tests have the potential to improve screening and triage pathways, increasing the percentage of patients referred for further investigation due to detected abnormalities. This heightened efficiency in the diagnostic process would mitigate delays in diagnosis and decrease associated costs (45).

1.3.1 Technologies of cf/ctDNA in plasma samples

Various DNA detection technologies for LB have been employed in both research studies and commercial settings. However, most of the approved techniques primarily revolve around traditional quantitative PCR (qPCR) mutational testing, ddPCR, or NGS.

In LB research studies, mutational testing via quantitative PCR (qPCR) has been utilized, relying on the effectiveness of mutation-specific primer design and optimization for the detection process. Subsequently, the studied sample is analyzed, and the frequencies of mutations are quantified. qPCR, a subtype of real-time PCR, exhibits the capability to accurately determine the initial number of template DNA copies with high sensitivity across a

broad dynamic range (46). On one hand, real-time qPCR data evaluation eliminates the need for gel electrophoresis, resulting in decreased bench time and enhanced throughput. On the other hand, it reduces contamination and the necessity for post-amplification manipulation because reactions and data are assessed within a unified, closed-tube qPCR system. In qPCR reactions, the focus is on detecting the initial amplification of a target rather than on measuring the accumulated amount of the target after a fixed number of cycles. The cycle at which fluorescence becomes detectable is termed the quantitation cycle (Cq), which is the fundamental outcome of qPCR. Lower Cq values indicate higher initial copy numbers of the target, representing the fundamental principle of the quantitative approach offered by real-time PCR. In contrast, an endpoint assay, also referred to as a "plate-read assay," quantifies the amount of enriched PCR product once the PCR cycle has concluded (47).

The next generation of qPCR is dPCR (digital PCR): one type herein is ddPCR (droplet digital PCR), which is a method for performing digital PCR that is based on water-oil emulsion droplet technology. A DNA sample is partitioned into 20.000 droplets, with each compartment containing zero to many target molecules that undergo individual PCR reactions. This implies that ddPCR demonstrates greater sensitivity compared to qPCR; while ddPCR can typically detect mutant abundances as low as 0.1%, qPCR achieves a frequency of around 1% (48). Droplets are generated within a water-oil emulsion, serving as the partitions that isolate the template DNA molecules. They fulfill the same role as individual test tubes or wells in a plate where the PCR reaction occurs, even though in a significantly smaller format and this droplet division allows for the assessment of thousands of separate amplification occurrences within a single sample (49). After PCR, every droplet undergoes analysis or reading to ascertain the proportion of PCR-positive droplets in the initial sample.

Although ddPCR provides excellent sensitivity, it requires prior knowledge of the mutations to be screened for, limiting the study to only a few genetic alterations at a time. In NGS no prior knowledge of sequence information is necessary and it enables massively parallel analysis with high throughput across multiple samples. The majority of NGS methods entail a library preparation stage, during which DNA samples (or RNA samples, converted to double-stranded DNA) are fragmented and adapters are affixed to both ends (50). This technology integrates the benefits of distinct sequencing chemistries, diverse sequencing platforms, and bioinformatics technology. As a result, NGS enables the high-throughput sequencing of DNA or RNA sequences of varying lengths, or even entire genomes, within a comparatively brief timeframe. It represents a groundbreaking sequencing technology following Sanger sequencing (51).

Common steps of all DNA (or RNA) liquid biopsy-based analyses are: the first step is achieving pathologic diagnosis and getting suitable blood draws. Subsequently, plasma separation and nucleic acid sample extraction, followed by one of previously outlined analytical approaches. The last step consists of data analysis, quality evaluation and interpretation of pathogenic variants (52). Compared to ddPCR/qPCR, NGS can detect multiple known and unknown genetic mutations at once, has the potential for detection of novel gene alterations at single nucleotide resolution. But its limitations are longer turnaround times, lower sensitivity, higher costs and the requirement of bioinformatic analysis (50).

In LB analysis, NGS and dd/qPCR could be considered as complementary methods. NGS offers a comprehensive profiling of ctDNA, dPCR is often more suitable for routine testing, such as monitoring therapy etc. over time. This is attributed to the robustness and cost-effectiveness of dPCR technology in analyzing limited numbers of predetermined markers or mutations. Additionally, it offers simple absolute rather than relative quantification of circulating DNA (50).

Regardless of the method utilized for ctDNA detection, DNA extraction and sample preparation are crucial steps in the integration of assays into a system. Sample preparation frequently involves four steps: cell disruption, removal of proteins and lipids, purification of DNA, and subsequent DNA concentration. Currently, there are different premade extraction kits on the market to simplify nucleic acid extraction from human blood plasma as the QIAmp MinElute circulating_cell_free_DNA Mini Kit (Qiagen) used in this study (48).

1.3.2 Clinical use of Liquid Biopsy

LB analytes include CTCs, ctDNA, and cfDNA in blood or other fluid samples (53). A thorough and inclusive understanding of these diverse components and molecules present in body fluids within specific cancers will facilitate early and enhanced cancer detection, improved cancer management, prognostic prediction, and effective monitoring of therapeutic response (54). At present, the clinical application of LB is primarily based on peripheral blood detection. It presents a supplementary method to traditional tissue biopsies, enabling the continuous monitoring of disease progression and treatment in real time (55).

Various clinical applications of LB using CTCs and other tumor-derived materials are shown in the Figure 1 below. In a single blood sample, various cell types and cell products from multiple tumor sites throughout the body can be present. Liquid biopsy assays analyzing these tumor-derived factors can fulfill multiple roles in cancer management (56).

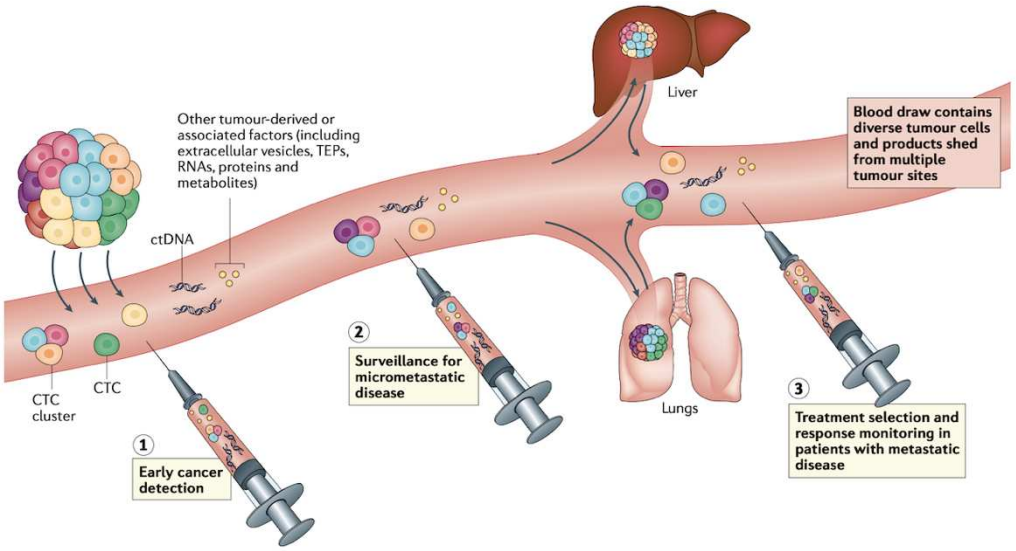


Figure 1. Various clinical applications of LB using CTCs, circulating nucleic acids or other tumour-derived materials in the bloodstream

