# HPV After chemoRadioTherapy

# (HART)

VFN PRAHA
VŠEOBECNÁ FAKULTNÍ NEMOCNICE

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|---|---|
| ***Protocol version*** | 1.0 |
| ***Date*** | 10.11.2025 |
| ***Sponsor*** | General Faculty Hospital in Prague |
| ***Principle investigator*** | MUDr. Lukáš Dostálek, Ph.D. |
| ***Full title of the trial*** | Implementation of HPV Testing in Patients After Radiotherapy for Cervical Cancer |
| ***Contact*** | Apolinářská 18<br>Praha 2, 12800<br>Czech republic<br>tel.: +420777980048<br>lukas.dostalek@vfn.cz |

## Research team

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|---|---|
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# Principal Investigator's Statement

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|---|---|
| Full title: | HPV After Radiotherapy |
| Acronym: | HART |
| Protocol version: | 1.0 |
| Principal Investigator's Statement: | By my signature below, I confirm that I will conduct the study in accordance with the protocol and in compliance with the accepted standards of Good Clinical Practice and the Declaration of Helsinki. I will adhere to all applicable laws and regulations governing the conduct of clinical studies. All information and data related to the study will be treated as confidential and will not be disclosed to third parties or used for any purpose other than the conduct of this study. |

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| Centre: | Department of Gynaecology, Obstetrics and Neonatology, General Faculty Hospital in Prague |
| Principal investigator: | MUDr. Lukáš Dostálek, PhD. |
| Date: | Signature: |
| 14.11.2025 | [redacted] |

**Synopsis**

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| **Acronyme** | HART |
| **Full title** | Implementation of HPV Testing in Patients After Radiotherapy for Cervical Cancer<br>HART: HPV After chemoRadioTherapy |
| **Trial type** | Prospective multicenter observational |
| **Primary objectives** | Sensitivity of recurrence detection in patients after (chemo)radiotherapy (CRT) for cervical cancer during two-year follow-up using:<br>1. Real-Time PCR of HPV DNA in a sample taken from a cervical swab<br>2. Detection of HPV ctDNA from a patient's peripheral blood sample<br>*Sensitivities will be determined separately with respect to the occurrence of recurrence in the year following the sampling or throughout the entire two-year follow-up - see chapter Statistical Analysis for more details.* |
| **Null hypotheses** | There is no difference in recurrence rates between patients who are HPV-positive vs. HPV-negative during follow-up after CRT for cervical cancer:<br>1. using PCR of HPV DNA in a sample taken from a cervical swab<br>2. using the detection of HPV ctDNA presence in a patient's peripheral blood sample |

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| **Secondary Objectives** | 1. Rate of PCR HPV DNA negativity from a swab after CRT (at Visit 1)<br>a. In case of HPV persistence, comparison of HPV genotype before and after completion of treatment<br>2. Rate of HPV ctDNA negativity after CRT (at Visit 1)<br>3. Comparison of the sensitivity of recurrence detection using PCR of HPV DNA in a sample taken from a cervical swab with the method of HPV ctDNA detection from a patient's peripheral blood sample<br>4. Evaluation of the course of further treatment (especially the possibility of curability) in patients who were detected with recurrence during the study<br>5. Analysis of HPV swab and cfDNA collection performed upon recurrence detection |
| **Porpose of the trial** | 1. Implement HPV testing into clinical practice for patients after primary CRT for cervical cancer in the Czech Republic.<br>2. Contribute to the introduction of the ctDNA monitoring method into clinical practice in the Czech Republic.<br>3. Compare the prognostic significance of HPV positivity after CRT detected by HPV DNA PCR from a cervical swab with detection by determining the presence of HPV ctDNA from serum.<br>4. Create a basis for prospective follow-up of patients who underwent a secondary surgical procedure based on the detection of recurrence in this study. |
| **Inclusion criteria** | 1. Patient indicated for primary RT for cervical cancer<br>2. FIGO stage IB - IVA<br>3. Signed informed consent<br>4. Age ≥ 18 years<br>5. Administration of RT with curative intent |
| **Exclusion criteria** | 1. Clinical stage FIGO IA<br>2. Clinical stage FIGO IVB<br>3. History of radiotherapy in the pelvis |

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| | 4. Hysterectomy performed before the start of radiotherapy (adjuvant RT)<br>5. History of HPV-associated malignancy in personal history<br>6. HIV or other significant immunodeficiency |
| **Time schedule** | ● duration: 4 years<br>● study initiation: 1/2026<br>● study completion: 1/2030 |
| **Sample size** | 120 patients |

## Contents

**Abbreviations**

| Abbreviation | Full Name/Description (English) |
|---|---|
| RT | Radiotherapy |
| CRT | Chemoradiotherapy |
| FIGO | International Federation of Gynecology and Obstetrics |
| HPV | Human Papillomavirus |
| SOP | Standard Operating Procedure |
| DFS | Disease-free survival |
| CR | Complete Response |
| SCC | Squamous Cell Carcinoma Antigen |
| ROC | Receiver-operating Curve |
| ddPCR | Digital Droplet Polymerase Chain Reaction |
| ctDNA | circulating tumor DNA |
| HPV-seq | Sequencing of circulating HPV genome |
| EBRT | External Beam Radiotherapy |
| BRT | Brachyradiotherapy |
| cfDNA | Cell-free DNA |
| CT | Computed Tomography |
| PET/CT | Positron Emission Tomography/Computed Tomography |
| MRI | Magnetic resonance imaging |
| PET/MRI | Positron Emission Tomography/Magnetic resonance imaging |
| D90 CTV HR | Dose covering 90% of the target volume (High-Risk Clinical Target Volume) |
| EQD2 | Biologically equivalent dose at 2 Gy fractionation (Equivalent Dose in 2Gy Fractions) |
| IMRT | Intensity-Modulated Radiotherapy |
| SIB | Simultaneous Integrated Boost |

