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# HPV AND POTENTIAL PROGNOSTIC MARKERS IN PRIMARY VAGINAL CARCINOMA

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# HPV AND POTENTIAL PROGNOSTIC MARKERS IN PRIMARY VAGINAL CARCINOMA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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MD

The thesis will be defended in public at Torsten Gordh Auditorium, Solna, 5th of May 2022.

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To my amazing family,

# POPULAR SCIENCE SUMMARY OF THE THESIS

Vaginal cancer is a rare disease that accounts for about 2% of all gynecological malignancies. Knowledge concerning this disease is limited, in part because it is so uncommon. The disease mainly affects older women, mean age about 65 years, although about 15% of such patients are under age 50. Squamous cell carcinoma is the most common type and is usually located in the upper part of the vagina. About one third of patients are diagnosed with metastatic disease and such cases have a worse prognosis. Generally, the prognosis in vaginal cancer is worse than that associated with other gynecological tumor diseases, with a five-year survival rate of about 50%-60%.

The disease is usually treated with a combination of radiation therapy and chemotherapy. Treatment strategies are based on those for cervical cancer since vaginal cancer is similar in many ways. No recommendations for customized individual treatment have been formulated based on known prognostic factors, for which reason overtreatment and undertreatment may contribute to adverse outcomes.

Vaginal cancer is often caused by a human papillomavirus (HPV) infection. Previous studies indicate that about 50% to 80% of cases are positive for HPV, but there is a gap in knowledge concerning the mechanisms underlying HPV-negative cancers. Many studies indicate that HPV-positive tumors may have a better prognosis than HPV-negative tumors, while established prognostic factors include clinical manifestations such as tumor stage, size, and patient age. Although no biological markers are currently used as prognostic factors, several have been proposed and are under study. Additional knowledge concerning prognostic markers in vaginal cancer is needed in order to improve diagnostics and provide a rational basis for more individualized treatment.

The purpose of this thesis is to identify potential prognostic factors in vaginal cancer. The first study investigates expression of two biomarker proteins, p16 and Ki67, in relation to the presence or absence of HPV in tumor tissue from vaginal cancer. In brief, p16 may act as a tumor suppressor and Ki67 is a protein marker for increased cell division. The study included tumor tissue samples obtained between 1978 and 1995 from women with vaginal cancer who were diagnosed, treated, and followed up in the County of Stockholm. Data pertaining to clinical features and tumor characteristics, as well as treatment and follow-up for possible relapse and/or survival, were collected from the medical records of the included patients over a period spanning up to 8 years from time of diagnosis. The cases were divided according to short- and long-term survival in order to compare the outcomes in relation to presence or absence of HPV and expression of p16 and Ki67 in tumor tissue. The presence of HPV was determined using the Polymerase Chain Reaction (PCR), which can be used to detect the genome (DNA) of HPV virus in tumor tissue. Ki67 and p16 expression was investigated through staining of these proteins in patient tissue samples (immunohistochemical staining) and subsequent microscopic examination by a pathologist.

In this material, prevalence of HPV positivity was found to be 43%, which was lower than in several previous studies. High expression of Ki67 was associated with poor differentiation (degree to which tumor cells resemble original tissue) and small tumor size; however, no correlation was found with improved survival. High expression of p16 was also associated with poor differentiation and to the presence of HPV. Moreover, p16 positivity alone was associated with long-term survival in the initial analysis, but not when additional factors, such as patient age, tumor stage, and tumor size, were taken into account. In summary, the results

show that p16 and Ki67 can be used to grade tumors in vaginal cancer and p16 may be useful as a marker for HPV-positive cancer. This study did not find HPV status or Ki67 to be useful as prognostic markers for better survival in vaginal cancer whereas p16 needs to be studied in larger cohorts than ours to clearify a possible association with survival. The main factors associated with survival in our material were tumor size and degree of differentiation.

The second study examined three proteins known as Leucine rich repeats and immunoglobulin-like domains (LRIG) 1, 2, and 3. The LRIG1-3 genes are prone to undergo changes in cancer cells. These genes encode proteins that are involved in the development of various cancers, although their exact functions are not yet clear. LRIG1 appears to exert an inhibitory effect on the development of tumors, while LRIG1-3 have been found to be valuable as prognostic markers for cancer, including cervical cancer. This study used immunostaining of previously collected tumor material from women with vaginal cancer in order to study expression of LRIG1, LRIG2, and LRIG 3, and to investigate whether they correlate with clinical manifestations and survival. This study was conducted on tumor samples from women diagnosed with vaginal cancer in Örebro, Karlstad, Eskilstuna, and Västerås between 1975 and 2002, while also considering previously collected data on clinical manifestations and HPV status as mentioned above. The LRIG1-3 proteins were analyzed using immunohistochemical staining and microscopic examination.
Most tumors in this study expressed both LRIG1 and LRIG2, while little or no LRIG3 expression was found. LRIG2 and LRIG3 expression in tumor tissue showed no association with clinical patient data, tumor characteristics, or survival. But the study did show that high expression of LRIG1 significantly correlated with better survival. It was therefore concluded that LRIG1 may possibly serve as a prognostic marker for vaginal cancer in the future.

The third study investigated the dyskerin and WRAP53ß biomarkers. These two proteins are components in an enzyme that protects chromosomes from shortening and as such, may play a role in the development of cancer. Expression of these two proteins was evaluated by immunohistochemical staining in 69 tumors. Low to moderate expression of dyskerin was found in most of these tumors, but those showing high dyskerin expression were associated with significantly worse prognosis, as well as with worse overall and cancer-specific survival. Although WRAP53ß was expressed by most tumors, no correlation with clinical manifestations or prognosis was found.

The fourth and final study aimed to assess the immunological response to tumors by analyzing them for infiltration of white blood cells, specifically CD4+ T cells (T-helper cells) and CD8+ T cells (killer or cytotoxic T cells). These T cells provide a defense against viruses and bacteria, but also against tumor cells. It has previously been shown that high infiltration of T cells into tumor tissue is important for slowing tumor development and may contribute to better survival in various cancers. In this study, we wanted to test the hypothesis that high tumor infiltration of CD4+ and CD8+ T cells is associated with more favorable clinical variables and tumor characteristics, as well as with HPV-positivity, p16 expression, and better prognosis. CD4+ and CD8+ T cell infiltration was higher in tumors that were both HPV-positive and p16-positive. Patients with tumors having high T cell infiltration appeared to have a better survival rate, an association that reached statistical significance in regard to tumors that displayed both high p16 expression and high CD8+ T

cell infiltration. The strongest statistically significant association found in this study was that of worse survival among patients with tumors that were both HPV-negative and lacking in expression of p16.
In conclusion, the goal of this thesis was to explore how HPV status and other biomarkers in vaginal cancer relate to survival. We found the most interesting markers for further study to be p16, LRIG1, dyskerin, and CD8+ T cells. The combination of HPV status and p16 expression emerge as clinically relevant for prognosis in vaginal cancer. Expanding our knowledge of prognostic markers may enhance our understanding of how vaginal cancer develops and progresses, ultimately leading to improved diagnostics and treatment options.

# ABSTRACT

The overall aim of this PhD thesis is to evaluate HPV status and immunohistochemical expression of different biomarkers, including tumor suppressor p16, proliferation marker Ki67, molecular markers in the LRIG family, WRAP53ß, and dyskerin as well as immune markers CD4+ and CD8+ TILs, and their correlation to clinical manifestations and survival as part of a search for potential prognostic factors in women diagnosed with primary vaginal cancer.

**Paper I** evaluates the presence of HPV in vaginal cancer tumor samples, as well as immunohistochemical expression of p16 and Ki-67. This study includes 68 short-term and long-term survivors diagnosed with vaginal cancer. The results, which have been correlated with both clinical parameters and survival, show presence of HPV in 43% of patients, with HPV16 found in 63% of the HPV-positive cases. HPV-positivity did not correlate with improved survival but did correlate with low histopathological grade. High expression of p16 was found in 54% of cases and correlated with low histopathological grade ($p=0.004$), HPV-positivity ($p=0.032$) and long-term survival ($p=0.047$). High expression of Ki-67, found in only 34% of patients, correlated negatively with histopathological grade ($p<0.001$) and tumor size ($p=0.047$). The results suggest that evaluation of p16 and Ki67 may be of value in tumor grading, while expression of p16 may also serve as a possible marker for HPV-positivity. In this study, high p16 expression, in contrast with positive HPV status and presence of Ki-67, was associated with improved survival in the univariate analysis, whereas multivariate analysis indicated that only histopathological grade and tumor size remain as independent prognostic factors.

**Paper II** focuses on the LRIG (leucine-rich repeats and immunoglobulin-like domains) proteins – LRIG1, LRIG2 and LRIG3. Expression of these three proteins is often altered in cancer and has significance for cancer progression. The LRIG1 protein acts as a tumor suppressor, while the function of the remaining two is still unclear. We evaluated immunohistochemical expression of LRIG1, LRIG2, and LRIG3 in tumor samples from a cohort of 70 patients, diagnosed with vaginal cancer between 1975 and 2002, in order to find out whether such expression relates to clinical manifestations and survival. Our results show high (>50% of the cells) expression of LRIG1 and LRIG2 in 72% of tumors, but conversely, little or no expression of LRIG3. The latter two markers did not correlate with any clinical manifestations or survival, while high expression of LRIG1 correlated with HPV positivity and with improved cancer-specific survival (HR 0.35: 95% CI 0.68-0.73) in vaginal cancer patients.

**Paper III** addresses the molecular factors dyskerin and WRAP53ß in vaginal cancer. These two proteins play a role in the telomerase holoenzyme complex and are upregulated in different cancers. Expression of dyskerin and WRAP53ß was assessed by immunohistochemistry in 68 tumor samples drawn from the same study population as in study II. Most of the tumor samples demonstrated low to moderate expression of dyskerin. This protein is associated with shorter survival time and worse cancer-specific survival (HR 3.701: CI 95% (1.094-12.517). WRAP53ß was also expressed in most cells from the tumor samples, albeit without any association to clinical manifestations or prognosis.

**Paper IV** is concerned with immune response as it relates to presence of CD4+ (Tumor Infiltrating Lymphocyte) TILs and CD8+ TILs in vaginal cancer tumor samples and also addresses the potential association between TILs, p16 expression and HPV status with clinical manifestations and survival. Once again, immunohistochemistry staining was used to evaluate CD4+ and CD8+ TIL infiltration along with p16 expression in 69 tumor samples from the same study cohort used for the two previous studies. The results

showed higher density CD4+ and CD8+ TIL infiltration in both HPV-positive and p16-positive tumors. High infiltration of CD4+ and CD8+ TILs in tumor samples implies better prognosis. Tumors demonstrating p16 overexpression in addition to high CD8+ TIL infiltration were associated with statistically significant ($p=0.033$) improvement in prognosis. In contrast, absence of p16 in HPV-negative tumors correlates with a substantially worse prognosis ($p= 0.010$).

In summary, the studies in this thesis, which is concerned with exploring potential prognostic markers in vaginal cancer, identify p16 as a prognostic marker of interest, especially when considered in light of HPV status. Moreover, LRIG1 and dyskerin may be novel prognostic markers of potential interest, while LRIG2, LRIG3, and WRAP53ß appear to fall short in this regard. Furthermore, CD8+ TILs may also be of interest as a prognostic factor, especially when considered together with HPV status and p16 expression. Although this thesis implicates p16 expression together with HPV status as clinically relevant prognostic factors in vaginal cancer, future studies, using larger study cohorts, will be needed to validate these results for improved diagnostics and treatment strategies for women diagnosed with vaginal cancer.

# LIST OF SCIENTIFIC PAPERS

I Hellman K, Lindquist D, Ranhem C, Wilander E, Andersson S. **Human papillomavirus, p16(INK4A), and Ki-67 in relation to clinicopathological variables and survival in primary carcinoma of the vagina.** British journal of cancer. 2014;110(6):1561-70.

II Rannhem C, Lillsunde Larsson G, Hedman H, Lindquist D, Karlsson MG, Hellström AC, Östensson E, Sorbe B, Hellman K, Andersson E. **Expression of LRIG proteins as possible prognostic factors in primary vaginal carcinoma.** PloS one. 2017;12(8):e0183816.

III Ranhem C, Larsson GL, Lindqvist D, Sorbe B, Karlsson MG, Farnebo M, Hellman K, Kovalevksa L, Kashuba E, Andersson S. **Evaluation of dyskerin expression and the Cajal body protein WRAP53β as potential prognostic markers for patients with primary vaginal carcinoma.** Oncol Lett. 2022;23(1):30.