SOURCE: Ignatiadis M, Sledge GW, Jeffrey SS. Liquid biopsy enters the clinic — implementation issues and future challenges. Nat Rev Clin Oncol. 2021;18:297–312. Available from: https://www.nature.com/articles/s41571-020-00457-x

In contrast to conventional tissue biopsy, LB is a safe, quick, and a minimally invasive method for the diagnosis of cancer (57). Circulating metabolites can offer a direct reflection of the pathological status in cancer patients, tumor characteristics, such as tumor location, disease burden, rates of proliferation and apoptosis, extent of necrosis, inflammation, tumor microenvironment, host-related factors (58). It possesses the potential to function as biomarkers for personalized cancer therapy. According to quantity and quality of cfDNA, a differentiation can be made between healthy persons and cancer patients in which the overall level of cfDNA tends to be elevated (59). It rises further with disease progression and the occurrence of metastases. The cellular alterations detected through LB using ctDNA are closely aligned with the findings of traditional genetic testing performed on tissue biopsies. Moreover, monitoring the dynamics of ctDNA changes facilitates the evaluation and prediction of treatment response and clinical outcomes, enabling timely adjustments to treatment protocols (60). Specifically, a reduction in ctDNA following therapy has been linked to a minimal risk of tumor advancement and a prolonged survival whereas, increased levels of ctDNA have been associated with progression, relapse, and unfavorable survival outcomes (61). These advantages speak for a good clinical application of LB regarding early detection, monitoring and progression of the tumor.

However, there are also restrictions that the LB entails. Up to now, the main limitation is the lack of cancer-specific markers and on the other hand the extremely low plasma concentrations of CTCs and ctDNA which makes in particular the detection of early-stage cancers often difficult (54). Apart from that, ctDNA assays are not able to detect any histological features, so a tissue biopsy is always presupposed. Another disadvantage of LB is that tumor-derived material may not be shed homogeneously from all sites or may not be shed at all (62). This phenomenon is referred to as a "non-shedding" tumor. In this case, no CTCs or ctDNA can be detected at any timepoint, so they cannot be used as marker for disease progression or treatment response. Currently, there is no singular ctDNA detection assay that meets the requirements for all clinical applications in tumors, which encompasses early tumor screening, minimal residual disease (MRD) analysis, gene mutation detection, genetic heterogeneity assessment and the detection of treatment resistance (63).

Over the past few years, LB has developed rapidly and achieved new successes in its clinical applicability, but it still requires even further investigations and larger studies in order to presumably replace the tissue biopsy.

2 OBJECTIVES

2.1 Aim of the study

Firstly, the major aim of this study was to perform a comprehensive evaluation of the clinical applicability of LB in therapy monitoring and prognostic prediction of HPV16 associated anal tumor relapse that is primarily based on an extensive literature research from online databases. Secondly, a pilot study was analyzed in order to assess the feasibility of detection of HPV16 copy levels in LB gained in clinical practice at the local hospital. For this purpose, anonymized data from a small cohort of patients from the Departments of Radiation Oncology and Pathology at the Coburg Cancer Centre, Germany, were used. This aimed to explore the potential of cfDNA as a cancer biomarker for anal carcinomas in clinical practice, particularly its utility in therapy monitoring and prediction of recurrence following CRT.

2.2 Hypothesis

In patients with HPV-associated anal carcinoma detection of HPV16 DNA in plasma allows monitoring of treatment response and reversion to seronegativity represents a surrogate marker for recurrence-free survival after CRT.

3 MATERIALS AND METHODS

3.1 Systematic Literature Search

The major part of this study involved a comprehensive literature review on the research topic. An electronic search of online databases was conducted to obtain key literature on HPV-associated anal (and cervical) cancer and LB. A systematic search identified relevant approaches, experimental procedures, and materials from similar research. The process began with defining the research question, focusing on the value of cfDNA in monitoring HPV-associated anal carcinoma treatment effects and detecting disease relapses post-CRT. Keywords and search terms were selected to capture key concepts and terms relevant to the study. PubMed and Google Scholar were chosen for the search. Titles and abstracts were screened for relevance, with irrelevant sources excluded and potentially relevant articles retained for full-text review. The content of selected articles was analyzed to extract information on materials and methods. Common methodologies and materials were synthesized to inform the study design. The search process, including search strings, databases, dates, and results, was documented, along with records of included and excluded sources and reasons for exclusion.

3.2 Pilot study

The minor part of this study was conducted in form of a pilot study that aimed to evaluate the feasibility of LB in monitoring HPV-induced anal (as well as three cervical) carcinomas before and after CRT. The study was associated and grounded on data from the Departments of Radiation Oncology and Pathology of the hospital in Coburg, Germany. The patients which were tested positive for HPV16 via tissue biopsy received CRT as their definite treatment. All data were used in a completely anonymized version. The patients' history was collected for data regarding their disease and its course via patients' records of the practice over the course of several years and evaluated for this study for the Thesis for the University of Split, School of Medicine. Inclusion criteria were patients with anal and to a small amount cervical carcinoma with a positive tissue biopsy test for HPV16. Before, during and after CRT, their response to the therapy was monitored by regular blood samples and the subsequent test results were used to determine the clinical applicability of LB regarding treatment monitoring and disease prognosis.

Data used in this study included serum levels of HPV-ctDNA in patients with HPV-associated anal or cervical carcinoma at time of diagnosis, during and after CRT. Clinical data were possessed in an anonymized form. Traceability of any kind of patient data is not possible.

All data were provided by the Departments Radiation Oncology and Pathology of the Hospital in Coburg, Germany.

3.3 Independent variables

Independent variables being collected included age, sex, date of diagnosis, age at diagnosis, cancer stage, date of blood samples, number of HPV16 DNA copies, duration, type and dose of radiation and chemotherapy, number in this study, and abbreviation in this study.

3.4 Outcome measures

The most important outcome was the evaluation of the patients´ clinical progression based on LB analyzing the blood samples regarding HPV16 DNA copy number per milliliter of blood plasma before, during and after receiving CRT.