## Summary

Human Papillomavirus infection is a causal factor for at least 95% of cervical cancers [1]. Surgical treatment and radiotherapy (RT) represent two fundamental modalities for the therapy of this disease, provided there is no distant metastasis. While surgical management is preferred in stage (FIGO) I, primary curative RT with concomitant chemotherapy (CRT) [2] plus targeted therapy with pembrolizumab is indicated in higher stages [3].

Follow-up of patients after surgical treatment for cervical cancer is codified by a number of recommended procedures and is based mainly on clinical examination, cytology collection, and HPV testing. The HPV test is mainly used in women who have retained their uterus after conservative surgery [4]. Especially in microinvasive carcinomas, the HPV test shows high sensitivity and a nearly 100% negative predictive value. This means that its negativity almost excludes the future development of recurrence [5].

**Follow-up of Patients after Primary CRT**

In patients after primary CRT, the uterus (including the cervix) is also preserved. However, the follow-up is governed by different principles. According to the EMBRACE-II protocol, therapeutic response in the tumor and lymph node area is assessed 3 months after completion of treatment using imaging (in Czech conditions, by performing PET/CT or PET/MRI) and gynecological examination. Regular follow-up is then started, which includes only gynecological examination. Imaging is repeated at 12 months after completion of treatment or at any time in case of suspected recurrence [6]. There is no consensus on the implementation of SCC antigen sampling into routine follow-up [2].

Implementation of imaging methods in later follow-up is not routinely recommended. Their indication is based on symptoms that lead to suspicion of recurrence [7]. For patients after CRT for cervical cancer, there is a clear recommendation not to collect a cervicovaginal cytology smear [2]. The implementation of the HPV test in the follow-up of these patients is not mentioned in current recommendations.

Five-year disease-free survival (DFS) in patients after primary CRT is stage-dependent and ranges between 76% in stage IB and 47% in stage IVA [8]. Local (pelvic) recurrences in patients after CRT can be treated by surgical procedure with curative intent. Overall survival and quality of life then depend on the size of the recurrence, its location, and the extent of the surgery. However, even with highly complex procedures (extended exenteration), overall survival reaches almost fifty percent [9]. It follows from the above that the early detection of recurrence after primary CRT significantly increases the patient's chance of survival and quality of life. However, due to limited follow-up

options, recurrences are often not detected early in many cases, and patients can only be offered palliative treatment [10]

**HPV PCR from a cervical swab**

It has been repeatedly proven that the elimination of HPV after primary CRT (the so-called HPV clearance) represents one of the most fundamental positive prognostic factors arguing against the development of recurrence. Traditionally, achieving HPV clearance is determined using a swab from the preserved cervix [11–13]. In a significant proportion of patients, HPV DNA is not detectable in the cervical swab shortly after the completion of CRT. With increasing follow-up duration, the proportion of HPV-positive patients further decreases. In a 2011 study, the proportion of HPV-positive patients after one, three, and twelve months from the completion of treatment was 28%, 22%, and 18%, respectively [11]. In this study, the development of recurrences (78%) was clearly associated with HPV persistence. The sensitivity and specificity of the HPV test taken from the cervix are 85% and 54%, respectively [12].

**HPV PCR from a blood sample**

Another possibility for detecting persistence or recurrence is the detection of tumor DNA in the patient's circulation. Tumors associated with viral infection have long served as a model for developing methods for determining circulating tumor DNA (ctDNA) and for testing its clinical use [14].

HPV DNA is detected in the peripheral blood of patients with cervical cancer both at the time of diagnosis and at the relapse of the disease. Although it has been shown that HPV ctDNA levels reflect the probability of the presence of persistence or recurrence of an HPV-associated tumor, routine deployment has been hindered by low sensitivity. This was only 57% compared to PCR performed from a cervical swab [15].

In contrast, digital droplet PCR (ddPCR) brings a significant refinement to the detection of molecules at low concentrations [16]. ddPCR detected the presence of HPV ctDNA in 100% of patients with advanced cervical carcinoma before primary CRT. In an analysis performed after its completion, HPV ctDNA determination showed a sensitivity of 75% and a specificity of 80%. Furthermore, HPV ctDNA determination at three months after the completion of CRT showed higher accuracy compared to PET/CT in detecting recurrence after 18 months [17].

Another possibility for increasing sensitivity is next-generation sequencing (NGS), whose sensitivity in determining circulating viral DNA (HPV-seq) surpasses the capabilities of ddPCR [18].