IV Ranhem C, Larsson GL, Näsman A, Hellman K, Kashuba E, Andersson S. **High p16 expression, presence of HPV and elevated number of infiltrated lymphocytes indicate an improved prognosis in vaginal carcinoma.** *Manuscript*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AJCC | American Joint Committee on Cancer |
| CAR-T | Chimeric antigen receptor |
| CDK | Cyclin-dependent kinases |
| CD4 | Cluster of differentiation 4 |
| CD8 | Cluster of differentiation 8 |
| CIN | Cervical intraepithelial neoplasia |
| CT | Computer tomography |
| DNA | Deoxyribonucleic acid |
| EBRT | External beam radiotherapy |
| EGFR | Epidermal growth factor receptor |
| FFPE | Formalin-fixed paraffin-embedded |
| FIGO | International Federation of Gynecology and Obstetrics |
| HIF | Hypoxia-Inducible Factor |
| HPV | Human papillomavirus |
| HSIL | High grade intraepithelial lesion |
| hTERC | Human telomerase RNA component |
| hTERT | Human telomerase reverse transcriptase |
| hTR | Human telomerase RNA |
| IARC | International Agency for Research on Cancer |
| IFNγ | Interferon-γ |
| LRIG | Leucine-rich repeats and immunoglobuline-like domains |
| LCR | Long control region |
| MHC | Major histocompatibility complex |
| MRI | Magnetic Resonance Imaging |
| PCR | Polymerase Chain Reaction |
| PET | Positron emission tomography |
| pRB | Retinoblastoma protein |
| PVC | Primary vaginal carcinoma |
| Rb | Retinoblastoma |
| RNA | Ribonucleic acid |
| SMN | Survival of motor neuron |
| snRNP | Small nuclear ribonucleoprotein |
| TIL | Tumor infiltrating lymphocyte |
| TNFα | Tumor necrosis factor-α |
| TNM | Tumour, nodal and metastasis |
| VAIN | Vaginal intraepithelial neoplasia |
| VEGF | Vascular endothelial growth factor |
| VLP | Virus like particle |
| WHO | World Health Organization |
| WRAP53ß | WD40 encoding RNA Antisense to p53 |

# PREFACE

The idea to have my thesis based on an HPV-related gynecological malignancy was a result of my clinical work at a Women´s Department at a County hospital in Sweden. As a specialist in Obstetrics and Gynecology, I have had the opportunity to participate in the engaging implementation of preventive strategies for cervical cancer. I have also had the opportunity to meet women of every age, at every stage of gynecological cancer disease: premalignant disease, curable disease, and palliative stage of the disease. A few of them, I have followed through every step their progression of disease: from first outpatient consultation, through medical investigation, diagnosis, surgery, health controls, relapse, palliative treatment, complications of treatments, and on their way to hospice care. The countless aspects of a cancer disease, not within reach to encompass for a mere clinician, astounds me every day.

This thesis barely touches upon a few aspects of cancer cell biology, such as viral oncogenesis, molecular processes and cancer immunology, and their relation to prognosis in vaginal cancer. Hopefully, I have learnt a little more for continued clinical work with patients that I am so grateful to meet and for future research collaborations within the field of gynecological cancers.

# 1 BACKGROUND

## 1.1 PRIMARY VAGINAL CARCINOMA

### 1.1.1 Definition and classification

Primary vaginal carcinoma is defined as tumor growth from a primary site originating in the vagina. Tumors involving the cervix, vulva, or urethra are differently classified. Moreover, an invasive squamous cell carcinoma in the vagina arising more than 5 years after diagnosis of cervical cancer, with similar histology, will be regarded as a new primary cancer (1).

Squamous cell carcinoma is the most common histological type of invasive malignancy in the vagina (80%-90%), presenting as a moderately differentiated, non-keratinizing usual-type squamous cell carcinoma. Most often they are exophytic, ulcerating tumors; an endophytic growth pattern is less common. Adenocarcinomas, such as clear cell adenocarcinoma, occur at a significantly lower incidence, while cancers of non-epithelial cell origin, such as mesenchymal, melanocytic, lymphoid, or secondary types, are very rare (2). It is noteworthy that vaginal metastases are much more common than primary vaginal cancer. Approximately 80-90% of all vaginal neoplastic lesions are metastases that usually result from invasion by tumors from adjacent organs such as the vulva, cervix, endometrium, ovary, bladder, and rectum (3).

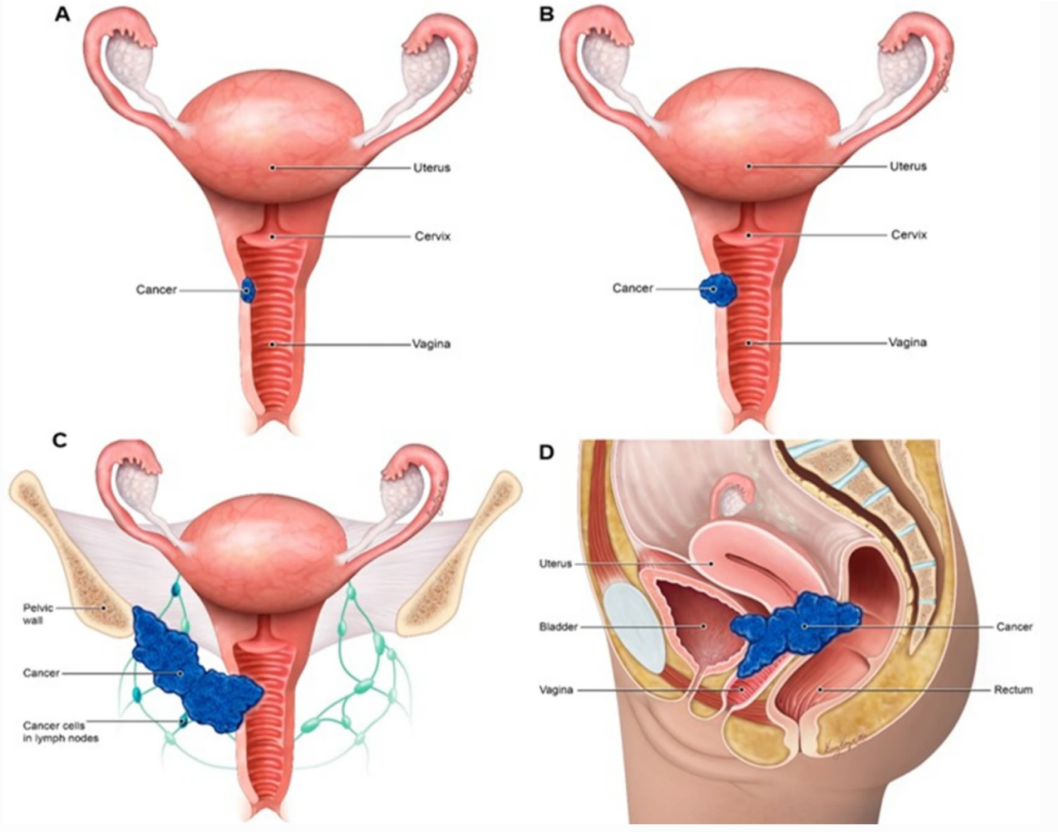



*Figure 1*. FIGO Staging of Vaginal Tumors. (A) Limited to vaginal wall (Stage I). (B) Beyond vaginal wall (Stage II). (C) Extends to the pelvis side wall (Stage III). (D) Direct extension into bladder and/or rectum (Stage IVa) (4). Reprinted by permission from Springer Nature; Stein, R., Ganeshan, D., Gopireddy, D.R. *et al.* Current update on vaginal malignancies. *Abdom Radiol* **46,** 5353–5368 (2021).

The most common site of vaginal cancer is the proximal third of the vagina, while approximately 20-25% will involve the lower third and 20% the entire vagina (5, 6). The disease primarily spreads by direct extension to adjacent organs such as the bladder, urethra, or rectum, or indirectly via lymph node metastasis. Lymphatic spread of vaginal cancer is dependent on tumor location. Lymphatic drainage of the lower third of the vagina is to the inguinal nodes, while the upper two-thirds drain to the pelvic lymph nodes. Distant metastases are less frequent, but may include the lungs, liver, and skeleton (1). The most common classification system used for staging in vaginal cancer is the International Federation of Gynecology and Obstetrics (FIGO) system (Figure 1) (7), which is primarily based on clinical parameters such as physical exam, biopsy, and diagnostic radiology (1, 8). The American Joint Committee on Cancer (AJCC) Staging Classification combines FIGO staging with the TNM (tumor size, node involvement, distant metastasis) system, the latter of which also takes into account histological assessment of the specimen, including surgical lymph nodes (Table 1) (9).

| FIGO stage | AJCC stage | Stage grouping | Stage description |
|---|---|---|---|
| Stage I | | | The carcinoma is limited to the vaginal wall. |
| | IA | T1a<br>N0<br>M0 | Tumor size ≤2 cm but confined to the vagina (T1a)<br>Tumor size >2 cm but confined to the vagina (T1a) |
| | IB | T1b<br>N0<br>M0 | |
| Stage II | | | Tumor size ≤2 cm and is beyond the vaginal wall without pelvis sidewall involvement (T2a)<br>Tumor size > 2 cm and is beyond the vaginal wall without pelvis sidewall involvement (T2b) |
| | IIA | T2a<br>N0<br>M0 | |
| | IIB | T2b<br>N0<br>M0 | |
| Stage III | III | T1-3<br>N0-N1<br>M0 | The carcinoma has extended to the pelvic wall, extends into the lower 1/3 of the vagina, and/or causes hydronephrosis (T3).<br>Pelvic/inguinal nodal metastasis (N1). |
| Stage IVA | IVB | T4<br>Any N<br>M0 | Tumor invades bladder and/or rectal mucosa and/or direct extension beyond the pelvic wall.<br>Any nodal metastasis. |
| Stage IVB | | Any T<br>Any N<br>M12 | Spread to distant organs. |

*Table 1*: FIGO nomenclature and AJCC staging for vaginal carcinoma (2009) (7, 9).

### 1.1.2 Epidemiology

Vaginal cancer accounts for approximately 2-4% of all genital tract malignancies (10-13). The age-standardized incidence rate is 0.4 and the mortality rate is 0.2 per 100 000 women (14). Globally, the incidence is estimated at almost 18 000 cases annually, with the highest age-standardized incidence rate found in South-Central Asia, followed by southern part of Africa (15). Several Caribbean countries also have high incidence (16). Incidence in the United States is reportedly higher among the black and Hispanic populations than among white or Asian/Pacific Islander women (17).

The incidence in Sweden has been stable over the last 50 years at approximately 30-40 cases annually, with an estimated age-standardized rate of 0.7/100 000 women (crude rate) (18). Worldwide, however, there appears to be a slight increase in the total number of estimated cases (19). Given the increasing prevalence of high-risk human papillomavirus (HPV) infection, a trend toward an increased incidence of vaginal cancer may be anticipated, but considering its rarity, may be difficult to detect (16).

### 1.1.3 Etiology and risk factors

Since vaginal cancer is rare, the biology and progression of this disease has neither been well studied nor well understood. Vaginal cancer is among the cancers related to HPV infection (20). A majority of cases are associated with a history of HPV, with a prevalence between 43% and 78% in various studies (19, 21-24). Thus, HPV is less commonly found with vaginal cancer than in cervical cancer (>90%) but is still more common than in vulvar cancer (40%) (23, 24). The most common type of high-risk HPV is HPV16, followed by HPV18 (23-26). The relative contribution of HPV16/18 to vaginal cancer has been estimated at 64%, which is lower than in other HPV-related cancers (27).

Vaginal intraepithelial neoplasia (VAIN) is a dysplastic lesion of the vaginal epithelium, regarded as a precursor to vaginal cancer. It is graded into three stages (VAIN1-3), and is associated with an extremely high prevalence of HPV (23). High-grade VAIN (VAIN2/3) has potential for malignant progression, but in previous studies the risk is only between 2% and 12% (28-30). In one study of 1478 patients diagnosed with VAIN, 28 (1.9%) progressed to vaginal cancer (28). The dominant HPV type was HPV16 (85.7%), followed by HPV18.

Additional risk factors for vaginal cancer, related to persistent HPV infection, include prior cervical dysplasia and cervical cancer, for which as many as one fifth to one third of the vaginal cancer vaginal cancer patients had previously been treated (6, 26). A Swedish study showed that women treated for high grade squamous intraepithelial lesion (HSIL) of the cervix were at almost seven times the risk of developing vaginal cancer – a risk which decreased over time, but still remained 25 years after treatment (31). Today, the Swedish national screening program for cervical cancer recommends that women treated for high-grade cervical dysplasia should have lifelong cervical screening with both cytology and HPV analysis (32). Furthermore, women who underwent hysterectomy for prevalent cervical intraepithelial neoplasia (CIN) are at relatively higher risk long-term of being diagnosed with vaginal cancer due to persistent HPV infection (33, 34). Similarly, women with prior or prevalent HSIL or adenocarcinoma in situ of the cervix at time of hysterectomy should be monitored with cytology, with the aim of further reducing the number of vaginal cancers (32).