The basic principles of LB were already explained in detail in the Introduction section. Blood sample analysis for HPV16 DNA detection was performed at the Department of Pathology of the Hospital of Coburg. The blood samples sent to Pathology Department were analyzed for the presence of HPV16 positivity in the blood plasma using highly sensitive digital PCR (ddPCR). Hereto, after separation of corpuscular blood components, free circulating DNA was isolated using the QIAmp MinElute circulating_cell_free_DNA Mini Kit (Qiagen) and its concentration was determined by fluorescence photometry. The PCR analysis was performed in comparison to the GNAS wild-type DNA detected in parallel. There was a minimum required detection limit of 500 copies/ml_plasma of wildtype GNAS DNA as internal positive control.

In order to achieve standardization for HPV16 LB tests, an internal standard operating procedure (SOP) was established at the Pathology Department. Figure 2 shows the different sequences of LB tests which were performed for the detection of HPV16 DNA in anal carcinoma patients. The first step involved drawing blood from a patient into a 10ml "Streck" tube. The plasma was then separated, with 2ml stored as a reserve at -80°C and 2ml used for DNA isolation into a 50 µl eluate. Following this, the ddPCR assay (HPV16-GNAS assay in triplicates) was performed. The results were interpreted as positive if there were more than 8 dots in 3 assays, marginal if there are 3 to 8 dots, and negative if fewer than 3 dots are detected across the 3 assays.

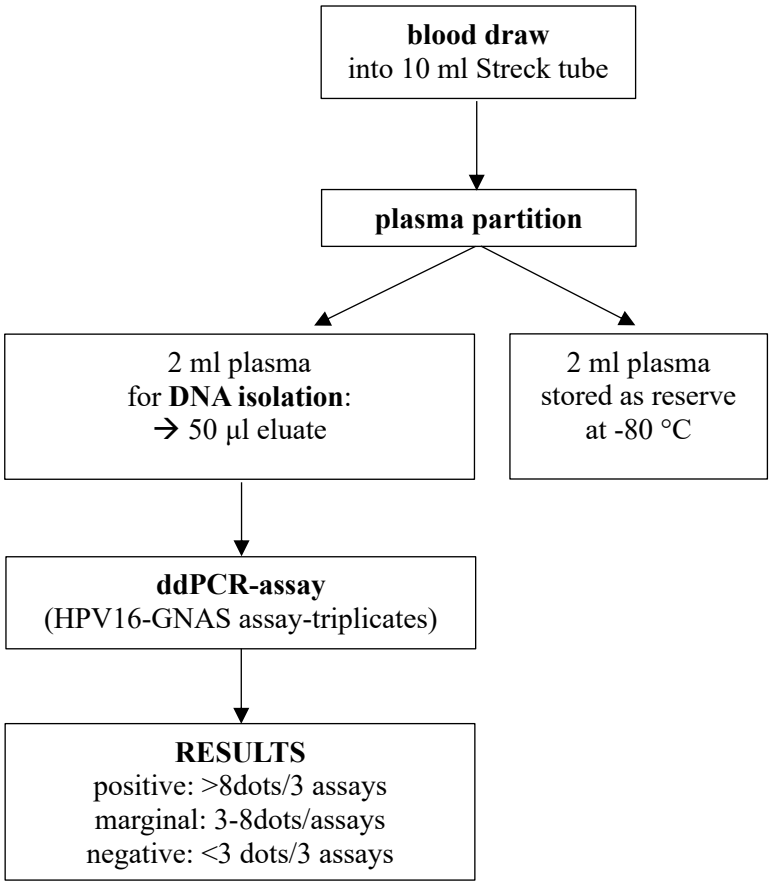


Figure 2. Standard operating procedure (SOP) for HPV16 LB

3.5 Ethical approval

Ethical approval was obtained from the Ethics-Committee of the University Hospital of Erlangen (Report No 23-316-B) on September 29, 2023.

3.6 Statistical tests

IBM SPSS Statistics (Version 29.0.2.0) for macOS was used to analyze the results. The Kolmogorov-Smirnov test was used to analyze the normality of the data distribution. Qualitative data are expressed as whole numbers and percentages, while quantitative data are expressed as mean ± standard deviation (SD) or mean and interquartile range (IQR). Results are presented as tables and figures with a 95% confidence interval (CI).

For analysis of the treatment response using HPV16 DNA copies/ml plasma, all data were summarized in descriptive statistics. To compare differences between two groups, the t-test was performed and to measure the linear correlation between two sets of data, the Pearson

correlation coefficient (PCC) was determined. Frequencies were reported for categorical variables. The significance of all tests was set at 0.05.

4 RESULTS

4.1 Literature review

Extensive literature analysis was conducted and constitutes the primary component of the study. The procedure of systematic literature search was already explained in detail in Materials and Methods. Some of the relevant articles were provided by the Department of Pathology in Coburg. In addition, PubMed and Google Scholar were used for further searches. The materials were then categorized into the following subgroups: HPV, HPV-associated anal carcinoma, HPV-associated cervical carcinoma and LB. The main findings of literature analysis are summarized in Table 4 below. The majority of studies investigated HPV16 and used ddPCR. Three studies also included other HPV-associated tumors such as cervical, oropharyngeal and vulval. The main focus of published studies was the investigation of the clinical applicability of LB regarding treatment response and disease progression monitoring. Cohort size and the post-therapy observation time were the main limitation factors in the described studies.

Table 4. Summary of Literature

Literature	**Tumor types**	**HPV subtypes** **Technology**	**Major findings**
Chatfield-Reed et al. 2021 (64)	Cervical Anal Oropharyngeal	HPV16 ddPCR qPCR	– median cps/ml of HPV cfDNA was >32x higher in T4 tumors than in T1 – SCCC patients with >20 HPV cfDNA positive droplets at baseline: increased risk for disease progression and mortality – most promising clinical application of LB: monitoring treatment response & disease progression
Bernard-Tessier et al. 2018 (65)	Anal	HPV16 ddPCR	– 91% of HPV16 anal carcinomas positive in serum – baseline copy number not correlated with patient outcome – ddPCR more sensitive than qPCR – after CRT: HPV DNA Median = 0 – one year OS (without detectable HPV DNA) after CRT: 87% – residual HPV ctDNA of 2,662 copies/ml after completed chemotherapy
Cabel et al. 2018 (66)	Anal	HPV16, HPV18 ddPCR	– baseline copy number dependent on stage (not prognostic for relapse) – reduction after CRT is very prognostic for DFS and relapse – residual HPV ctDNA after CRT showed rapid metastatic relapse – lower detection rates of HPV ctDNA in stage II (64%) than stage III (100%)
Jeannot et al. 2016 (67)	Anal Cervix Oropharyngeal	HPV16, HPV18 ddPCR qPCR	– no tumor DNA is released at detectable level in blood with non-invasive tumors – viral load in tumor tissue is an important marker when assessing baseline levels – higher viral load in HPV16 than HPV18