In a prospective clinical study examining the relationship between HPV ctDNA detection and the incidence of recurrences in patients after CRT for advanced cervical carcinoma, both methods were used—standard ddPCR and HPV-seq. It was again demonstrated that the detection of HPV ctDNA after the completion of CRT is associated with a poorer prognosis. Consistent with earlier work, an increase in sensitivity upon repeated

**Situation in the Czech Republic and Project Context**

In the Czech Republic (as in most other developed countries), HPV testing in the form of a simple cervicovaginal smear is currently not routinely performed for patients after CRT for cervical cancer. Also, monitoring of HPV ctDNA levels is limited to individual academic experiments, currently exclusively in the field of oropharyngeal carcinoma [22].

Testing for the presence of HPV DNA in the cervix and/or the patient's circulation can be an effective tool to identify a cohort of women who are at increased risk of recurrence *20*. These women can be subjected to more intensive monitoring for the development of recurrence, which should enable timely indication of surgical management or systemic treatment. Conversely, it is possible to define a group of negatively tested patients who should represent a cohort with an exceptionally good prognosis, in whom less intensive follow-up with a lower burden of imaging examinations could, conversely, be performed.

The aim of this study is to document data that will enable the individualisation of follow-up based on HPV detection in patients after primary CRT for cervical cancer in the Czech Republic; to establish the relationship between the presence of HPV in the cervical smear or the patient's circulation and the occurrence of recurrence; to determine the rate of HPV infection elimination depending on the tumour characteristics and the administered treatment, and to evaluate treatment options for patients in whom recurrence was detected thanks to the study.

## Study Objectives

Main Study Objectives:

1. Sensitivity of recurrence detection using HPV DNA detection via Real Time PCR from a cervical swab in patients after (chemo)radiotherapy administered for cervical cancer

2. Sensitivity of recurrence detection using detection of HPV DNA fragments circulating in the peripheral blood of patients after (chemo)radiotherapy administered for cervical cancer

Sensitivities will be determined separately with respect to the occurrence of recurrence in the year following the sampling or throughout the entire two-year follow-up - see the Statistical Analysis chapter for more details.

**Secondary Study Objectives:**

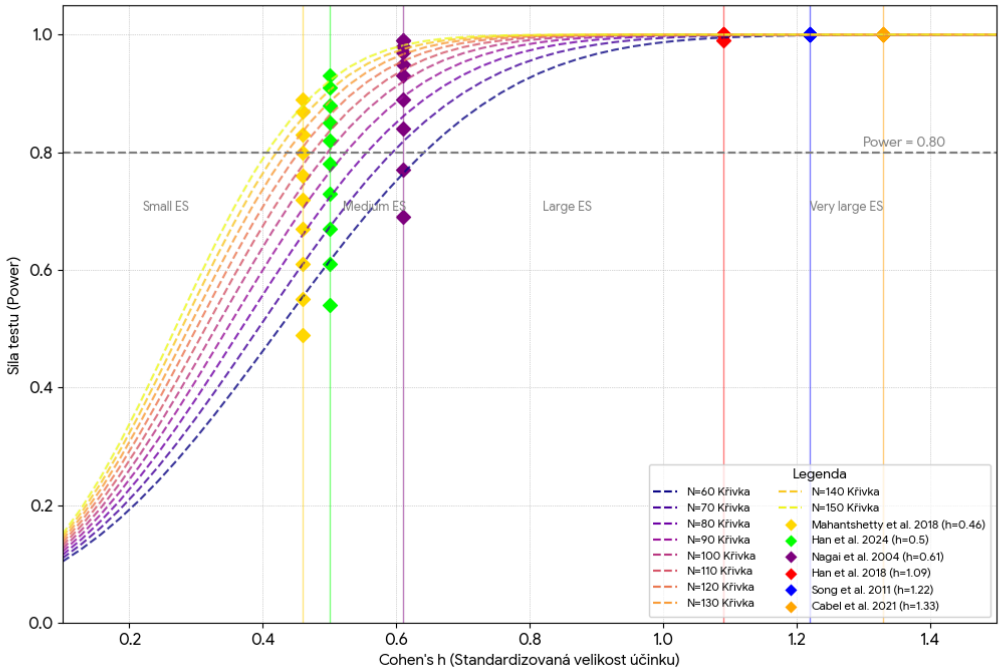
1. Rate of PCR HPV DNA negativity from a swab after CRT administration (comparison of HPV testing results within Screening with Visit 1)
    a. In case of HPV persistence, comparison of HPV genotype before the start of treatment and its completion
2. Rate of HPV ctDNA negativity after CRT administration (comparison of HPV testing results within Screening with Visit 1)
3. Comparison of the sensitivity of recurrence detection using PCR HPV DNA in a sample taken from a cervical swab with the method of HPV ctDNA detection from a patient's peripheral blood sample
4. Evaluation of the course of further treatment (especially the possibility of curability) in patients who were detected with recurrence during the study
5. Analysis of HPV swab and cfDNA collection performed upon the Unscheduled recurrence visit

## Sample Size

*The sample size calculation was performed by Mgr. Martin Komarc, PhD, Department of Biomedical Statistics (Biostat), 1st Faculty of Medicine, Charles University*

The significance of the difference in recurrence rates will be assessed using a one-sided test. To evaluate the effect size, graphs were constructed comparing Cohen's h with the power of the study for hypothetical cohorts of size 60 -150 patients (see Figure 1)

**Figure 1. The power of a one-sided test for N=60 - 150 with points corresponding to the studies cited in Tables 1 and 2 (alpha=0.05, one-sided).**




Into these, the values of Cohen's h calculated from the studies listed in Tables 1 and 2 were entered based on specific initial proportions. The resulting powers for individual studies and the mentioned sample sizes are presented in Table 3.