An etiologic pathway other than persistent HPV infection has been proposed (6, 35, 36). Although it is difficult to prove significant associations, several predisposing risk factors have been proposed. Late menarche, nulliparity and early menopause are more common in patients with vaginal cancer. Smoking, gynecological infections, prior hysterectomy for either benign or malignant indications, and previous pelvic radiation therapy are examples of risk factors for vaginal cancer (6). Prolapse with pessary treatment, vaginal surgery, and multiparity > 5 are also risk factors. In short, hormonal factors and vaginal trauma may be associated with a risk of developing vaginal cancer. It has been proposed that, in a setting of chronic inflammation, non-HPV-related squamous cell vaginal cancer p53 mutations are the driving oncogenic mechanism (37).

Few genomic and proteomic studies have been conducted. A high genomic instability with a pattern of genomic alterations similar to that seen in advanced cervical cancer has been demonstrated (38). A proteomic study on tissue-specific protein markers found in vaginal cancer, cervical cancer, and normal vaginal tissue identified three proteins that were uniquely altered in vaginal cancer, while different proteins were similarly altered in both vaginal and cervical cancers (39). These findings support previous evidence of a common etiologic pathway. In summary, risk factors and etiology in vaginal cancer are often linked to those of cervical

cancer by a number of disease similarities. Still, it is important to note that cervical cancer and vaginal cancer affect different age groups.

### 1.1.4 Diagnosis, treatment and prognosis

Vaginal cancer most commonly affects postmenopausal women at a median age of 63-67 years (6, 40). Most patients present with symptoms such as vaginal bleeding, discharge, or dysuria (41). Diagnosis is confirmed by biopsy of the tumor lesion. It is important to distinguish between vaginal cancer and metastatic lesions from other organs. Clinical staging of the lesions is based on examination with the patient under anesthesia. Tumor spread and staging are established by abdominal and thoracic computer tomography (CT), as well as by magnetic resonance imaging (MRI) of the pelvis (42)**.** Positron emission tomography **(**PET)-CT is superior to other imaging modalities for detecting spread of vaginal cancer to lymph nodes and for detecting recurrent disease (8).

When vaginal tumors occur in close proximity to adjacent pelvic organs, such as the urethra, bladder, and rectum, surgical treatment has a limited role. Primary surgical treatment may be an option in selected cases, especially for stage I disease involving small lesions (less than 2 cm) confined to the vaginal mucosa, or in a few very specific situations beyond stage I (8, 43).

Radiotherapy has been and remains the treatment of choice for vaginal cancer. A wide variety of techniques and dosages involving a combination of EBRT and interstitial brachytherapy are used by different institutions to treat stage II-IV disease, depending on tumor location and size (44). The optimal or lower threshold dose is 70 Gy, while doses higher than 70 Gy may result in significant grade 3 and 4 toxicities (5). EBRT to the pelvis includes the external iliac and obturator lymph nodes. Should the tumor be located in the lower third of the vagina, the inguinal nodes may be included. Brachytherapy, used to deliver a boost of high-dose radiation to the residual tumor, is associated with longer overall survival (45). Image-guided or adaptive brachytherapy with CT or MRI offers the potential to administer higher doses of radiation to the residual tumor, while decreasing the radiation dose to surrounding healthy tissues (46). More advanced stages require a more individualized treatment plan (1).
The use of chemotherapy has greatly increased over the past two decades based on knowledge gained from small retrospective studies and extrapolated from treatment of cervical cancer. Use of cisplatin-based chemotherapy is similar to treatment of cervical cancer but has been expanded to include palliative treatment of stage IV and recurrent disease. Concurrent chemoradiation has been shown to be an independent prognostic factor for improved survival (47, 48).

Five-year relative survival is estimated to be 41%-70% (16, 49, 50). When compared with other lower genital tract cancers, vaginal cancer appears to be associated with lower survival rates (17, 50-53). Notably, reported survival rates vary considerably depending on region, stage, and time period. Although some studies have shown that survival has improved over time (54, 55), other studies report no such improvement (49, 56, 57). Five-year overall survival rates differ by country: 74%-78% in stage I, 52%-69% in stage II, 43%-68% in stage III and less than 20%-47% in stage IV (58, 59). A recent study reported 58% disease-specific 5-year survival (60), while a different series reported disease-specific survival rates ranging from 76%-85 % in stage

I, 62%-84% in stage II, 57%-75% in stage III, and 22%-65% in stage IV (57, 59, 60). Recurrent disease is associated with an extremely poor prognosis, with 5-year survival rates reported to be 12%-15% (6).

## 1.2 PROGNOSIS AND PROGNOSTIC STUDIES

The word "prognosis" is borrowed from the ancient Greek (pro-, "before" and gnôsis, "foreknowledge, perceiving beforehand, prediction"), referring to a forecast of the future, a prediction, or estimation of a probability of future events or conditions. Along with diagnosis and treatment, prognosis is at the core of medicine, since it relates to likely future health outcomes, including complications, disease progression, or death, based on the individual patient's risk profile, condition, circumstances, treatment, and additional factors. In clinical practice involving care of cancer patients, prognosis is required in order to make predictions and to stratify patients into different groups to facilitate decisions concerning treatment strategies and to aid in patient counseling.

The evolving field of personalized medicine, which relies on individual prognostic and predictive markers, is playing an increasingly important role in cancer prevention, diagnosis, and treatment. Through use of molecular analysis, personalized medicine is gradually replacing the prevailing organ-centric concept of cancer diagnostics and therapeutics. This new approach is becoming increasingly more integrated into routine clinical practice, as illustrated by the introduction of individualized, molecular targeted therapies, which offer the benefits of increased efficacy and/or reduced toxicity.

Prognostic factors may be classified into three groups – host-related, environment-related, and tumor-related (61). Tumor-related prognostic factors concern tumor pathology, anatomic disease extent, and tumor biology. The last factor relates to translational research, a field currently undergoing explosive expansion as a result of advances in high throughput "omics" techniques, including development of tools such as next-generation sequencing and ribonucleic acid (RNA) sequencing, which now allow for simultaneous detection and evaluation of both mutations and gene expression. However, how to apply this information for the benefit of patients still remains largely unsolved.

Identification of a new prognostic marker involves different phases of studies (62, 63). As a start, exploratory studies are performed, aimed at identifying novel prognostic markers and formulating new hypotheses. Exploratory research may help to define the natural history of a disease, but in some cases, it may also help to clarify causal relationships related to the disease (61, 64). This initial phase is followed by assay development and qualification to help identify markers in clinical samples. Further exploratory investigations assess the values of prognostic markers in stratifying patients based on degree of risk for progression of disease or death. Ultimately, confirmatory studies are needed as a complement to identify important markers and to validate prior findings. Regrettably, exploratory studies far outnumber confirmatory studies, largely because the latter are saddled with requirements for reproducibility pertaining to clinical practice, including the need for widely available quality controls related to the prespecified marker, as well as larger sample sizes to increase study power and a requirement to confirm that the novel marker is associated with a uniquely independent capability that exceeds available pre-existing clinical markers.

Another concern relating to prognostic research is the paucity of prospective studies. Retrospective studies allow for identification of a cohort with a defined follow-up time to assess for a number of outcome events, such as deaths or recurrences. However, retrospective studies are associated with several limitations, including uncertainty concerning whether the study cohort is complete and correctly defined, lack of standardization of diagnostic and therapeutic procedures, as well as often incomplete baseline and follow-up data.

Translational research has long focused on a single biomarker to identify putative prognostic factors. However, given the limitations concerning single genes, which are rarely capable of providing an accurate estimate of prognosis, the need for multivariate research is obvious. Prognostic models, which entail different combinations of variables, are under study to provide outcome probabilities for different combinations of prognostic factors. However, as more variables come under study, demands increase for a larger study population and number of events to scrutinize (65).

Systematic reviews and meta-analyses of prognostic studies may serve as important tools to synthesize study findings based on small sample sizes and inconsistent results. It is important to consider that several methodological problems pertaining to prognostic marker studies may result in bias, which will be reflected in the scientific literature. Such problems may include selectively or non-reported information, issues related to study quality, and a focus on finding and reporting only statistically significant results.

In an effort to address the research difficulties encountered in the field of prognostic markers, the REMARK guidelines for reporting tumor marker studies were presented in 2005 (66). With a checklist of twenty items pertaining to methodology (including study design, study procedures, and analysis of tumor markers), the guidelines provide a comprehensive framework for prognostic studies relating to tumor markers. The guidelines are aimed at more transparent and complete reporting, including both strengths and weaknesses, to enable readers to assess the quality and relevance of tumor markers. However, the difficulties encountered when conducting prognostic studies still remain, for which reason many prognostic studies fail to comply with guideline recommendations (67).

The above summary describing the complexities of prognostic research serves to illustrate the limitations of the studies included in this thesis, and may also contextualize the challenges of translational research, as well as cancer management and treatment.

## 1.3 PROGNOSTIC FACTORS IN VAGINAL CANCER

Since vaginal cancer is rare, knowledge about this disease and its prognostic factors are mainly based on retrospective studies with relatively small study populations. Important factors that impact survival include advanced stage of disease and old age (5, 40, 57, 68), but tumor size >4 cm, although not included in FIGO staging, has repeatedly been shown to be significant (41, 57, 68). Tumor location in the upper third of the vagina has been associated with significantly better prognosis (69, 70), although research findings are divided on this point (71). Patients with inguinal lymph node involvement at time of diagnosis had worse 5-year disease-free survival (33.3%) than patients without lymph node involvement (55.5%) (72).

Additional factors that have come under investigation include morphology and lymphatic involvement, both of which have been associated with vaginal cancer prognosis. Adenocarcinoma of the vagina has a worse prognosis than squamous cell carcinoma (73). Studies are inconsistent concerning the importance of parameters such as histological grade, anemia, and prior hysterectomy (44).

Biomarkers that have been studied and proposed as holding significance for prognosis include p16, Ki67, p53, p21, cyclin A, E-cadherin, laminin-5 expression, EGFR, and VEGF (44). With the exception of p16 as will be discussed further below, the results regarding these biomarkers are inconsistent or insignificant (22, 44, 74). One study involving 72 vaginal cancer patients showed that all tumors contained DNA aneuploidy, though the value of this finding in clinical assessment is uncertain (75).

## 1.4 PROGNOSTIC FACTORS OF THIS THESIS

Biomarkers are biological factors related to biological and pathogenic processes and/or to response to treatment that may be measured accurately and reproducibly. When searching for prognostic factors related to cancer, it is reasonable to investigate the biomarkers involved in its various processes. However, carcinogenic processes are notoriously multifaceted and heterogenic, and biomarker research is vast.

In 2000, Hanahan and Weinberg organized the processes of cancer formation by rationalizing their complexities into six underlying principles, now known as the "hallmarks of cancer" (76). The list of capabilities that cells must acquire in their stepwise journey toward cancer transformation was elaborated in 2011 and includes the following: (1) sustained proliferative signals, (2) evasion of growth suppressors, (3) resistance to cell death, (4) replicative immortality, (5) induction of angiogenesis, (6) activation of invasion and metastasis, (7) avoidance of immune destruction, (8) deregulation of cellular energetics, (9) genome instability and mutation, and (10) tumor-promoting inflammation (77). In this thesis we have focused on a number of factors involved in the "hallmarks of cancer" and investigated their potential as prognostic factors in vaginal cancer (Figure 2).

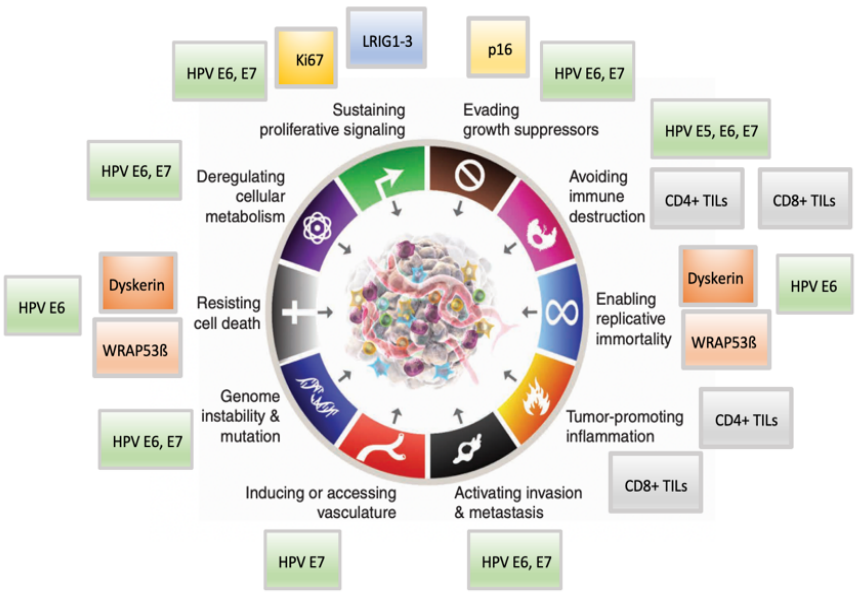



*Figure 2.* Prognostic factors investigated in this thesis and their relation to the hallmarks of cancer (78-81). Reprinted by permission from Elsevier. Douglas Hanahan, Robert A. Weinberg. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar 4;144(5):646-74.