Malusecka et al. 2022 (68)	Anal	Plasma DNA	– no relevance of base-line levels for staging/therapy – strong correlation between concentration of total cfDNA and tumor volume (PET/CT) but not the SUVmax of primary tumor or lymph nodes – increase in cfDNA levels after initiation of therapy, a peak at end of therapy, a decrease during follow-up period, after 2 years: levels below pre-treatment values – primary tumor breakdown is responsible for increase in cfDNA – no relationship between cfDNA and treatment response – cfDNA has short half-life (1 hour)
Liauw et al. 2021 (69)	Anal	HPV16	– clearance in blood may occur prior to completion of therapy, early response assessment, selection of patient for de-escalation of therapy – early detection of disease recurrence possible by LB compared to standard approaches (exam, anoscopy with biopsy, PET/CT)
Lefevre et. al 2021 (70)	Anal		– three elimination patterns defined: fast responders with no local/distal failures, slow responders with high risk of local failures, but no distal failures, persistent high risk of distal failures, but no local failures
Lee et. al 2020 (71)	Anal		– positive HPV copy number 12 weeks after therapy showed relapse
Sastre-Garau et al. 2021 (72)	Cervical Oropharyngeal Anal Vulva	HPV16, HPV18 ddPCR	– NGS-based technology possible which allows to include integrations site analysis – full characterization of HPV status: genotyping, complete sequence, insertion pattern → identification of specific tumor marker – “personalized HPV strain”
Mazurek et. al 2023 (73)	Anal	HPV16	– HPV detectable in blood t=0 prognostically good – HPV disappears after CRT

4.2 Pilot study

Data related to anal (n=12) carcinomas patients were extracted from their files at the Departments of Radiation Oncology and Pathology of the Hospital in Coburg. The characteristics are summarized in Table 5. Data related to cervical (n=3) carcinoma patients are explained in more detail in the Attachment section.

Table 5. Patient characteristics

Variables		
Total number of patients		15 (100%)
Anal Carcinoma		12 (80%)
Cervical Carcinoma		3 (20%)
Sex	Female	12 (80%)
	Male	3 (20%)
Age at diagnosis	Mean	59 years
	Minimum	43 years
	Maximum	84 years
TNM[†]	T1	1 (6.7%)
	T2	8 (53.3%)
	T3	3 (20%)
	T4	1 (6.7%)
	N0	8 (53.3%)
	N1	6 (40%)
	N2	1 (6.7%)
	M0	15 (100%)
	G0	9 (60%)
	G2	4 (26.7%)
	G3	2 (13.3%)
FIGO[‡]	<IIIB	0
	≥IIIB	3 (100%)
HPV16 DNA detection	Yes	10 (66.7%)
at diagnosis	No reliable evidence	5 (33.3%)
HPV16 DNA copies/ml plasma	Mean	1254 cps/ml
at diagnosis	Minimum	65 cps/ml
	Maximum	9157 cps/ml
Dose primary tumor	Mean	53.629 Gy
	IQR	53.5-56 Gy
Dose regional LN	Mean	50.469 Gy
	IQR	50.4-52.1 Gy
Total dose of BT[§]	Mean	13 Gy
	IQR	4-28 Gy
Duration of CRT and BT	Mean	43.67 days
	IQR	38-45 days
Chemotherapy[‖]	5-FU + Mitomycin	8 (53.3%)
	Capecitabine	3 (20%)
Complete remission	Yes	14 (93.3%)
	No	1 (6.7%)
Recurrence	Yes	0
	No	15 (100%)

[†] Tumor stage classification for anal carcinoma

[‡] Classification for cervical carcinoma

[§] One patient received EBRT instead of Brachytherapy

[‖] Three patients did not receive chemotherapy

The study population consisted of 12 patients and was predominantly female (80%) with a mean age at diagnosis of 59 years. Of these patients, 80% (n=12) were diagnosed with a HPV16 positive anal carcinoma (3 additional cases represented HPV16-positive cervical carcinoma, all with FIGO ≥IIIb). The majority of anal carcinomas were classified as T2 (53.3%) or T3 (20%), N0 (53.3%) or N1 (40%) and M0 (100%). No patient had any metastases at the time of diagnosis. Regarding the grade of differentiation, most of the anal tumors were G1 (60%) and therefore well differentiated, 4 (26.7%) patients with G2 and 2 patients (13.3%) with G3.

In respect of therapy, the median dose administered as external radiotherapy was 56.0 Gy for the primary tumor, with a maximum total dose of 58.0 Gy. For the regional lymph nodes (LN) the median dose delivered was 50.4 Gy. The standard chemotherapeutic regimen for anal carcinoma consisting of 2 cycles 5-FU (1000mg/m^2) and Mitomycin (10mg/m^2) was given in 53.3% of anal cancer patients, whereas the remaining 3 (20%) received Capecitabine instead of 5-FU. Postoperative chemoradiation therapy was only necessary in 2 patients (13.3%). In cervical cancer patients, the median dose of BT administered was 7 Gy. The chemoradiation therapy (CRT) together with brachytherapy (BT) lasted a median time of 38.0 days.

The basic strategy for taking blood samples of the carcinoma patients that was elaborated in the Department of Pathology in the Hospital of Coburg was already explained in Materials and Methods. Blood draws were not conducted at specific time points, but related to the therapeutic time course, most importantly before and after therapy. Figure 3 illustrates the time intervals at which blood was taken. The first blood sample was taken on average 3 days before the start of therapy and the longest observation time after the completion of CRT were 2 years (post-therapy).