The power analysis was performed based on the following:

**Table 1. Background data for power analysis of the part of the study analyzing the implementation of a cervical swab**

| HPV status after CRT | Total | Recurrence yes | Recurrence no |
|---|---|---|---|
| **Song et al.** [11] | | | |
| **HPV negative** | 127 | 4 | 123 |
| **HPV positive** | 29 | 14 | 15 |
| **Total** | 156 | 18 (12%) | 138 |
| **Nagai et al.** [12] | | | |
| **HPV negative** | 42 | 3 | 39 |
| **HPV positive** | 50 | 17 | 33 |
| **Total** | 92 | 20 (22%) | 72 |

| | | | |
|---|---|---|---|
| **Mahantshetty et al.** [21] | | | |
| **HPV negative** | 43 | 5 | 38 |
| **HPV positive** | 89 | 29 | 60 |
| **Total** | 132 | 34 (26%) | 98 |

**Table 2. Background data for power analysis of the part of the study analyzing the implementation of dPCR**

| HPV ctDNA after CRT | Total | Recurrence yes | Recurrence no |
|---|---|---|---|
| **Cabel et al** [22] | | | |
| **Nedetekována** | 10 | 2 | 8 |
| **Detekována** | 4 | 3 | 1 |
| **Total** | 14 | 5 (36%) | 9 |
| **Han et al** [17] | | | |
| **Nedetekována** | 13 | 1 | 12 |
| **Detekována** | 6 | 3 | 3 |
| **Total** | 19 | 4 (21%) | 15 |
| **Han et al** [23] | | | |
| **Nedetekována** | 37 | 9 | 28 |
| **Detekována** | 25 | 12 | 13 |
| **Total** | 62 | 21(34%) | 41 |

Given that a power of ≥ 0.8 was achieved, the minimum sample size was determined to be 110 patients (Table 3). After taking into account the anticipated study exclusion rate (drop-out rate), the target sample size was set at 120 patients.

**Table 3. Power values of the study for individual sample sizes**

| Study | *Cohen h* | Power for N= 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Song et al. 2011** | *1,2* | 1,0 | 1,0 | 1,0 | 1,0 | 1,0 | **1,0** | 1,0 | 1,0 | 1,0 | 1,0 |
| **Nagai et al. 2004** | *0,7* | 0,7 | 0,8 | 0,8 | 0,9 | 0,9 | **1,0** | 1,0 | 1,0 | 1,0 | 1,0 |
| **Mahantsh. et al. 2018** | *0,5* | 0,5 | 0,6 | 0,6 | 0,7 | 0,7 | **0,8** | 0,8 | 0,8 | 0,9 | 0,9 |
| **Cabel et al. 2021** | *1,2* | 1,0 | 1,0 | 1,0 | 1,0 | 1,0 | **1,0** | 1,0 | 1,0 | 1,0 | 1,0 |
| **Han et al. 2018** | *1,0* | 1,0 | 1,0 | 1,0 | 1,0 | 1,0 | **1,0** | 1,0 | 1,0 | 1,0 | 1,0 |
| **Han et al. 2024** | *0,5* | 0,6 | 0,6 | 0,7 | 0,7 | 0,8 | **0,8** | 0,9 | 0,9 | 0,9 | 0,9 |

## Study Procedure - SOP

Patients who meet all inclusion criteria and none of the exclusion criteria may be enrolled. The study will proceed according to the scheme.

**Table 4. Visit schedule**

| | Screening | Visit 1 | Visit 2 | Visit 3 | Unscheduled recurrence visit |
|---|---|---|---|---|---|
| ***Interval since CRT*** | *max 1 month before the start of CRT* | *3 months ± 14 days* [1] | *12 months ± 30 days* | *24 months ± 30 days* | |
| **Informed consent** | + | | | | |
| **Imaging** | | + | + | + | + |
| **HPV swab** | + | + | + | | +/- |
| **ctDNA sample** | + | + | + | | +/- |
| **SCC sample** | +/- | +/- | +/- | +/- | +/- |
| **Symptoms** | | + | + | + | + |

*+ compulsory; +/- optional*
[1]*In case of necessity to postpone the date of Visit 1 (for example, due to the coincidence of imaging with a visit in another study), the principal investigator must be consulted.*

Examinations indicated before the administration of radiotherapy are described below within the description of the Screening visit.

**Radiotherapy**

Radiotherapy must be indicated with curative intent. It must be primary radiotherapy for cervical carcinoma. It must not be adjuvant radiotherapy - the uterus must be left in situ. The indispensable standard treatment for locally advanced cervical carcinoma is external beam radiotherapy (EBRT) with a recommendation for concomitant chemotherapy and subsequent brachytherapy (BRT). Patients for whom only EBRT was indicated may also be included in the study.