## 1.4.1 Human papillomavirus

### 1.4.1.1 HPV genome and classification

HPV is a small, circular double-stranded capsid-containing DNA virus of icosahedral symmetry, which is structured into three regions having different functions in the viral life cycle: (1) the long control region (LCR) regulates viral gene expression and replication; (2) the early (E) region, which encodes proteins E1, E2, E4, E5 and the oncoproteins E6 and E7, also essential for viral gene expression and replication, as well as for survival; (3) the late (L) region, which encodes the viral capsid proteins L1 and L2 that are responsible for maturation and assembly of the virus particle (82).

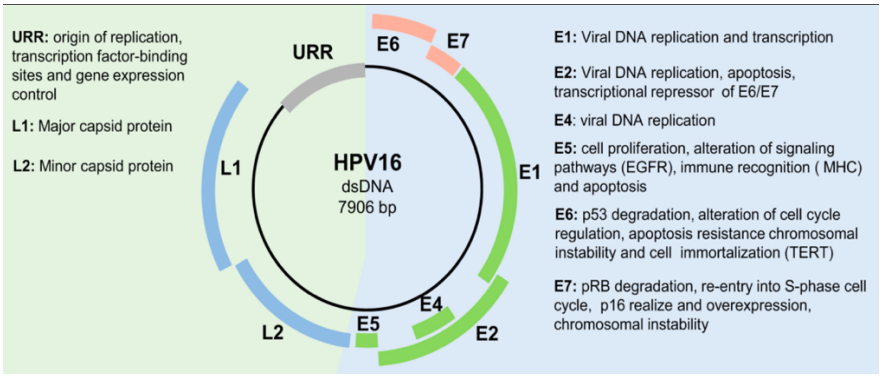



*Figure 3*. HPV16 and structure and viral proteins (83). Reprinted by permission from Elsevier. de Sanjosé S, Brotons M, Pavón MA. The natural history of human papillomavirus infection. Best Pract Res Clin Obstet Gynaecol. 2018 Feb;47:2-13.

HPV belongs to the *Papillomaviridae* family. Classification of HPV is based on DNA sequence homology of the L1 gene, which is the most conserved region of the genome (84). Today, over 220 different HPV types are known and new types are continually being identified. The *Papillomaviridae* phylogenetic tree is subdivided into five genera (alpha, beta, gamma, my and nu). HPV behavior and carcinogenic potential correlate with the phylogenetic genera. The alpha-genus, which infects mucosa, contains all 13 carcinogenic HPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 (82, 84). The most prevalent type, also having the strongest carcinogenic potential, is HPV16, followed by HPV18 (20).

### 1.4.1.2 HPV infection

Genital HPV infection is the most common sexually transmitted infection worldwide (85). The global burden of HPV-related cancer is estimated at about 4.5% (630 000) of all new cancer cases (19). Vaginal cancer accounts for approximately 1.6% of all HPV-related cancer in women (24). Other cancers attributable to HPV infection have received more attention in recent years. The 2009 International Agency for Research on Cancer (IARC) list of cancers for which there is evidence of HPV as a causative factor includes vulva, vagina, anus, penis, and oropharynx (86).

HPV preventive strategies, including HPV-based cervical cancer screening and HPV vaccination programs, are expected to reduce the global burden of HPV-related disease and cancer in the coming decades. It is worth noting that vaginal cancer is among the cancers diagnosed at the oldest median age, for which reason a decrease in the number of cancers following screening and vaccination may not be realized for many decades. (87, 88).

HPV infection is most common among women <25 years and declines with increasing age, although on many continents there is an unexplained second peak in HPV prevalence among middle-aged women (89, 90). In general, HPV infection clears within 12-24 months as a result of cell-mediated immunity response (83, 91). During the interim, the virus is maintained in the host and replicates as an episome, progressing through a normal viral life cycle.

However, 10%-20% of women fail to clear the virus, at which point the HPV infection becomes persistent (83, 89) and the viral genome often integrates into the human genome, resulting in an accumulation of genetic and epigenetic events (92, 93). Integration of the viral genome has been suggested as an important, albeit not a prerequisite, step for progression to invasive cervical cancer (82, 94). The published literature is consistent and it is now widely accepted that HPV genome integration occurs in approximately 90% of cervical cancer cases.

When HPV integrates the host cell genome in squamous cells, the result is over-expression of E6 and E7 proteins. These proteins interact with a number of specific cellular proteins to initiate neoplastic transformation, through inhibition of essential steps in the human cell cycle (Figure 4). The E6 protein affects the tumor suppressor protein p53, which normally mediates cell cycle arrest, and inhibits apoptosis (95, 96). The E7 protein binds to pRB (retinoblastoma protein), another tumor suppressor protein, thereby blocking binding of the latter to E2F. Through a series of steps this results in up-regulation of cyclin-dependent kinases (CDK)4 and CDK6. In turn, they cause the inactivation of pRB, allowing activation of genes necessary for cell cycle progression. In other words, the activity of E6 and E7 appears to in vivo studies result in a number of cellular events that contribute to an accumulation of genetic abnormalities and cancer development through immortalization, inhibition of DNA damage response, genomic instability and inhibition of differentiation (96). HPV integration may also disrupt tumor suppressor genes or be localized at fragile sites for DNA breakage which may be linked to cancer development (97, 98).

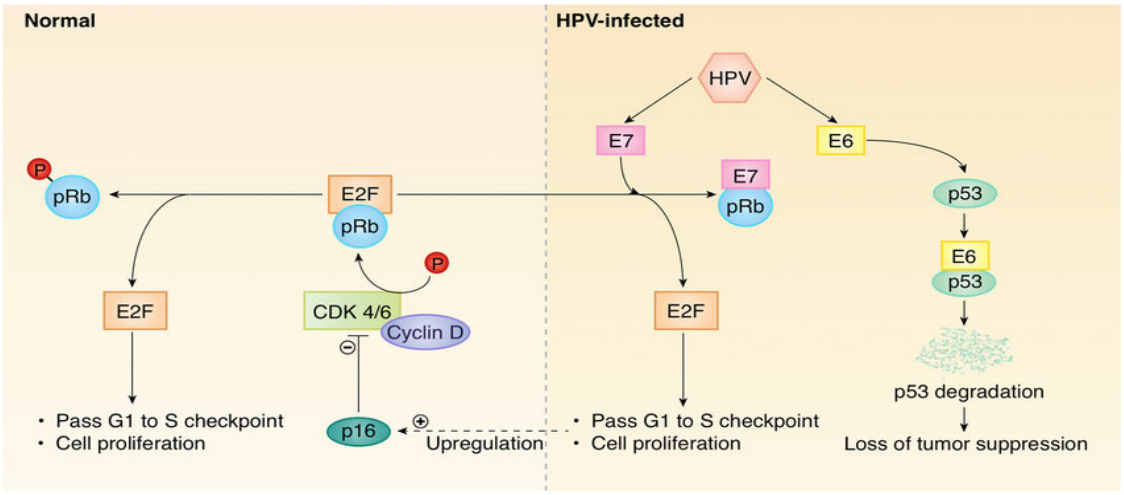



*Figure 4.* Increase of p16 expression in HPV-positive cancers (99). Reprinted from Wai KC, *et al*. Molecular Diagnostics in Human Papillomavirus-Related Head and Neck Squamous Cell Carcinoma. Cells. 2020 Feb 22;9(2):500.

Little is known about the specific characteristics of the epithelium at sites that are vulnerable to infection with oncogenic HPV types, but this knowledge gap has been most studied in the transformation zone of the cervix

where the stratified epithelium of the ectocervix meets the columnar epithelium of the endocervix, an area known to be susceptible to HPV infection. An updated model considers how HPV infection targets cervical reserve cells, a specialized form of stem cells. Depending on where in the cervical epithelium HPV infection occurs, three different routes of progression, each associated with a different risk of cancer, may occur; however, the risk of persistent infection and deregulation of viral gene expression may be present in all three scenarios (100). When infection occurs in stratified epithelial sites outside the transformation zone, it is unclear whether infection of a reserve cell is required for persistent infection, or whether this is caused by HPV-encoded genes that modify the infected basal cell (101).

Yet one other model has been proposed to explain development of HPV-related multicentric disease of the lower genital tract that considers three hypothetical pathogenic pathways (Figure 5) (102). These pathways relate to: (A) independently transformed cell clones, characterized by synchronous lesions, identical HPV type, and different levels of oncogene expression; (B) susceptibility to HPV infection through repeated infections with different HPV types and frequently deregulated oncogene expression; or (C) clonal lesions stemming from an initial cervical lesion, with development of metachronous lesions with aberrant oncogene expression caused by an identical HPV type, although some cases may be explained by clonal propagation of already transformed cervical cells.
In summary, it would appear that HPV-related cancers may depend on a variety of HPV types and their deregulation of genes, in addition to the site of epithelial origin, but our knowledge remains limited.

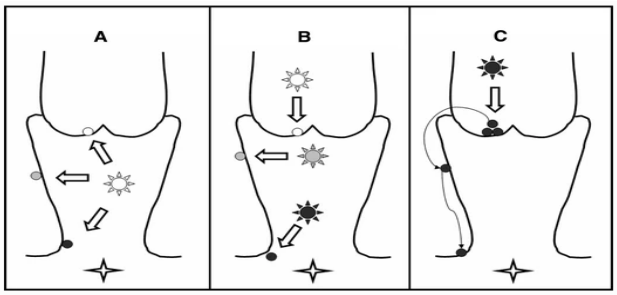



*Figure 5.* Hypothetical pathways for development of multicentric intraepithelial lesions of the lower genital tract (102). Reprinted by permission from Springer Nature. Hampl M, *et al*. Comprehensive analysis of 130 multicentric intraepithelial female lower genital tract lesions by HPV typing and p16 expression profile. J Cancer Res Clin Oncol. 2007 Apr;133(4):235-45.

#### 1.4.1.3 HPV and immune response

Although most HPV infections are cleared within 1-2 years, the nature of the HPV life cycle allows for infections to remain undetected by the immune system and thereby become persistent, with an attendant risk for development of cancer. HPV is recognized for strategies to evade the adaptive immune system, which allows for latency and completion of the viral replication cycle (101, 103).
The HPV life cycle is localized to the keratinocytes of the epithelium where immune cells are scarce, rather than infiltrating through the basal membrane to stroma, where immune cells are more abundant by comparison. HPV remains an intra-epithelial pathogen throughout its life cycle without a viremic phase, well hidden from the host immune response. Low viral expression in the cells adjacent to the basal membrane significantly limits viral antigen presentation. HPV replication and assembly do not result in lytic cell death; instead, the keratinocytes involved in HPV replication are simply shed from the upper parts of the epithelium,

well away from immune activity. As a result, no inflammatory response with release of proinflammatory cytokines occurs to activate antigen-presenting cells.
HPV may also compromise the inherent defense mechanisms of keratinocytes by, for example, causing downregulation of pathogen recognition receptors and adhesion molecules in infected host cells that would otherwise normally induce an innate and adaptive immune response. HPV is endowed with mechanisms that inhibit interferon synthesis and signaling. Through altered gene expression and disturbed protein and cytokine functions, induction of antigen presenting cells is thwarted.
Nevertheless, HPV infection is cleared in 80%-90% of cases in response to cell-mediated immunity against early viral proteins, a mechanism involving accumulation of CD4+ and CD8+ T-cells in and around the lesion (101, 103, 104). An understanding of the immune system response to HPV infection and of the mechanisms leading to HPV persistence is crucial for understanding carcinogenesis, and for adoption of novel treatment strategies, including immunotherapy.