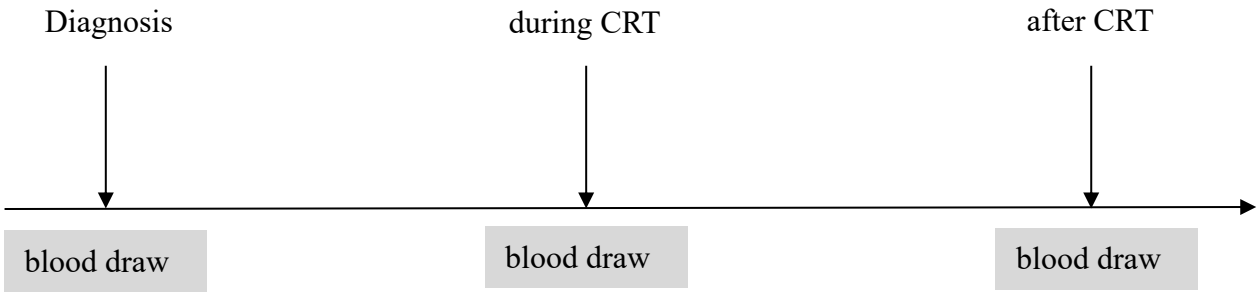
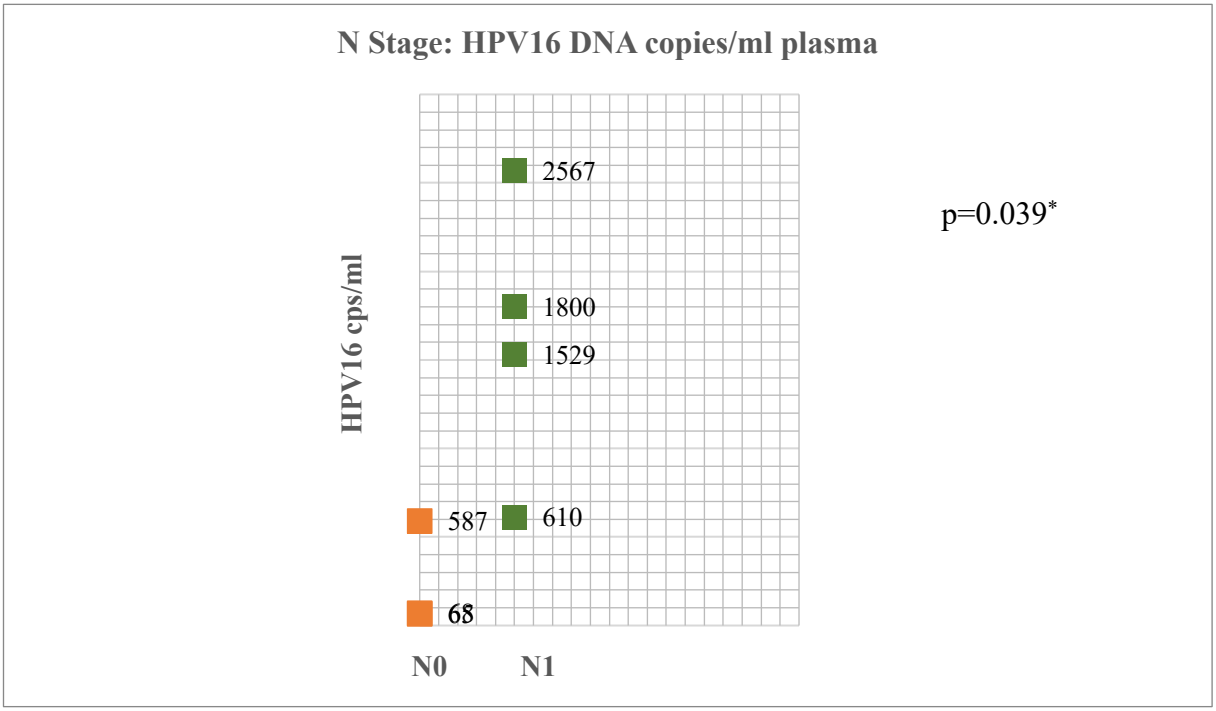


Figure 3. Flow chart of blood draw timepoints

HPV16 DNA detection by LB at the time of diagnosis was possible in 9 patients (75%) whereas in the remaining 3 patients (25%) no reliable evidence for HPV16 DNA was found in plasma samples. The median HPV16 DNA copy number at the time of diagnosis was 1254 cps/ml. There was no statistically significant correlation between copy number and tumor stage (p=0.657) (the weak negative correlation observed is most likely due to chance). A significant positive correlation between copy number and lymph node involvement (p=0.039) was discovered. Thus, the main differences between different tumor stages according to TNM classification and the associated HPV16DNA copy numbers were seen in lymph node involvement (N). Figure 4 illustrates the prevalent HPV16 DNA copy numbers relative to lymph node involvement (N) before the treatment.



* Pearson Correlation Coefficient

Figure 4. N Stage: HPV16 DNA copies/ml plasma before start of treatment

All HPV16-associated anal carcinoma patients (n=12) reached complete remission after completion of CRT; however, for the DFS the observation period was only two years at most. Tumor stages and the progression of HPV16 copies before, during and after CRT are summarized in Table 6. The values in brackets either indicate on which day of therapy or how many days after completion of CRT blood was taken. In non-shedding tumors the baseline HPV16 copy number within blood plasma was (per definition) 0 cps/ml_plasma: thus, in these cases (n=4) ctDNA could not be used as a therapy monitoring value as there is no baseline value as reference during CRT.

For the HPV16-shedding tumors after the first 15 days of therapy, a significant reduction in the copy number was observed. The patients whose blood samples were taken during the therapy already achieved a value of 0 HPV16 copies per ml plasma during the therapy, which indicates a significant reduction in the tumor load and a response to the therapy.

Table 6. Anal carcinoma stage and progression of HPV16 copy numbers during therapy

Patient	Stage	pre-Therapy	during Therapy	post-Therapy	Clinical picture
1	T2N+M0	1529	275 (9/43) †	0 (+ 56) ‡	CR§
			86 (16/43)	0 (+ 73)	
			74 (29/43)		
			0 (37/43)		
			0 (42/43)		
2	T2N1M0	2567	10692 (7/38)	0 (+ 40)	CR
			20 (15/38)	0 (+ 85)	
			0 (37/38)		
3	cT2cNXcM0	0	0 (22/43)	0 (+ 35)	CR
			0 (42/43)		
4	T3N0M0	587		0	CR
5	T2N0M0G2	68		0 (+ 5)	CR
6	T2N1M0	1800		0 (+ 56)	CR
7	T2N1M0	610		0 (+ 1)	CR
8	T2N0M0G3	1179	0 (26/42)		CR
9	cT3cN2cMXG2	0	0 (9/38)		CR
			0 (36/38)		
10	rpT3pN1aM0R1	0			CR
11	T1N0M0G2	0			CR
12	T2N0M0G2	65		0	CR

† Day of therapy

‡ Days post-therapy

§ Complete remission

The detection frequency of HPV16 copies/ml plasma at different time points is shown in the following Figure 6 for the patients with positive basic values (the non-shedding cases were excluded as their detection levels remained 0 cps/ml_plasma throughout all blood draws). HPV16 DNA values are shown in $\log_2$(HPV16 DNA cps/ml plasma).