**External Beam Radiotherapy** to the pelvis, including pelvic and potentially para-aortic and inguinal lymph nodes, is a fundamental part of the treatment strategy with the main goal of achieving local and nodal control. The total dose of EBRT must be 45–50.4 Gy delivered in 23–28 fractions (1.8–2.0 Gy per fraction). Affected nodes will be treated with a Simultaneous Integrated Boost (SIB) so that the total dose (including the dose contribution from brachytherapy) reaches 60 Gy EQD2. Radiotherapy will be performed using one of the following techniques with daily image verification and potential correction of the patient's position:

- IMRT (Intensity-Modulated Radiotherapy)
- VMAT (Volumetric Modulated Arc Therapy) or

- Tomotherapy

In case the patient is not indicated for brachytherapy or is unable to undergo it, an external beam radiotherapy boost will be performed to the maximum achievable dose, taking into account the tolerance of healthy tissues.

**Brachytherapy** (if indicated) will be performed after the completion of external beam radiotherapy or during the 5th week of EBRT, with the aim of utilizing maximum tumor regression. BRT contouring and planning will be based on 3D image documentation obtained using CT or MRI. The total brachytherapy dose will be 25–30 Gy delivered in 4–6 fractions (5–7 Gy per fraction). To achieve adequate coverage of the target volume (CTV) and simultaneous adherence to dose limits for organs at risk (OAR), brachytherapy will be performed intracavitarily or as a combined intracavitary and interstitial technique. The sum of the EBRT and BRT doses will be evaluated using the calculation of the biologically equivalent dose at 2 Gy fractionation (EQD2), based on the linear-quadratic model with parameters $\alpha/\beta = 10$ Gy for tumor effect and $\alpha/\beta = 3$ Gy for late healthy tissue damage. The D90 value for CTV-HR will be $\geq 85$ Gy.

Contouring of target volumes, organs at risk, and recommended fractionation will follow the EMBRACE II protocol [6]. The total treatment time, including both EBRT and BRT, should not exceed 56 days. Any interruptions of radiotherapy must be compensated according to local site standards. BRT administration is not a mandatory condition for study enrollment.

**Chemotherapy** (if indicated) will be administered concurrently with external beam radiotherapy (concomitantly). The treatment regimen includes cisplatin at a dose of 40 mg/m² administered once a week, always on the day of planned EBRT. In case of treatment prolongation and the need to continue chemotherapy after the completion of EBRT, cisplatin will not be administered on the days of brachytherapy application. The goal is to administer 5–6 cycles of concomitant chemotherapy. Dose reduction, reduction in the number of cycles, or complete omission of chemotherapy due to toxicity or contraindications on the patient's side is permissible and will be managed by the treating physician in accordance with local standards of care.

**Concomitant and Adjuvant Immunotherapy** - if the criteria of the KEYNOTE-A18 / ENGOT-cx11 study [3] are met, pembrolizumab may be administered concomitantly and subsequently as adjuvant therapy according to the recommended dosing. The management of administration and toxicity treatment will be led by the treating physician in accordance with current international and local recommendations.

**Course of Visits**

- During the Screening visit, which will take place before the start of CRT, the study will be thoroughly explained to the patient and informed consent will be signed. The visit must not precede the start of CRT by more than 1 month. Furthermore, an HPV DNA test will be taken from the cervix and a blood sample will be collected to determine the presence of ctDNA HPV. Patient characteristics, tumor properties, and the result of clinical staging (FIGO) will be recorded in the eCRF.
- Visit 1 will follow 3 months after the completion of CRT ± 14 days. (In case of necessity to postpone the date of Visit 1 (for example, due to the coincidence of imaging with a visit in another study), the principal investigator must be consulted.) Imaging will be performed during this visit. Furthermore, an HPV swab and a blood sample for ctDNA determination will be collected. The SCC marker may also be collected during Visit 1. Its value will be determined locally and the result will be recorded in the eCRF. Any symptoms of the patient will also be recorded.
- Visit 2 will follow 12 months after the completion of CRT ± 30 days. Imaging will be performed during this visit. Furthermore, an HPV swab and a blood sample for ctDNA determination will be collected. The SCC marker may be collected during Visit 2. Its value will be determined locally and the result will be recorded in the eCRF. Any symptoms of the patient will also be recorded.
- Visit 3 will follow 24 months after the completion of CRT ± 30 days. Imaging will be performed during Visit 3. The SCC marker may also be collected. Its value will be determined locally and the result will be recorded in the eCRF. Any symptoms of the patient will also be recorded.
- In case of recurrence detection, an Unscheduled Recurrence Visit will be performed. Imaging will be performed during this visit. During this visit, an HPV swab and a blood sample for ctDNA determination will be collected. The SCC marker may also be collected. Its value will be determined locally and the result will be recorded in the eCRF. Any symptoms of the patient will also be recorded. This visit will also be completed if recurrence is detected outside of Visits 2 and 3 (for example, by performing imaging for other reasons).