#### 1.4.1.4 HPV vaccination

HPV prophylactic vaccines are based on recombinant expression of the L1 gene and self-assembly into virus-like particles (VLP), without viral genome. Vaccine recipients produce high levels of neutralizing antibodies that prevent HPV from entering cells. The initial bivalent and quadrivalent vaccines registered in 2006 are gradually being replaced by a nonavalent vaccine covering HPV6, 11, 16, 18, 31, 33, 45, 52, and 58 (105). Vaccination programs are increasingly incorporating HPV vaccination of girls, which is gradually being extended to boys. However, as of June 2020, 55% of the World Health Organization's (WHO) 194 member states were a long way from meeting the 90% vaccination target (106). Encouragingly, the first studies containing real-world data have now been published, revealing the anticipated effectiveness of vaccination in terms of a substantial reduction of both high-grade cervical lesions in young women and incidence of cervical cancer (107-109). A decrease in high-grade VAIN among young women, likely attributable to vaccination, has already been described (54). Similarly, the anticipated effect of HPV vaccination on incidence rates of HPV-related cancers involving noncervical sites, including vaginal cancer, is also significant (110, 111).

### 1.4.2 Molecular markers as prognostic factors

#### 1.4.2.1 Tumor suppressor p16

Tumor suppressor protein p16 is frequently used as a biomarker in gynecological malignancies. This tumor protein is involved in cell-cycle regulation, which is tightly controlled in normal noninfected cells, while the cell cycle is upregulated in HPV-infected cells (112). Its mode of action is through suppression of cycline-dependent kinase activity (CDK). CDK regulates cell cycle progression and cell division by facilitating binding of pRb to transcription factor E2F, resulting in cell cycle arrest and attendant decrease in p16 expression.
In the case of HPV-infected cells, as previously described, the HPV DNA encodes for the E7 protein, which binds to and inactivates pRb, which results in continuous cell cycle progression and overexpression of p16 (Figure 4). Since oncogenic HPV infection causes accumulation of p16 in the cell nucleus (96, 113), overexpression of p16 is of interest as a potential marker for HPV-related cancers (114-116).
Studies on other squamous cell carcinomas have shown that p16 expression, as measured by immunohistochemistry, has high sensitivity and specificity for identification of HPV-positive tumors (36, 117, 118), which also makes it a useful biomarker for HPV-related oncogenic activity in the lower female

genital tract (36, 112, 113, 118). As a prognostic marker, p16 has been evaluated and demonstrated to be of significant value in cervical, vulvar, penile, anal, and oropharyngeal cancers (119-123). Concerning oropharyngeal cancers in particular, p16 has now become established as a significant clinically relevant marker for treatment decisions (124, 125).

Although immunohistochemical evaluation has shown that p16 can serve as a valuable surrogate marker for HPV-induced transformation in the cells of precancerous and cancerous tissues, it is important to note that immunohistochemical detection of p16 does not equate to evidence that HPV is the etiological factor since tumors may even overexpress p16 in the absence of HPV infection (126).

#### 1.4.2.2 Proliferation marker Ki67

Ki67 is an antigen widely used as a marker of cell proliferation, but its functions remain unclear. The MIB-1 antibody is a monoclonal antibody used in immunostaining for identification of the Ki67 antigen, expressed in the nuclei of mitotically active cells. It allows us to discriminate between proliferating and resting cells, since it is present in the active phases ($G_1$, $G_0$, $G_2$, and mitosis) of the cell cycle, but not in resting cells in $G_0$ phase (127). In the clinical setting, the Ki67 immunostaining pattern serves as a proliferation index within a cell population, where higher staining intensity indicates increased proliferation activity. Such activity is associated with an unfavorable clinical outcome, increased tumor size, and more advanced stage of disease in a number of different human cancers (75, 128).

Ki67 has proved to be valuable in clinical application in different gynaecological pre-cancerous lesions and cancers. Since HPV infection may induce cell proliferation in epithelium, increased expression of Ki67 may be regarded as an indicator of HPV infection (129). In cervical screening, dual staining of Ki67 and p16 in cytological low-grade lesions will detect high-grade lesions with equally high sensitivity and increased specificity compared to HPV testing (130). It is very often used in used in grading of cervical pre-cancerous lesions (131). Ki67 has also been suggested as a useful prognostic factor in early-stage endometrial cancer (132).

#### 1.4.2.3 LRIG1, LRIG2 and LRIG3

The LRIG (leucine-rich repeats and immunoglobulin-like domains) gene family, discovered in 2001, comprises LRIG1, LRIG2, and LRIG3. The LRIG genes encode transmembrane proteins involved in the regulation of growth factor signaling and cell proliferation. The human LRIG1 gene is localized to chromosome 3p14, the LRIG2 gene to 1p13.1, and LRIG3 gene to 12q14.1 (133). The structure of the corresponding proteins is similar with a transmembrane or luminal part containing fifteen leucine-rich-repeats and three immunoglobulin-like domains, a transmembrane glycoprotein, and a cytosolic domain which differs between the three members (Figure 6). A wide variety of human tissues express the three LRIG genes (134-136), but the subcellular localization of the LRIG proteins appears to be specific to different cell types (137). Nuclear, perinuclear, cytoplasmic, or plasma membrane localization may influence their function and thus have important clinical implications.

The LRIG proteins have been found to be regulators of growth factor signaling and cell proliferation through interaction with receptor tyrosine kinases and modulation of their signaling. However, the role of dysregulated LRIG proteins in the genesis and progression of human cancers remains controversial.

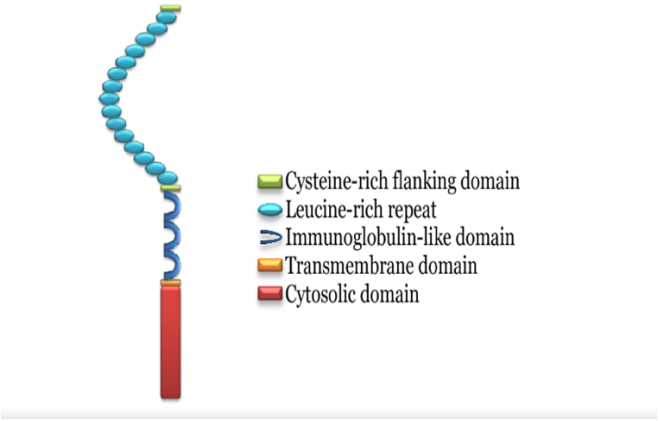



*Figure 6*. Characterization of LRIG protein (138).
Reprinted by permission from Eureka Science. Malik U, Javed A. LRIGs: A Prognostically Significant Family with Emerging Therapeutic Competence against Cancers. Curr Cancer Drug Targets. 2017;17(1):3-16.

LRIG1 protein has been proposed to have a tumor suppressor function (139). In a variety of different human cell types, LRIG1 inhibits cell proliferation in both normal and transformed cells. LRIG1 negatively regulates growth factor signaling mediated by several oncogenic receptor tyrosine kinases, acting by various mechanisms including ubiquitination, degradation, and destabilization, as well as through paracrine signaling, just to illustrate the complexity of its function (133, 140-143).
Although LRIG1 is highly expressed in specific cells within various human tissues (144), expression is downregulated in several human cancers, such as renal cell cancer (145), glioma (146) and breast cancer (147). However, certain cancers, including those involving breast, colorectum, and prostate, may display either up- or downregulation of LRIG1 (80, 148, 149). In light of its endogenous down-regulation of growth factor signaling, one might postulate that LRIG1 could be associated with improved survival in cancer. High expression of LRIG1 in tumor tissues, as measured through gene copy number, mRNA levels, or protein levels, has in fact been linked to better prognosis in breast cancer (80, 139, 150), skin cancer (151), non-small cell lung cancer (152), head and neck cancer (153), hepatocellular carcinoma (154), high-grade glioma, and malignant melanoma (155). LRIG1 also appears to increase sensitivity and/or reduce resistance in tumor cells toward chemotherapy (138), which suggests a possible future role for LRIG1 in cancer treatment.
To summarize, there is no consistency in LRIG1 expression among different cancers, which may possibly relate to subcellular protein localization, or specific protein interactions (137), but staging and cancer subtype are also certainly important (138).

Less is known about LRIG2 and LRIG3, with respect to both their function and to their impact on cancer prognosis (137, 156). The LRIG2 gene is localized to the 1p13 region, which is frequently deleted in various cancers (138). Although expressed in all tissues, expression is highest in the skin, ovary, and uterus (136). LRIG2 has been proposed to play a stimulatory role in regulation of the EGFR signaling pathway by increasing tyrosine kinase receptor signaling, which, through mitogenic activity, promotes downstream proliferation in glial cells (157). In skin, LRIG2 is associated with activated pro-oncogenic pathways that accelerate tumor progression *in vivo* (158). High LRIG2 expression is linked to better survival in meningioma (138), but with poor survival in other cancers such as oligodendroglioma (159), non-small cell lung carcinoma (160), and esophageal cancer (161). Thus, LRIG2 is thought to act as an oncoprotein, but as with LRIG1, both level of expression and subcellular protein localization of LRIG2 may determine its impact in cancer (162).

The mechanism of action of LRIG3 is not extensively studied. Diverse roles have been proposed; it is known to play a significant role in formation of the neural crest during embryonic development in Xenopus (163), as well as in morphogenetic formation of the lateral canals of the vestibular organ, and is also involved in regulation of heart function and blood cholesterol levels (138).
The effect of LRIG3 on clinical outcomes in cancer is not clear, but it appears to act as a tumor suppressor in most cancers that have been studied thus far (138). As an example, LRIG3 has been shown to be a potent tumor suppressor, with a role in invasion, proliferation, and apoptosis in glioblastoma cells (164). Again, the subcellular localization of this protein impacts survival in oligodendroglioma (162). LRIG3 interacts with, and possibly opposes, the function of LRIG1, which further illustrates the complex functions of the LRIG protein family in cells (165).
To conclude, prior studies show that LRIG1 and LRIG3 generally play a tumor-suppressive role, while LRIG2 usually functions as a tumor initiator.

#### 1.4.2.4 Dyskerin

Cellular immortality is a hallmark of cancer and tumor cells rely on the telomerase holoenzyme complex, where dyskerin is an important factor, for their unlimited replicative potential. Telomeres protect the end of the chromosome from DNA damage or from fusion with neighboring chromosomes. Telomerase compensates for the telomere loss that results from incomplete genome end replication by adding telomeric repeats of six-nucleotide repeating sequences (5'-TTAGGG) at chromosome ends. Replication in somatic cells stops when telomeres are critically short, but in 90% of cancer cells telomerase activity is reactivated and unregulated, resulting in continuous elongation of the telomeres, which permits continuous cell division and proliferative immortality (166). Telomerase activity is an important pathway in HPV-related oncogenic progression (167).

The telomerase holoenzyme complex comprises human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR), along with dyskerin (Figure 7). Dyskerin, along with small nucleolar RNAs (Nhp2, Nop10, and Gar1), is a member of the H/ACA family of ribonucleoproteins, which helps regulate protein stability and activity in the telomerase holoenzyme complex. WRAP53ß is also a member of this protein complex, as will be discussed in further detail below. Several additional factors (14-3-3, Hsp90, p23, pontin, reptin, NAT10, GNL3L, and hnRNPs) are associated with the telomerase holoenzyme complex and play transient roles in enzyme activity (168).

Dyskerin, a nucleolar protein encoded by the DKC1 gene at Xq28 and possessing pleiotropic functions, is involved in protein expression, growth, and proliferation. It interacts with hTERC by binding to hTR to help stabilize the telomerase complex. Dyskerin is also necessary for pre-RNA splicing and ribosome biogenesis (169). It catalyzes formation of pseudouridine (RNA modification by an isomer of the nucleoside uridine) in ribosomal and certain small RNAs (170, 171). Defective ribosomal uridine modification will result in a defect in translation of mRNAs, including those encoding for the antiapoptotic factors and for tumor suppressors p53 and p27 resulting in cell immortality and lack of tumor suppression (172-174).

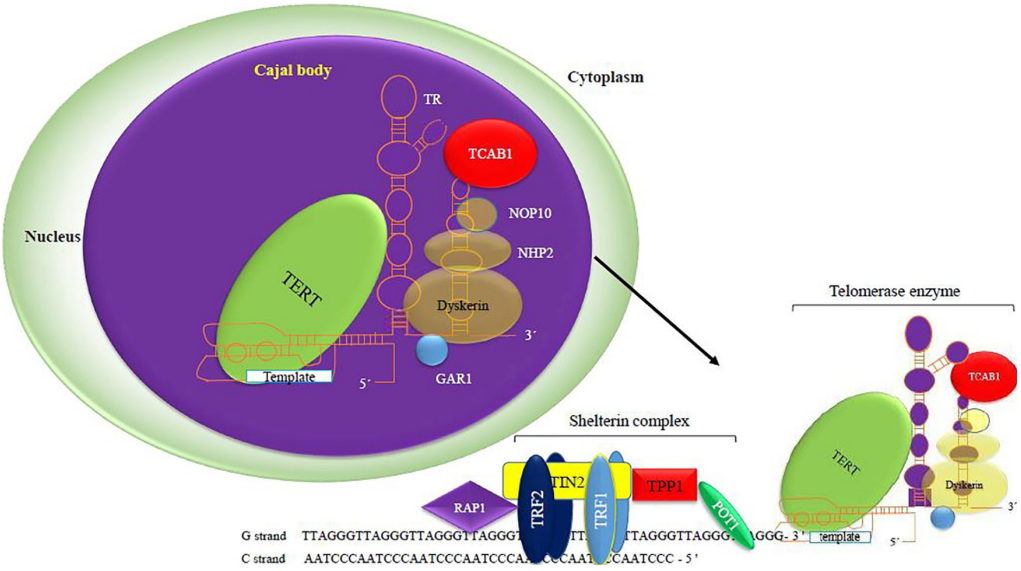



*Figure 7:* Schematic structure of the telomerase complex assembled in Cajal bodies including hTERT, hTERC, dyskerin, NOP10, NHP2, GAR1 and TCAB1/WRAP53ß (175). Reprinted by permission from Elsevier. Brazvan B *et al*. Telomerase activity and telomere on stem progeny senescence. Biomed Pharmacother. 2018 Jun;102:9-17.