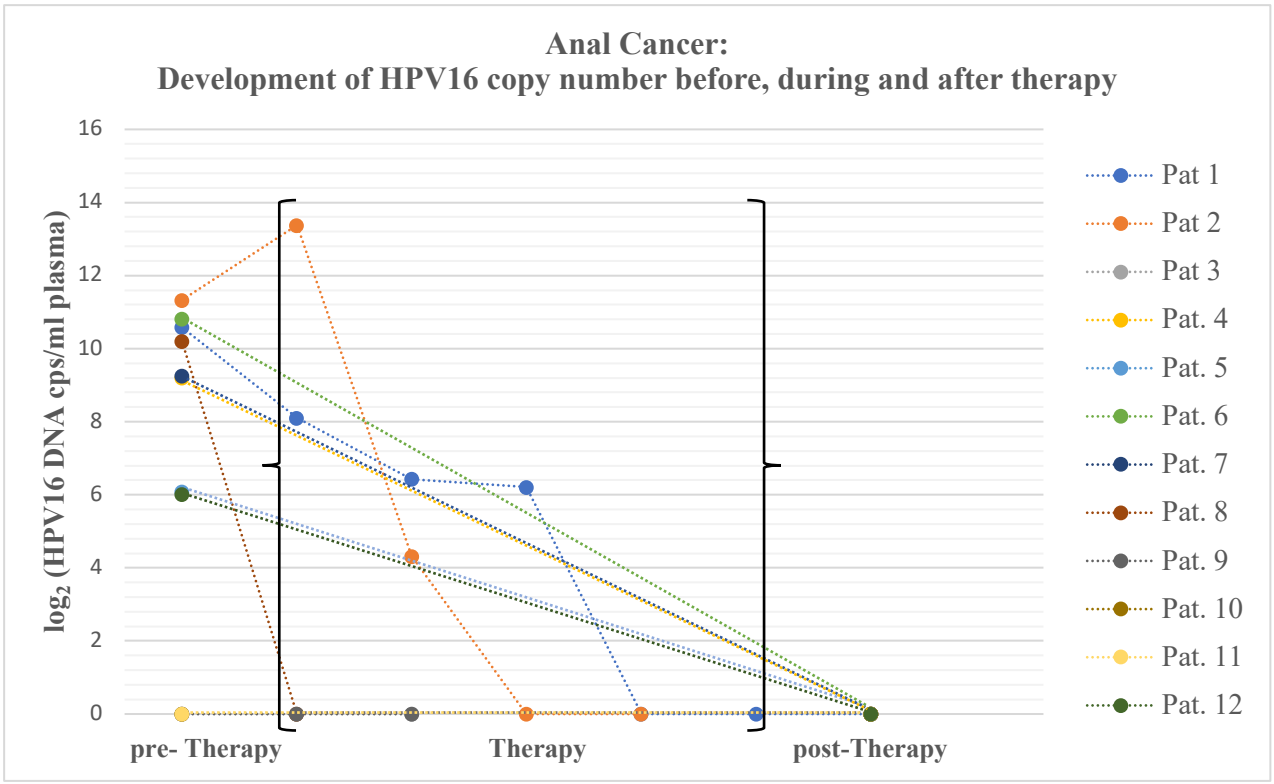


Figure 6. Development of HPV16 copy number before, during and after therapy

4.2.1.1 Attachment: Outlook cervical carcinoma

The characteristics of patients with HPV16 cervical carcinoma (n=3) have been detailed in Table 5 above. The three cases of HPV16 cervical cancer displayed similar patterns to those observed in HPV16 anal carcinoma. Detection of HPV16 DNA at time of diagnosis was possible in 2 out of 3 (66.7%) cases. The measured HPV16 ctDNA values correlated to the disease follow-up.

Further characteristics of HPV16-associated cervical cancer patients and the progression of their ctDNA before, during and after CRT are shown in Table 7 and Figure 7 below. In these cases, 2 patients achieved complete remission after completion of CRT and 1 patient (Patient 3) still showed a positive HPV16 ctDNA value in the post-therapeutic measurement. After undergoing 35 days of external radiotherapy followed by 10 days of brachytherapy, her last measurement taken 97 days post-completion of CRT and brachytherapy showed a positive result (3670 cps/ml plasma). Consequently, she will undergo additional therapy.

Table 7. Cervical carcinoma stage and progression of HPV16 copy numbers during therapy

Patient	Stage	pre-Therapy	during Therapy	post-Therapy	Clinical picture
1	FIGO IIIb	9157	3581 (6/38) [†] 504 (13/38) 0 (20/38) 25 (26/38)	0	CR[§]
2	FIGO IIIc	0	0	0	CR
3	FIGO IVA cT3-4N+G3		14703 (15/45)	3670 (+ 97) [‡]	ongoing therapy

[†] Day of therapy
[‡] Days post-therapy
[§] Complete remission

The development of HPV16 copy number before, during and after therapy is shown in Figure 7 below. HPV16 DNA values are shown in $\log_2$(HPV16 DNA cps/ml plasma).

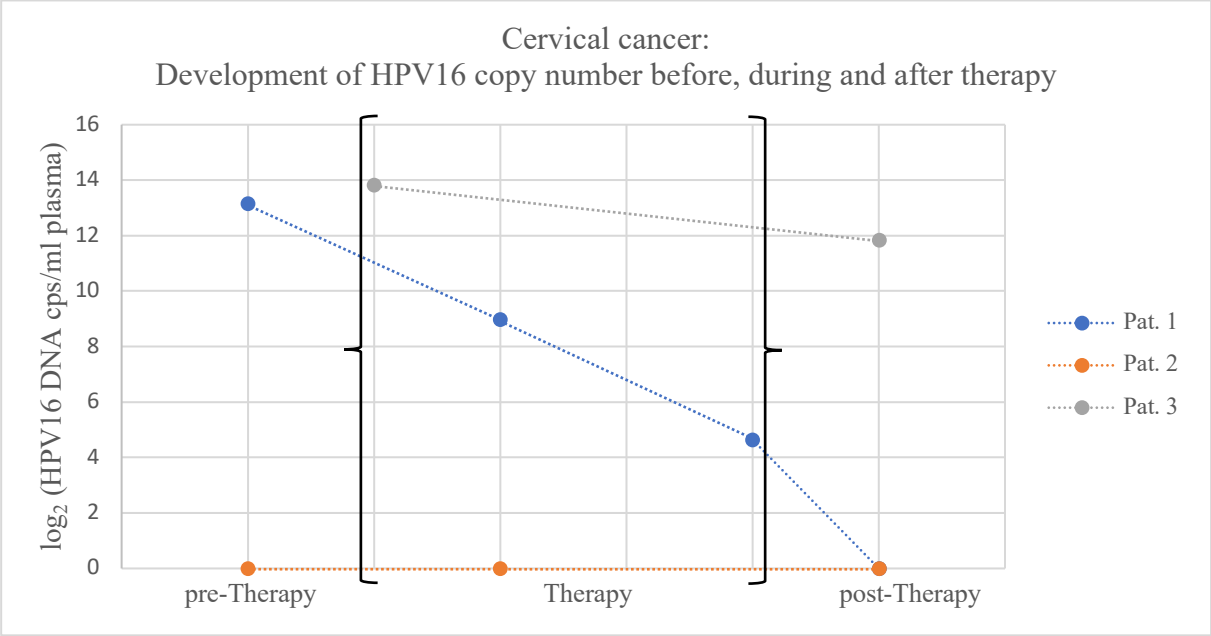


Figure 7. Development of HPV16 copy number before, during and after therapy

5 DISCUSSION

The application of ctDNA as a biomarker for monitoring treatment effects and early detection of disease relapses in HPV-associated anal carcinomas represents a promising approach for improving patient outcomes in oncology. Given the challenges associated with traditional methods of disease monitoring and surveillance, such as tissue biopsy and imaging modalities, the identification of minimally invasive and sensitive biomarkers holds considerable value in clinical practice and is under continuous research.