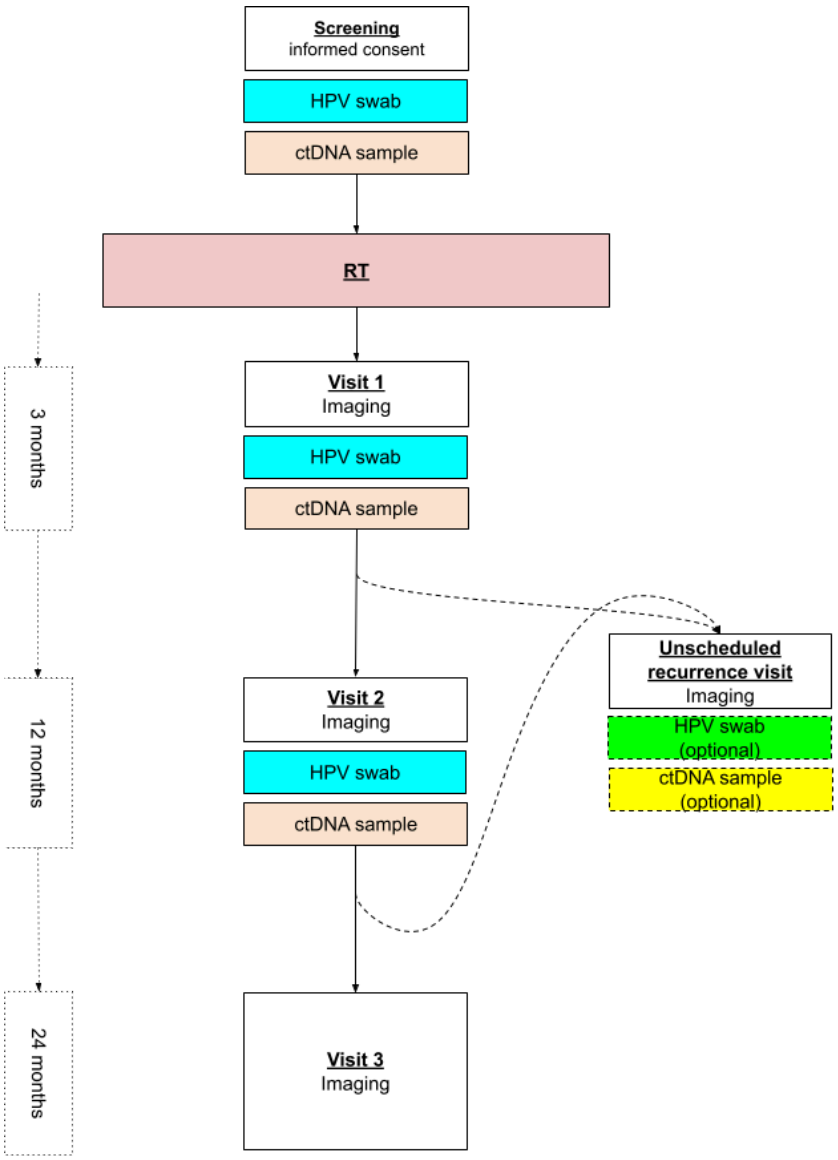
Treatment of a potential recurrence depends on the decision of the multidisciplinary oncogynecological board. The patient will be further monitored in the prospective study registry. The type of recurrence (local, combined, distant), location of recurrence, proposed treatment, and the result of this treatment (CR vs. non-CR) will be recorded.

In case the patient does not want to or cannot continue (for example, due to withdrawal of consent, death, etc.), the EoT Visit will be completed.

**Imaging**

PET/CT or PET/MRI is accepted as imaging. In case only CT or MRI is performed, the principal investigator will be contacted, who will decide on the possibility of accepting this imaging. The reading will be performed locally. Imaging performed during Visit 1 will serve for the assessment of therapeutic response to CRT and the achievement of CR. Imaging performed during Visits 2, 3, and during the Unscheduled Recurrence Visit will serve to identify patients with recurrence - see the chapter.

**Figure 2. Study scheme**




**Samples**

The Evalyn self-sampling set (ROVERS MEDICAL DEVICES, Lekstraat 10, NL-5347 KV Oss., Netherlands) will be used to perform the cervical swab. The sets will be distributed in sufficient numbers to individual workplaces. The test will be taken by the examining clinician according to the instructions, or the patient may take it herself (the method of collection will be recorded in the eCRF). After collection, the set will be sent according to the instructions below.

Full genotyping of all 14 high-risk HPV genotypes will be performed.

Venous blood will be drawn into a tube intended for ctDNA/cfDNA collection and preservation (e.g., Streck Cell-Free DNA BCT, PAXgene Blood ccfDNA Tube, AmoyDx® Cell-free DNA Protection Vacuum Tube). The sample will be processed according to the manufacturer's protocol. DNA isolation will be performed manually from 10 ml of blood for one examination using the QIAamp MinElute cfDNA Midi Kit (Qiagen, (Qiagen, QIAGEN Straße 1, 40724 Hilden, Germany), quantified and stored at -80°C until subsequent processing.

cfDNA analysis will be performed using panel NGS with target enrichment technology utilizing a custom panel targeting high-risk HPV genomic DNA and selected human ctDNA targets. Library preparation will be done using the KAPA EvoPrep Kit (Roche) with UMI adapters. Sequencing will take place on the Illumina platform (NexSeq/NovaSeq; 5200 Illumina Way, San Diego, CA 92122 USA). Bioinformatics processing will be performed using an optimized in-house bioinformatics pipeline (Laboratory of Oncogenetics)

**Sending of Samples**

1. Envelopes for sending samples will be distributed to individual centers. Please send the samples immediately after collection (the same day)! Procedure for sending samples:
2. Mark the samples (Evalyn brush and tube/tubes for cfDNA) with the patient identifier - use the same one you selected for entry in RadCap - CENTERNAME_XXX
3. Place the samples in the envelope
4. Order transportation at DHL:

**+420 220 300 111**

5. Use following details:

account number:
**304117963**
owner name:
**Všeobecná fakultní nemocnice v Praze**

Address for sending:

Adam Buryanek
Klinika gynekologie, porodnictví a neonatologie
Apolinářská 18
Praha 2
128 00
Czech republic

The results of the HPV testing will be provided to the attending physician. Further procedure will be under the sole responsibility of the attending physician.