Dyskerin expression and function affect aging, cell proliferation, bone marrow, and cancer development. Knowledge concerning dyskerin has been derived from studies on premature aging syndromes, such as Dyskeratosis Congenita, which is caused by mutations in the DKC1 genes. Moreover, ribosomal disorders may also be related to defective dyskerin expression or function. Patients with such conditions may be more susceptible to development or progression of cancer (176).

In view of these findings, it is not surprising that altered dyskerin expression has been linked with tumor formation and multiple types of cancer. Expression of dyskerin varies and is deregulated in various human tumors, where it has been proposed to act as a tumor suppressor in some instances, but also as a factor associated with poor prognosis in for example breast cancer, neuroblastoma, and hepatocellular carcinoma (174, 177, 178).

#### 1.4.2.5 WRAP53ß

The WRAP53 (**W**D40 encoding **R**NA **A**ntisense to **p53**) gene, also known as WDR79 or TCAB1, is linked to the pathogenesis of various cancers and to diseases like spinal muscular atrophy (179) and dyskeratosis congenita (180). As a natural p53 antisense transcript, WRAP53ß is located on the opposite DNA strand, with overlap to p53 in a head-to-head fashion and transcribed in the opposite direction in relation to p53 (Figure 8A). Three variant start exons (α, β, γ) result in three alternative transcripts of WRAP53: WRAP53α, WRAP53β, and WRAP53γ (function of the last one is unknown). WRAP53α, regulates p53 function in DNA repair. WRAP53ß is in focus of this thesis; it has emerged as a significant protein factor linked to various processes, one of which is its role in carcinogenic transformation (181).

WRAP53ß is involved in three diverse processes: (1) formation of nuclear organelles known as Cajal bodies, (2) telomere elongation and (3) DNA damage repair (Figure 8B-C). WRAP53β acts as a scaffold protein that facilitates simultaneous interaction with multiple proteins, which enables WRAP53β to carry out various functions independently of one another (78). WRAP53β directs factors to the Cajal bodies, which are nuclear

structures that enhance essential biological cellular processes by assembling the necessary factors. Maintenance and stabilization of Cajal bodies is one of the main functions. In similarity to dyskerin, WRAP53β is also an important component of the telomerase holoenzyme complex with a role in telomere elongation and telomerase trafficking (182). Finally, WRAP53β is also involved in DNA damage response and repair where, once again, it acts as a scaffold for DNA repair proteins, which stabilizes protein interactions at the site of DNA double strand breaks (183) .

It has been suggested that the subcellular localization of WRAP53β is integral to its normal function (81). It is localized to both the cytoplasm and the nucleus. Little is known about the subcellular trafficking mechanisms of WRAP53β, but it is suggested that deviant localization may cause dysfunction.

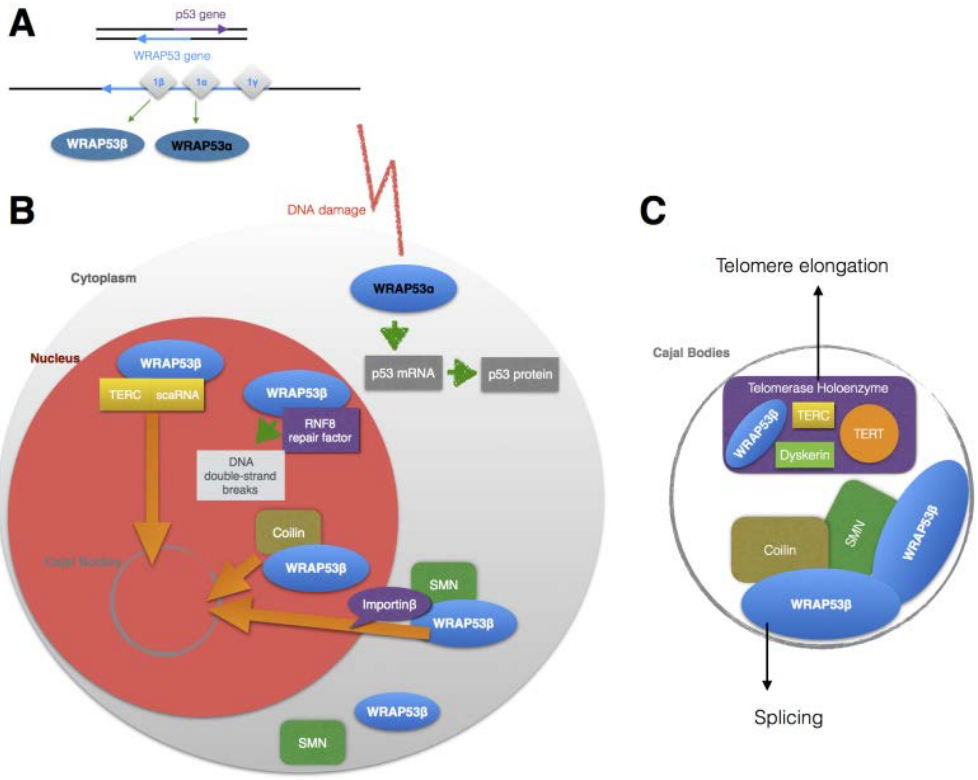



*Figure 8*. A) The transcription of WRAP53 gene in an antisense fashion to p53 and the gene products of WRAP53. B) The functions of WRAP53α and β. C) The role of WRAP53β in Cajal bodies. Adopted from Tiefenböck-Hansson, K. (2017). The impact of Survivin, WRAP53β, and Hypoxia on treatment response in Head and Neck Cancer (PhD dissertation, Linköping University Electronic Press). https://doi.org/10.3384/diss.diva-142112.

## 1.4.3 Immune markers

### 1.4.3.1 Tumor microenvironment and tumor editing

The tumor microenvironment is complex and evolves continuously; in addition to adjacent cancer cells, it refers to a mix of infiltrating and resident cells, as well as secreted factors and the extracellular matrix. The composition varies between tumor types, but hallmark features include stromal cells, endothelial cells, and non-cellular components of the extracellular matrix such as collagen, fibronectin, and laminin, as well as a diversity of adaptive and innate immune cells with both pro- and anti-tumorigenic functions.

A complex network of intercellular communication involving cytokines, chemokines, and growth factors contribute to the interactions (184, 185). Furthermore, circulating tumor cells, exosomes, cell-free DNA, and apoptotic bodies all are involved in complex crosstalk between cancer cells and distant target cells (186). With support from the surrounding environment, tumor cells may stimulate significant molecular, cellular, and physical changes that facilitate invasion, local progression, and metastasis.

Immunoediting is a dynamic process that occurs between tumor cells and the immune system. Through mutations and selection of immune-resistant cancer cells, the immune response in tumors change may be divided into three different phases: elimination, equilibrium, and escape. In the initial elimination phase, also known as immunosurveillance, the immune system is able to identify transformed cells for elimination. During the following equilibrium phase, tumor cells acquire, through mutations, features to escape immunosurveillance. Most of the transformed cells are still eliminated by the innate and adaptive immune response, but the eradication is without entire success. In the final phase, by an accumulation of adaptations, tumor cells manage to escape the immune system (187).

T cells are the most important lymphocytes for adaptive antitumor immunity and perform various effector functions depending on subtype. The two subtypes of interest in this thesis are (1) T helper cells with surface expression of CD4 (CD4+ TILs) and (2) cytotoxic T cells with surface expression of CD8 (CD8+ TILs).

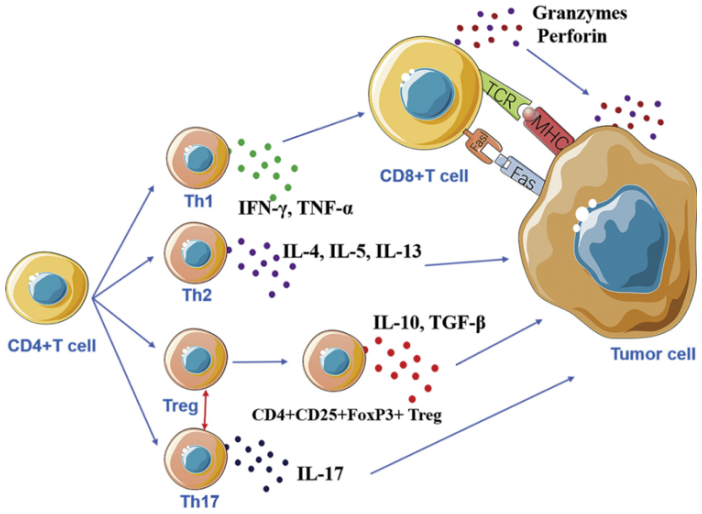



*Figure 9*. Schematic illustration of immune functions of $CD4^+$ and $CD8^+$ TILs (188). Reprinted by permission from Elsevier. Tang Y, *et al*. Prognostic and therapeutic TILs of cervical cancer-Current advances and future perspectives. Mol Ther Oncolytics. 2021 Jul 21;22:410-430.

#### 1.4.3.2 CD4+ TILs

$CD4^+$ T cells can differentiate into several diverse functional subtypes. Firstly, they help to mediate anti-tumor response through direct and indirect means involving $CD8^+$ TILs (189) (Figure 9). Concerning indirect mechanisms, secretion of effector cytokines such as interleukin (IL)-2 activates CD8+ TILs. CD4+ also supports and maintains pro-inflammatory dendritic cells, which indirectly aids CD8+ TILs function. Secondly, they exert a direct anti-tumor effect through release of activation signals that induce acquisition of cytotoxicity and secretion of effector cytokines such interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) which stimulate differentiation of effector and memory CD8+ TILs. Thirdly, CD4+ TILs induce humoral immune response against tumor antigens by aiding in the differentiation and antibody production of B cells (189).

#### 1.4.3.3 CD8+ TILs

CD8+ TILs depend on CD4+ TILs for their response to cancer, via which they gain the ability to recognize and kill cells that are infected or transformed. CD4+ TILs will present foreign (or transformed) antigens on their surface, aided by major histocompatibility complex (MHC) class I molecules. CD8+ TILs kill enemy cells by establishing an immunological synapse with the antigen, thereby initiating signals that will lead to apoptosis through one of two possible mechanisms. In the first, the cytotoxic granules are induced to empty the perforin/granzyme B contents in the vicinity of the foreign target cell, where they are transported into the target cell through perforin pores, with a following cascade of events that induces apoptosis. Secondly, they may also use the FasL expressed on CD8+ TILs to form a cross-link with the Fas receptor on the target cell surface; the latter acts as a death receptor which induces intracellular mechanisms in the cell that ultimately culminate in apoptosis (190).

#### 1.4.3.4 CD4+ TILs and CD8+ TILs as prognostic factors

A growing number of studies have shown that the elements characterizing the host immune response to tumors, including TIL type, number, and ratio, can provide useful information for tumor prognosis. CD8+ TILs have mainly been recognized for their impact on tumor growth, while CD4+ TILs are known to play a more complex role in immunomodulation, since they may both mediate antitumor immunity and promote tumor growth. When considered together, however, their mutual presence in tumor tissue is regarded as a good prognostic indicator (79).

The tumor microenvironment in cervical cancer shares some common features with solid tumors, but also has some unique characteristics associated with HPV infection (191). To our knowledge, infiltrating T-cells have never been studied in relation to vaginal cancer, for which reason a brief review of the literature on cervical cancer may provide some clues concerning their value. A meta-analysis of infiltrating T-cells in cervical cancer showed a pattern of higher T-cell infiltration in both normal cervical tissue and cancerous tissue, while precancerous lesions had lower infiltration (192). This might be explained by the previously described mechanisms of immune activation and immune evasion. Elevated cytotoxic $CD8^+$ T cells are now known to be a good prognostic marker in cervical cancer (193) and activated memory $CD4^+$ T cells also indicate a favorable prognosis (194).