Anal carcinoma presents complex challenges in terms of diagnosis, treatment, and outcome assessment. While anal carcinoma is a rare tumor, comprising less than 5% of all malignant tumors in the gastrointestinal tract, 93% of cases are associated with HPV16 (3). Therefore, many studies focused on HR-HPV research in association with anal carcinoma. HPV16 is very prevalent in anal carcinomas and often used as biomarker. The majority of anal carcinomas are shedding viral HPV DNA into peripheral blood. In shedding tumors, ctDNA can be potentially used as a therapy monitoring tool and gives real-time information about the tumor progression. Ultrasensitive detection technologies, such as ddPCR, are being continuously tested. The most promising clinical application of LB is therapy response monitoring and disease progression observation (64). LB shows a greater accuracy regarding minimal residual disease (MRD), is lower in cost and less dependent on expert interpretation, and radiation exposure or invasive procedures are avoided (74). However, in order to be able to compare the ctDNA values with each other in a standardized way, cut-off values of HPV16 copy number for determination of positive results or recurrences are still missing (75). There were many studies regarding the significance of HPV16 DNA copy number values. The baseline copy number of HPV16 DNA seemed to be dependent of tumor stage but it was not prognostic for relapses (64,66). In one study, one patient with a low HPV16 ctDNA (10 cps/ml) at the end of CRT did not have a recurrence (76).

In cases presented in this pilot study, no recurrence has been detected. This is in line with published data that patients with undetectable HPV DNA at the end of CRT had a higher PFS compared to patients with still detectable at ctDNA at the end of CRT (74). Another study stated that persistent HPV ctDNA following CRT correlates independently with poorer progression-free survival (PFS) (77). According to many studies, regional lymph node involvement had an influence on the absolute value of HPV16 DNA which was also observed in our pilot study (75). The same applies for distant metastases, though this could not be validated in our data set as no patients with distant metastases were investigated (78). The mean age of 59 years at the time of diagnosis was described in many studies and a significantly higher prevalence of anal carcinoma in female patients (80%) was found (66,79). Different studies

proved an association between the detection of HPV ctDNA and tumor stage. This represents the primary constraint on the clinical utility of LB in premalignant lesions and early-stage cancers. Pre-malignant lesions are still often not detected due to their smaller size and their non-invasiveness (80). Regarding the invasiveness of tumors, one study stated a correlation between the detection probability and the invasiveness, as non-invasive tumors do not release ctDNA into circulation and therefore rarely can be detected by LB (67).

Another major issue is the definition of cut-off values described in published studies: 10 copies per 10^5 cells as positive result in tissue biopsy and a cut-off value for HPV positivity of >3 copies/ml plasma (81,82). After the initiation of therapy, the primary tumor breakdown was responsible for an increase of ctDNA levels. Other observations such as a peak of ctDNA at the end of therapy, a decrease of ctDNA levels during follow-up and after 2 years ctDNA levels which are below pre-treatment values were also described in another study (68).

Definitive chemoradiotherapy (CRT) is the current standard of care for patients diagnosed with locally advanced anal cancer. The median dose administered as external radiotherapy was 56.0 Gy for primary tumors, with a maximum total dose of 58.0 Gy. Corresponding to further studies, in anal cancer, CRT is a combination of external beam radiation therapy (ERBT) and chemotherapy usually with 5-FU and mitomycin (34). ctDNA reduction after CRT was described as a prognostic factor for disease free survival (DFS) or recurrences and patients with residual HPV ctDNA after CRT experienced a rapid metastatic relapse (66). The clearance of HPV DNA in blood prior to completion of therapy could be used as an early response assessment and for the selection of patients suitable for a de-escalation of therapy (69). The same study proved that compared to standard approaches, e.g. clinical examination, anoscopsy with biopsy or PET/CT, the early detection of disease relapse by LB is possible. In accordance to that, another study examined the accuracy of relapse prediction after 3 months with the following detection probabilities: HPV DNA 77% compared to FDG-PET: 60% (74). But no correlation between cfDNA concentration and survival time was found (79). The main limitation that was described in nearly all studies was the small sample size and the limited follow-up time.

In this pilot study, we investigated the potential utility of ctDNA analysis in HPV-associated anal carcinoma patients and to a small amount cervical cancer patient undergoing definitive chemoradiation therapy. The progression of HPV16 DNA copy number was analyzed during CRT by LB. The tumor volume correlated with the initial baseline ctDNA concentration in plasma, which was especially seen cervical carcinomas (67). 66.6% of cervical cancer patients with FIGO >IIIb had initial ctDNA baseline values of >9160 cps/ml. The majority of

anal carcinomas were classified as T≥2 (53.3%) or T≥3 (20%), which states a higher detection frequency with a larger tumor mass. This could not be investigated most likely due to the small number of patients, as none of the patients had proven distant metastases and the nodal involvement was classified as N0 (53.3%) or N1 (40%).

HPV16 DNA detection by LB at the time of diagnosis was possible in only 66.7% of patients whereas in the remaining 33.3% of cases no reliable evidence for HPV16 DNA was found. The median baseline HPV16 DNA copy number at the time of diagnosis was 1800 cps/ml. In this study, HPV positivity was confirmed by the detection of HPV16 DNA copies per ml of plasma. The result was considered to be positive if >8 dots in 3 assays were positive, borderline if 3-8 dots were positive and negative if <3 dots per assay were present. A complete remission was achieved in 93.3% of patients and 6.7% were still in therapy or did not reach complete remission. No recurrence has been detected so far. In 14 out of 15 patients, the HPV16 ctDNA progression was corresponding to therapy response and therefore worked in 93.3% of cases as monitoring parameter for therapy response.

This pilot study was limited by its small sample size and therefore many important aspects of the clinical applicability of LB could not be fully analyzed. Additionally, to its limited sample size, the study's scope was restricted by its recruitment solely from Hospital Coburg, Germany. Consequently, the applicability of the results to a broader population is constrained. Blood samples were not taken in standardized time intervals during therapy and not all patients could be diagnosed by LB because HPV16 DNA was not detected. Standardized comparison among all 15 patients was consequently restricted. Due to the limited post-therapeutic observation period, drawing conclusions about long-term tumor development is nearly impossible. The objective is to use LB both as a diagnostic and therapeutic tool in a greater patient cohort in order to determine the significance of ctDNA and thus the clinical applicability of LB. However, the discoveries illuminate the potential and effectiveness of employing ctDNA as a method for real-time monitoring of treatment response, providing valuable perspectives into the management of these malignancies.

In summary, our study has demonstrated the feasibility of LB for HPV16-associated anal and cervical carcinomas in the setup of Coburg Medical Cancer Center. The final effectiveness of this approach, however, needs further investigations in larger medical centers with longer observation periods of patient outcome.