In case of urgent issues/clarifications, please call the principal investigator:

**Lukáš Dostálek**
**+420 777 98 00 48**

## Statistical Processing

### Recurrence

Recurrences will be evaluated in patients who achieved CR after the completion of CRT.

Recurrence is defined as a clear evidence of avid tissue on PET/CT or PET/MRI (oncological probability scale = 5 - positive finding). In case of equivocal findings, recurrence must be verified by biopsy.

Recurrence may be detected in the following ways:

- by imaging indicated within Visit 2 or 3
    - (recurrence detected within Visit 1 will be labelled as persistence or non-CR)
- by imaging indicated outside the prescribed visits - "Unscheduled Recurrence Visit";
    - **Note: in case of recurrence detection outside the study but within the monitored follow-up (two years from the completion of CRT) (for example, during imaging indicated at another workplace, etc.), it is ***always*** necessary to complete the "Unscheduled Recurrence Visit"**
    - **If in this case PET/CT or PET/MRI was not performed as imaging and its result is clear (i.e., the treating physician does not indicate a supplementary PET/CT or PET/MRI), the principal investigator (MUDr. Lukáš Dostálek, Ph.D.) will be contacted, who will decide on further procedure**

Recurrences will be defined by temporal distribution as follows:

- Recurrence in the first year will be labelled as the one that occurs between Visit 1 and imaging indicated within Visit 2 inclusive
- Recurrence in the second year will be labelled as the one that occurs between Visit 2 and imaging indicated within Visit 3 inclusive; this applies to patients who did not experience recurrence in the first year
- Recurrence during the entire follow-up will be labelled as the one that occurs between Visit 1 and imaging indicated within Visit 3 inclusive

Recurrences will be defined by localization as follows:

- local, affecting only the cervix, including those that spread continuously to its surroundings
- regional, where the tumor occurs in the pelvis without connection to the cervix
- distant, where the tumor occurs outside the pelvis and does not occur in the cervix
- combined - a combination of local and distnat.

**Evaluation of the Primary Objective**

Only patients with confirmed CR within Visit 1 will be included in the evaluation of the primary objective (sensitivity).

Sensitivity in the periods defined below will be calculated as follows (see Figure

$$\frac{umber\ of\ HPV\ positive\ patients\ in\ whom\ recurrence\ occurred}{number\ of\ patients\ in\ whom\ recurrence\ occurred}$$

Sensitivity will be calculated separately as:

- Sensitivity of the entire follow-up - patients who tested HPV positive at Visit 1 related to recurrences detected throughout the entire follow-up.
- Sensitivity of the 1st year - patients who tested HPV positive at Visit 1 related to recurrences detected during the first year.
- Sensitivity of the 2nd year - patients who tested HPV positive at Visit 1 or Visit 2 related to recurrences detected during the second year.
- Sensitivities will be calculated separately in this way for HPV testing using a cervicovaginal swab and ctDNA.

**Exclusion of the Null Hypothesis**

The prognosis of HPV positive patients (by both tests) and HPV negative patients will be plotted using the Kaplan-Meier curve. Differences in prognosis (HPV positive vs. HPV negative) will be evaluated using the log-rank test.