The idea has been proposed that an effective T-cell response involving both CD8+ TILs and CD4+ IL-2, as well as IFNγ-producing TILs, are required to eradicate HPV-infected cells (195). This is consistent with the idea that HPV-induced progressive disease is associated with a lack of strong T-cell response against early viral proteins. Interestingly, though, tumors are often infiltrated with immunosuppressive cells such as regulatory T cells, macrophages, and IDO+ cells. It has been shown that in cervical cancer, T-cells do not produce granzyme B, which is known to be crucial for induction of target cell apoptosis. Instead, they express PD-1, which is a sign of immune exhaustion (196).

Accordingly, the presence of an HPV-specific CD4+ response in cervical cancer is associated with an improved survival outcome and response to treatment. In HPV-associated cervical cancers, the degree of CD8+ TILs have been shown to correlate with better clinical outcome (197, 198). Absence of lymph node metastases in primary tumors is linked to high intratumoral CD8+ T-cell infiltration. Notably, there is a strong correlation between infiltration with CD8+ T cells and the two CD4+ subtypes Foxp3-negative and Foxp3+ T cells, but currently, only the ratio between $CD8^+$ T cells and Foxp3+ Tregs has proven to be useful as an

independent prognostic factor in regard to cervical cancer (196). One analysis regarding the composition of tumor-infiltrating immune cells in cervical cancer, which was based on RNA expression data, showed that the immune infiltration profiles in normal tissues differed from those in cervical cancer; higher densities of activated memory $CD4^+$ T cells were present in cancer, a characteristic that was also found to be an independent factor for favorable overall survival (199).

The search for the ideal biomarker is becoming increasingly important in the effort to select those patients who are most likely to respond to the various emerging immunotherapeutic treatments, which now include chimeric antigen receptor T (CAR-T) cells, negative immune regulation-based therapies, adaptive cell therapy, and TIL-based therapy (188). Infiltrating lymphocytes have proven to be important to predicting progression of different types of solid tumors and currently appear to be the most interesting immune cells in cancer treatment.

The assessment of TILs in tumors is considered to be a robust and inexpensive approach in the quest for the ideal biomarker, where to date greater progress has been made in relation to breast cancer and colon cancer than in relation to HPV-related tumors. Comparing the immune profiles in HPV-positive and HPV-negative vaginal cancer will contribute to our understanding of the complex biology underlying these different tumors, each with its own unique tumor microenvironment, and pave the way for future implementation of novel immunotherapies.

# 2 RESEARCH AIMS

The overall objective of this PhD thesis was to identify potential prognostic factors in primary vaginal cancer with focus on HPV.

The specific objectives of the included studies were as follows:

*Study I*: To investigate presence of HPV and the expression of p16 and Ki67 and their correlation with clinical parameters and survival in vaginal cancer.

*Study II*: To evaluate the expression of the LRIG1-3 proteins, their correlation to clinical parameters and prognostic significance in vaginal cancer.

*Study III*: To evaluate the expression of dyskerin and WRAP53ß, their correlation to clinical parameters and prognostic significance in vaginal cancer.

*Study IV*: To assess the presence of CD4+ and CD8+ TILs in tumors and their correlation to clinical parameters and prognosis as well as the prognostic significance of HPV status and p16 expression in vaginal cancer.

# 3 MATERIALS AND METHODS

## 3.1 STUDY POPULATION AND TUMOR SAMPLES

The study population in *study I* derived from a cohort of 130 patients consecutively diagnosed with vaginal cancer between 1978 and 1995. All patients received treatment and follow up at Karolinska University Hospital, Stockholm, Sweden. In order to achieve significance for difference in survival, patients were divided into two groups: short-term (death ≤2 years after diagnosis) and long-term (survival ≥8 years after diagnosis). After excluding tumor samples failing to meet eligibility criteria, 68 of the initial 130 patients were categorized into the two predefined groups, 39 patients in the short-term and 29 in the long-term group. Data regarding clinical and tumor characteristics, as well as treatment and follow-up, were obtained from medical records (6). FIGO staging (1995) was undertaken based on clinical records and histopathological results. For the purposes of this study, recurrence was defined as reappearance of disease after three months or more in patients with complete remission after primary treatment.
*Studies II-III* are based on a consecutive cohort of 81 patients diagnosed with vaginal cancer at hospitals in Örebro, Karlstad, Eskilstuna, and Västerås between 1975 and 2002, who were subsequently treated and followed up at Örebro University Hospital. The various study populations differ slightly with respect to the quality of tumor samples and immunohistochemical studies. *Study II* included 70 patients, *study III* 68 patients and *study IV* 69 patients. Clinical data regarding patients, treatment, and follow-up, as well as tumor size, localization, characteristics, and FIGO staging (Montreal 1994) at time of diagnosis, were previously retrospectively recovered from medical records by Lillsunde Larsson (25). For the purposes of this study cohort, recurrence was defined as reappearance of cancer after six months or more following completion of primary treatment.

Archived formalin-fixed paraffin-embedded (FFPE) tumor biopsies were used for histological diagnosis and immunohistochemistry (thickness: 4.5-5 μm). Following hematoxylin and eosin staining, senior pathologists reviewed and confirmed histological diagnosis and identified representative tumor sections for subsequent analyses. Histological assessment followed the WHO-recommended nomenclature (2).

## 3.2 METHODS

The following sections briefly describe the methodology used in the studies of this thesis. Detailed information can be found in the method section of each paper.

### 3.2.1 HPV analysis

10μm thick sections from the same FFPE block sections used for morphological evaluation were dewaxed with xylene-ethanol. DNA was extracted with using a MagNA Pure LC Robot. ß-globin detection by real-time PCR was used to ensure sample adequacy. Presence of HPV DNA was detected using PCR with modified general primers amplification system (primer targeting L1). HPV detection was by Luminex technology, while genotyping was undertaken using multiplex bead-based hybridization. Probes for 14 high-risk HPV types and for 22 low and possible high-risk types were included in the Luminex.

Data concerning HPV status in papers II-IV were previously obtained by Lillsunde Larsson using Taqman real-time PCR with targeted E6/E7 genome segments from 12 high-risk and two low-risk HPV types (25).

### 3.2.2 Immunohistochemistry

The four studies are based on immunohistochemical staining using the relevant antibodies. Briefly, the immunohistochemical staining protocol includes the following steps: (1) deparaffinization with xylene and rehydration with ethanol, (2) hydrogen peroxide to block or irreversibly inactivate endogenous peroxidase in order to reduce non-specific background staining, which could lead to false positive results, (3) antigen retrieval so as to reduce or eliminate chemical modifications following preservation with formaldehyde in FFPE sections, since these may interfere with subsequent protein detection, (4) blocking with species-specific serum, if necessary, depending on subsequent steps and use of horseradish peroxidase, (5) incubation with primary antibody, (6) incubation with a secondary labeled chromogenic antibody for detection purposes, (7) counterstaining of tissue with hematoxylin to visualize cellular anatomy and orient the viewer with respect to the specific staining.

Table 2 presents a brief summary of the solutions. Detailed information concerning solutions, time intervals and temperatures are provided in the respective papers.

### 3.2.3 Evaluation of immunohistochemical staining

*Study 1*: Two observers independently performed each p16 immunostaining analysis. Cells with a clearly stained nucleus or with a distinct cytoplasmic reaction were regarded as positive. Both staining intensity and percent of immunoreactive cells were used for scoring purposes: no expression (negative); weak expression (<30% positive cells); moderate expression (30%-50% positive cells); strong expression (>50% positive cells). Samples scored as having moderate or strong staining were regarded as positive for p16.
Nuclear immunostaining for Ki67 was evaluated as follows: <10% positive cells; 10%-50% positive cells; and >50% positive cells. Microscopic evaluation was performed by two senior pathologists blinded to clinical data.
*Study II:* The percentage of positive cells, regardless of whether staining was cytoplasmic or nuclear, was assessed on a semi-quantitative scale: 0=0% positive cells; 1= 1%-25% positive cells; 2=25%-50% positive cells, and 3= >50% positive cells. The evaluation was carried out by a senior pathologist blinded to the clinical data.
*Study III:* Microscopic evaluation of dyskerin staining by a senior pathologist was quantified as follows: (i) negative (i.e. no positive cells observed), (ii) weak (1+), (iii) moderate (2+), and (iv) strong (3+). In addition, the location of the protein in the nucleoplasm and/or in nuclear bodies was noted.
The second protein of this study, WRAP53ß, was evaluated by two senior pathologists with respect to percent of stained tumor cells and staining intensity. Staining of tumor cells was semi-quantitatively grouped into four categories based on percentage: 0= 0 negative, 1=<25%, 2= 25%-50%, 3= >50%. Meanwhile, staining intensity was labeled as (i) negative (no positive cells observed), (ii) weak, (iii) moderate, or (iv) strong.
*Study IV:* CD4+ and CD8+ TILs were counted in 10 high-power fields (40x) for each tumor sample.
Assessment of p16 was categorized as either p16 overexpression (diffuse and strong cytoplasmatic and nuclear staining in >70% of tumor cells) or as p16-negative staining. Again, microscopic evaluation was performed by a senior pathologist.

| | Study I | Study II | Study III | Study IV |
|---|---|---|---|---|
| Deparaffinization | Xylene-ethanol | Xylene-ethanol | Xylene-ethanol | Xylene-ethanol |
| Antigen retrieval | Tris EDTA buffer | LRIG1 &2: Cell conditioning 1<br><br>LRIG3: Protease 1 | Heating in citrate buffer | CD8: EnV FLEX TRS, High pH<br><br>CD4: Cell conditioning 1 |
| Blocking | Endogenous peroxidase activity blocked w/ Dako REAL | Ultra View Universal DAB Inhibitor with 3% $H_2O_2$. | 2% $H_2O_2$ | CD8: EnV FLEX Peroxidase-Blocking Reagent<br><br>CD4: peroxidase inhibitor |
| Primary body incubation | Primary antibody against p16 clone E6H4<br><br>Clone MIB-1 | Rabbit primary antibodies LRIG1, LRIG2, LRIG3 | Primary anti-dyskerin ab,<br><br>Primay WRAP53 ab | Dako CD 8, clone C8/144B<br><br>Ventana CD4, clone SP35<br><br>Ventana CINtec® p16, clone E6H14 |
| Secondary antibody incubation for visualization | Visualization reagent: a horseradish peroxidase/goat anti-mouse ig-labeled dextran polymer | Ultra View Universal HRP, Multimer + Ultra View Universal DAB Chromogen + Ultra View Universal Copper | EnVision Detection Peroxidase/DAB system Kit | EnV FLEX/HRP together with EnV FLEX Substrate Working Solution |
| Staining | Harris hematoxylin solution | Hematoxylin and Bluing Reagent | Mayer´s hematoxylin | CD8: Hematoxylin<br><br>CD4 +p16: Hematoxylin and Bluing Reagent |
| Staining Kit | CinTec Histology Kit | | | CD8: EnVision FLEXCD4 +p16: U OptiView DB IHC version 6 detection kit |
| System used | Dako autostainer | Ventana Benchmark XT apparatus | Dako Omnis | Dako Omnis<br><br>CD4 +p16: Ventana Benchmark Ultra |

*Table 2*: Immunohoschemical solutions and antibodies used in the four studies of this thesis.

## 3.3 STATISTICAL ANALYSES

In *study I*, no power calculation was performed. The collected database is unique with respect to population size since it represents a consecutive 17-year accumulation of cases; a power calculation would not have provided a larger population size. Although the possibility that results would fail to reach significance was understood, it was still regarded as an interesting study to perform.

The four semi-quantitative groups in the p16 immunohistochemical analysis were dichotomized and analyzed for p16 expression in relation to clinical parameters, as categorized using ordinal variables, and then subjected to the *Pearson Chi-square test*, or in cases where sample size was less than five, to the *Fisher´s Exact test*. Ki67 staining was similarly analyzed, but without dichotomizing the groups. The same tests were used for analysis of HPV expression in relation to clinical parameters, and in relation to p16 and Ki67. To ascertain how p16 expression correlates with short-term or long-term survival, we subjected the results to the *Spearman rank correlation* test to obtain a coefficient reflecting the strength and direction of the association. The *Mann-Whitney U test* was used as a non-parametric test to compare variables such as mean age between the groups.