6 CONCLUSION

In conclusion of the first part of the study, the findings of the literature analysis underscore the potential of LB, particularly ddPCR-based HPV16 ctDNA analysis, in enhancing prognostication, treatment monitoring, and early detection of disease recurrence in anal squamous cell carcinoma patients. Integrating these insights into clinical practice promises to improve patient outcomes through more precise and timely interventions.

The experimental pilot study demonstrated that the initial detection of HPV16 ctDNA by LB predisposes for successful therapy monitoring and facilitates disease progression observation following CRT. Tumors can be subdivided into shedding and non-shedding. In case of non-shedding tumors, LB is clinically not applicable, which limits its practical use, especially regarding replacing conventional tissue biopsy completely. However, in cases with an initial detection of baseline HPV16 ctDNA, LB is superior to conventional methods in terms of real-time monitoring of disease progression. Because it is a minimally invasive method, easy to implement, cheaper compared to other procedures and displays no detrimental side effects (like radiation damage of side effects of chemotherapeutic agents). However, each ctDNA value should always be interpreted in the clinical context of the patient: in cases of HPV16 ctDNA values of 0 cps/ml plasma, the diagnosis of a (non-shedding) HPV16 positive tumor must be ruled out, and in case of positive HPV16 values the actual presence of the neoplasm needs to be verified by further conventional morphological examinations. This work successfully introduced LB-based HPV16 detection in patients with anal squamous cell carcinomas for therapy monitoring and outcome prediction in the Department of Radiation Oncology and Pathology in the Hospital of Coburg.

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8 SUMMARY

Objectives: The aim of this study was to characterize the potential of cfDNA via liquid biopsy (LB) in patients with HPV-associated anal carcinomas. Specifically, its use as cancer biomarker for therapy surveillance and as early indicator of recurrence after chemoradiation (CRT) was evaluated.

Materials and methods: The major part of this study consisted of a thorough literature analysis based on the research topic. An electronic search of online databases was performed to obtain key literature on the topics HPV-associated cancer and LB. The academic database PubMed and search engines such as Google Scholar deemed most appropriate for the research topic were selected. The systematic literature search was executed in selected databases and search engines using the predefined search strategies. The minor part of this study was conducted in form of a prospective pilot study that aimed to evaluate the feasibility of LB in monitoring HPV-induced anal and to a small amount cervical carcinomas before and after CRT. The study was associated and grounded on data from the Departments of Radiation Oncology and Pathology of the hospital in Coburg, Germany.

Results: Many studies investigated HPV16-associated anal carcinoma and used ddPCR technology. Some studies also included other HPV-associated tumors such as cervical, oropharyngeal and vulval. The main focus of published studies is the investigation of the clinical applicability of LB regarding treatment response and disease progression monitoring. Cohort size and the post-therapy observation time were the main limitation factors in the described studies. The experimental pilot study primarily focused on HPV16-associated anal carcinoma treated with CRT. After completion of CRT, all cases (n=12) reached complete clinical and biochemical remission. Short post-therapeutic observation time and small cohort size were the main limitations.

Conclusion: The findings of the literature analysis underscore the potential of LB, particularly ddPCR-based HPV16 ctDNA analysis, in enhancing prognostication, treatment monitoring, and early detection of disease recurrence in anal squamous cell carcinoma patients. The pilot study demonstrated that the initial detection of HPV16 ctDNA by LB predisposes successful therapy monitoring and facilitates disease progression observation following CRT. This work introduced LB-based HPV16 detection in patients with anal squamous cell carcinomas for therapy monitoring and outcome prediction in the Department of Radiation Oncology and Pathology in the Hospital of Coburg.

9 CROATIAN SUMMARY

Naslov: Značenje DNA cirkulirajućih tumorskih stanica u HPV-asociranom analnom karcinomu za praćenje učinka liječenja i detekciju recidiva bolesti nakon liječenja definitivnom kemoradioterapijom.

Ciljevi: Cilj ove studije bio je karakterizirati potencijal cfDNA putem tekuće biopsije (LB) kod pacijenata s HPV-povezanim analnim karcinomima. Konkretno, procijenjena je njegova upotreba kao biomarkera raka za nadzor terapije i kao rani pokazatelj recidiva nakon kemoradioterapije (CRT).

Materijali i metode: Glavni dio ove studije sastojao se od temeljite analize literature na temelju istraživačke teme. Elektronska pretraga online baza podataka obavljena je kako bi se dobila ključna literatura o temama raka povezanog s HPV-om i tekuće biopsije (LB). Odabrane su akademska baza podataka PubMed i tražilice kao što je Google Scholar koje su smatrane najprikladnijima za istraživačku temu. Sustavna pretraga literature provedena je u odabranim bazama podataka i tražilicama koristeći unaprijed definirane strategije pretrage. Manji dio ove studije proveden je u obliku prospektivne pilot-studije koja je imala za cilj procijeniti izvedivost LB u praćenju analnih karcinoma uzrokovanih HPV-om i u manjoj mjeri cervikalnih karcinoma prije i nakon kemoradioterapije (CRT). Studija je bila povezana sa podacima iz odjela za radijacijsku onkologiju i patologiju bolnice u Coburgu, Njemačka.

Rezultati: Većina studija istraživala je analni karcinom povezan s HPV16 i koristila ddPCR tehnologiju. Tri studije su također uključivale druge tumore povezane s HPV-om, kao što su cervikalni, orofaringealni i vulvalni. Glavni fokus objavljenih studija je istraživanje kliničke primjenjivosti tekuće biopsije (LB) s obzirom na reakciju na liječenje i praćenje razvijanja bolesti. Veličina kohorte i vrijeme postterapijskog praćenja glavni su ograničavajući faktori u opisanim studijama. Pilot-studija je prvenstveno bila usmjerena na analni karcinom povezan s HPV16 koji je liječen kemoradioterapijom (CRT). Nakon završetka CRT-a, svi slučajevi (n=12) postigli su potpunu kliničku i biokemijsku remisiju. Kratko postterapijsko vrijeme praćenja i mala veličina kohorte bili su glavni ograničavajući faktori.

Zaključci: Nalazi analize literature naglašavaju transformacijski potencijal tekuće biopsije (LB), osobito analize HPV16 ctDNA temeljene na ddPCR-u, u poboljšanju prognoze, praćenju liječenja i u ranom otkrivanju recidiva bolesti kod pacijenata s analnim pločastim staničnim karcinomom. Pilot-studija je pokazala da početno otkrivanje HPV16 ctDNA putem LB-a predisponira uspješno praćenje terapije i olakšava praćenje razvijanja bolesti nakon CRT-a. Ovaj rad je uveo otkrivanje HPV16 pomoću LB-a kod pacijenata s analnim pločastim staničnim karcinomom za praćenje terapije i predviđanje ishoda na odjelu za radijacijsku onkologiju i patologiju u bolnici Coburg.