**Evaluation of Secondary Objectives**

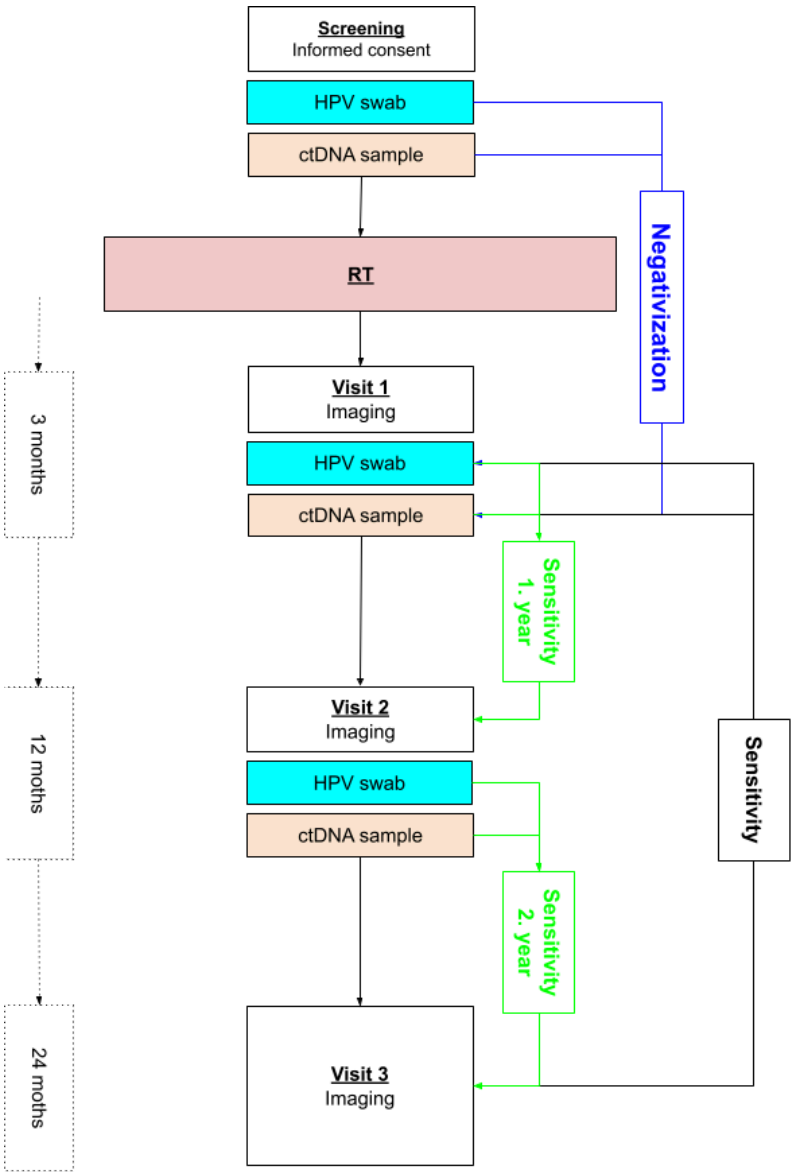
The rate of negativity will be evaluated separately for HPV testing using a cervicovaginal swab and ctDNA. It will be calculated as follows:

$$1 - \frac{number\ of\ HPV\ positive\ patients\ within\ the\ Screening\ visit}{number\ of\ HPV\ positive\ patients\ within\ Visit\ 1}$$

Furthermore, the relationship between the negativity of both tests and the achievement of CR on imaging within Visit 1 will be evaluated. This analysis will be performed using Chi-squared test.

Comparison of the sensitivities (and other parameters such as specificity, negative predictive value, etc.) of both tests will be performed using analysis of the ROC curve.

**Figure 3. Statistical Processing**




## Data Processing

The parameters obtained by this analysis (sensitivity, specificity) will be plotted on an ROC curve. The deviation of the ROC curve from the level of chance (AUC = 0.5) will be evaluated using the Z-test according to the method of Hanley and McNeil (1982). Other parameters will be processed using standard methods of descriptive statistics, including relative and absolute frequencies and arithmetic mean with standard deviation. The chi quare test will be used to compare the frequencies of demographic and clinical categorical variables. Continuous variables will be compared using the Mann-Whitney U test, which is a non-parametric alternative to the t-test for independent samples. The level of statistical significance will be set for all tests at alpha = 0.05

**Information System**

Data will be entered in pseudonymized form into an electronic database via eCRF. For this purpose, REDCap implemented under the 1st Faculty of Medicine of Charles University.

**redcap.lf1.cuni.cz**

Patients will be pseudonymized in the system - they will be entered into RedCap using identifiers chosen by each center. The recommended format is: CENTERNAME_XXX. Each center will maintain a table from which it will be possible to identify a specific patient by the identifier (patient log).

**Study Monitoring**

Oversight of the study conduct will be ensured by a monitor who will check compliance with basic legal norms and the quality of the collected data. Within the scope of centralized monitoring, they will supervise whether the rights and health of study subjects are protected, whether the recorded data is correct, complete, and verifiable, and whether the clinical study is being conducted in accordance with the latest approved version of the Protocol/amendments, Good Clinical Practice, and legal regulations.

**Regulatory and Ethical Aspects of the Study**

Participating sites are responsible for obtaining local ethical committee approval. Each patient will voluntarily agree to participate in the clinical trial and will sign an informed consent form before being enrolled in the study. The study will be conducted in accordance with the protocol terms and generally accepted standards of good clinical practice, in compliance with the guidelines of the International Conference on Harmonisation of Good Clinical Practice (ICH-GCP) and the Declaration of Helsinki. Investigators will comply with all applicable laws and regulations governing the conduct of clinical trials. Investigators will treat all information and data related to the study as confidential and will not disclose this information to any third parties or use it for any purpose other than the conduct of this study.

## Financial Support of the Study

Institutional support from the General Faculty Hospital will be requested in 2026.

## Multicenter Collaboration

The treatment of each patient is led in accordance with local standards. The local physician or principal investigator must explain the nature of the study and the associated procedures to the patient in detail. The local physician or principal investigator bears responsibility for any complications arising from the treatment, including radiotherapy toxicity. Users will have access to the REDCap system via major web browsers without the need to install any additional software. Local investigators will receive a username and password to access the REDCap system. Communication between the server and users will be secured by an encrypted protocol (HTTPS) and Secure Socket Layer encryption. Only pseudonymized data will be collected; local investigators will create a local identifier for local patient identification.

## Study Termination

The study will end with the completion of Visit 5 for the last monitored patient.

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