For purposes of the survival analysis, we used a *Multivariate logistic regression model* that included histopathological grade and tumor size (instead of FIGO stage), as well as expression of p16 and Ki67.
In *study II,* the *Pearson Chi-square test* and *Fisher´s Exact test* were similarly used to study associations between ordinal variables. *Kaplan-Meier* graphs were used to provide a graphical illustration of 10-year cancer-specific survival in relation to LRIG1-3 expression. *Log-rank test* was used as the statistical basis for comparison of hazard functions in the illustrated groups. The univariate survival analysis showed age, tumor size, FIGO stage, and HPV status to be significant parameters for survival and they were therefore included together with LRIG1 in the *Cox regression multivariate analysis* for hazard ratio calculation.
In *study III,* the same statistical analyses were performed. Once again, the semi-quantitative groups of the immunohistochemical analysis of dyskerin expression were dichotomized. The *Independent sample t-test* was used for comparison of means of the groups. The Cox regression model included age, tumor size, tumor stage, and histology, as well as dyskerin.
In *study IV*, the *Pearson chi-square test, Fisher's exact test,* and *student t-test* were similarly applied as in the previous studies. CD4+ and CD8+ TILs counts were analyzed using median values, or dichotomized into quartiles, based on quartile 1-3 (low infiltration) vs quartile 4 (high infiltration). The *median test* was used to test for differences in mean values. The Cox regression model included age, tumor stage, HPV status, and the relevant parameters of interest (CD8+TILs or p16 expression). The proportional hazards assumption was examined graphically for the Cox regression models, using Schoenfeld residual plots and tested including interaction terms between time and independent variables in the regression model. P-values corresponded to two-sided tests of the interaction term regression coefficients were considered an indication of non-proportionality.

## 3.4 ETHICAL CONSIDERATIONS

The first study of this thesis was approved by the Regional Ethical Review Board in Stockholm, Sweden (EPN, Dnr 01-194). The following three studies were approved by the Regional Ethical Review Board in Uppsala, Sweden (EPN, Dnr 2008/294/1+ Dnr 2008/294/2 + Dnr 2008/294/3). Specific informed consent from patients was not required, according to the ethical approval. Patients were verbally informed about the clinical research database. The studies were performed in accordance with the Declaration of Helsinki.

The women involved in the studies were diagnosed and treated for vaginal cancer. The studies are retrospective and their use for our purposes has no impact on the continued care of the involved women. Study participation and results will not directly benefit the patients involved.
Privacy is a necessary consideration in relation to any form of registration and the processing of information such as medical records or similar. The risk associated with this project is limited to perceived violation of privacy and a reduced confidence related to established registers within the healthcare system. Proper record management and anonymization of data reduce risk of a negative patient experience.
One challenge encountered by this project is the size of the study population. Research on rare cancers is often based on small study populations with the associated risk of type 2 errors (i.e. hypotheses about differences between groups may be rejected because of a low power study).
Patient risk in this study is very limited, and their inclusion in the study may provide future help for women diagnosed with this disease by adding to the knowledge basis, with the ultimate aim of reducing mortality, morbidity, and suffering; consequently, the benefits may be regarded as significantly outweighing the risks.

# 4 RESULTS AND DISCUSSION

The overall objective of this PhD thesis is to identify prognostic factors of potential significance in primary vaginal cancer, with special focus on HPV. To accomplish this goal, we investigated the prognostic significance of HPV status (*study I*) and expression of: p16 (*studies I and IV*), Ki67 (*study I*), LRIG1-3 (*study II*), dyskerin (*study III*), and WRAP53ß (*study III*), as well as CD4+ and CD8+ TILs infiltration, including the combination of HPV status and p16 expression (*study IV*) in relation to patient and tumor characteristics.

## 4.1 HPV STATUS (STUDY I)

### 4.1.1 Results

In *study I,* analysis for HPV was possible in 44 of 68 (65%) patients, 19 (43%) of whom were shown to be HPV-positive. HPV16 was predominant in this study, with 12 patients (63%) positive for HPV16, 3 (16%) for HPV45, and 1 (5.3%) for the following types: HPV18, HPV35, HPV56, and HPV68.
All these HPV-positive patients demonstrated moderate to strong expression of Ki67, albeit without statistical significance. No statistically significant correlation between HPV positivity and clinical or tumor characteristics was found, except for histopathological grade, where all HPV-positive tumors were moderately to poorly differentiated. HPV-positive patients were more likely to be diagnosed with stage II or III disease and tumor located in the upper third of the vagina than were HPV-negative patients. The two patients with adenocarcinomas who were included were both HPV negative. HPV status did not correlate with either risk of relapse or survival.

The results of the analysis concerning HPV expression in relation to p16 expression and survival will be presented below.

### 4.1.2 Discussion

The prevalence of HPV in our study cohort is lower than in most other prior studies, which generally report a pooled prevalence of 65.5%-69.9% in relation to vaginal cancers (23, 200, 201). Nevertheless, the studies included in the metanalyses demonstrate considerable variation in HPV prevalence.
As in other HPV-related anogenital cancers, including vaginal squamous cell cancers, HPV16 was found to be the predominant HPV type in *study I* (19). Among the few additional HPV types found, none appeared to be more dominant. Both patients with adenocarcinomas who were included in this study were HPV-negative. Prior studies have found considerably lower prevalence of HPV expression in cases of adenocarcinoma than in squamous cell carcinoma, for which reason different etiological pathways have been postulated (200).

The present study was unable to confirm our hypothesis that HPV-positive cancers may be associated with superior survival compared with HPV-negative cancers. In other types of anogenital and head and neck cancers, HPV-positive tumors are associated with better prognosis than HPV-negative tumors, which holds potential implications for treatment recommendations (125, 202). Studies on the impact of HPV infection on overall survival in vaginal cancer show inconsistent results, even though HPV positivity is associated with a more favorable prognosis. Alonso *et al.* demonstrate that HPV-positive patients in early-stage disease have a better prognosis than HPV-negative patients (35). Brunner *et al.* report that HPV-positive patients who are at

a more advanced tumor stage (≥ III) demonstrate improved survival compared with HPV-negative patients at comparable tumor stages (68). Lillsunde Larsson et al. also found that HPV-positive patients demonstrate significantly superior overall survival rates (25). In two other studies, HPV positivity was associated with a trend toward better overall survival, but the results were not significant (87, 203). Finally, two additional studies found no association between HPV status and survival (22, 87).

The prognostic value of HPV type also remains to be determined. In cervical cancer, improved survival was found among patients who were HPV16/HPV18-positive compared with those infected by other types; however, results from other similar studies were inconclusive (202, 204). HPV16-positive vaginal cancers have previously been shown to be associated with better survival compared with other HPV types, but this remains to be confirmed in larger cohorts (25). The results of *study I* add to the evidence that implementation of an HPV vaccination program could ultimately reduce the number of women diagnosed with vaginal cancer.

The insignificant results regarding the prognostic impact of HPV status in *study I,* as well as the variable results found in previous studies, might be explained by small sample sizes, retrospective study design, and/or variations in HPV DNA detection methods, PCR primer type, and other methodological differences used for HPV detection (88). The material in *study I* is not consecutive, and bias may have been introduced as a result of exclusion of patients who survived between 2 and 8 years. One study concerning the prognostic value of HPV status in cervical cancer cites other factors that potentially make HPV detection in tumors difficult, including: low viral load of HPV DNA in tumor samples, loss of the L1 region, poor sample quality, and partial loss of HPV because of genome integration (107). Their findings suggest that HPV DNA may become increasingly difficult to detect at later stages of disease.

Regarding the analyses and results of this thesis, it is important to note that the data concerning HPV status in *study IV* were obtained through HPV analysis with real-time PCR as in *study I,* but using a slightly different technology (25). The HPV prevalence in *study IV*, which was based on prior findings, was 53.6% (37/69), 70.3% of which were HPV16-positive. Contrary to the findings of *study* I, HPV-positivity in tumors was associated with a significant improvement in overall and cancer-specific survival. These factors may contribute to the difference in results between *studies I* and *IV* that considered p16 in relation to clinical characteristics, HPV, and survival.

## 4.2 TUMOR SUPPRESSOR P16 (STUDIES I AND IV)

### 4.2.1 Results

Immunohistochemical analysis showed that most vaginal tumor samples expressed p16; in *study I,* 23.5% of tumors displayed moderate expression and 54.4% strong expression. S*tudy IV* showed similar findings with 62% of tumors expressing p16.
High expression of p16 correlated significantly with moderate to poor differentiation (p=0.004) in *study I,* while this was not seen in *study IV.* Neither study showed any association between p16 expression and other prognostic factors such as age, tumor size, stage, histology, or tumor location.

In *study I*, moderate to strong expression (>30% of the cells) of p16 was found in 95% of HPV-positive patients, while only one HPV-positive tumor was p16-negative. Among HPV-negative tumors, 68%

demonstrated p16 overexpression. Among tumors that were HPV16-positive, all but one demonstrated overexpression of p16.
S*tudy IV* found that 84% of the HPV-positive tumors were also positive for p16 (>70% of cells), while 38% of the HPV-negative tumors were p16-positive.

Overexpression of p16 emerged as a factor of prognostic relevance in both *studies I* and *IV.* In *study I*, high expression of p16 was found in 90% of tumors from long-term survivors. The univariate logistic regression analysis showed a statistically significant correlation between survival and higher p16 expression (p=0.045). In the multivariate analysis, including histopathological grade, tumor size, and Ki67 expression, the correlation between p16 overexpression and survival no longer achieved statistical significance.
In *study IV*, p16-positive tumors were associated with statistically significant better 5-year cancer-specific survival (log rank, p=0.002; unadjusted HR 0.347; 95% CI 0.172-0.699) and overall survival (log rank test, p=0.005; Cox regression adjusted HR 0.398; 95% CI 0.214-0.742)

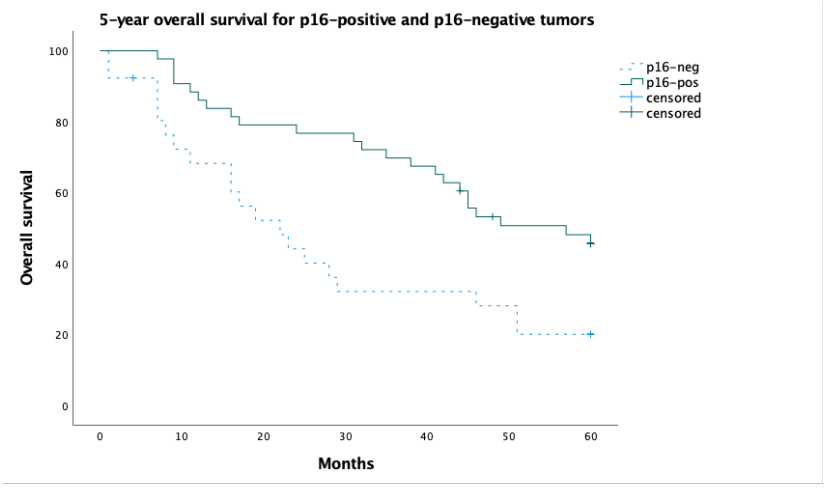
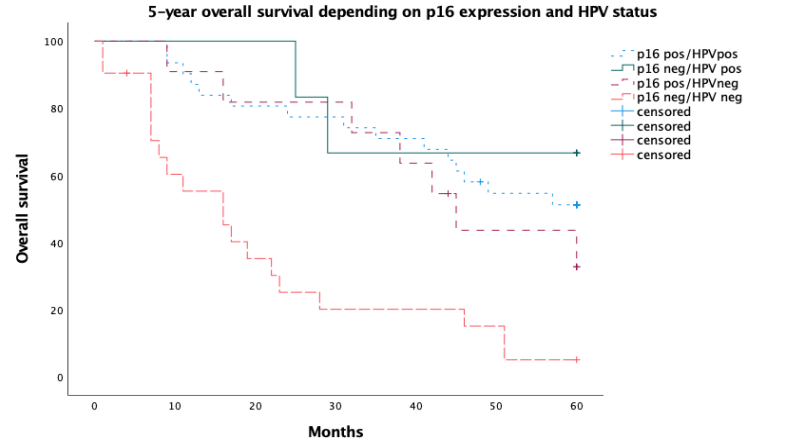



Figure 10: Kaplan-Meier curves showing 5-year overall survival for p16-negative and p16-positive cancers (log rank test, p=0.005; Cox regression adjusted HR 0.398; 95% CI 0.214-0.742).

In *study I*, patients with p16-positive and HPV-negative tumors had better survival (p=0.028). In *study IV,* however, 5-year overall survival differed significantly among tumors, depending on HPV status and p16 expression (log rank test <0.001, unadjusted HR 1.645; 95% CI 1.283-2.109) (Figure 11A). Survival was significantly worse among patients with p16-negative and HPV-negative tumors compared with those having p16-negative and HPV-positive tumors, or with those whose tumors were p16-positive, regardless of HPV status (log rank p<0.001; adjusted HR 5.125; 95% CI 2.497-10.520) ().

**A**




**B**

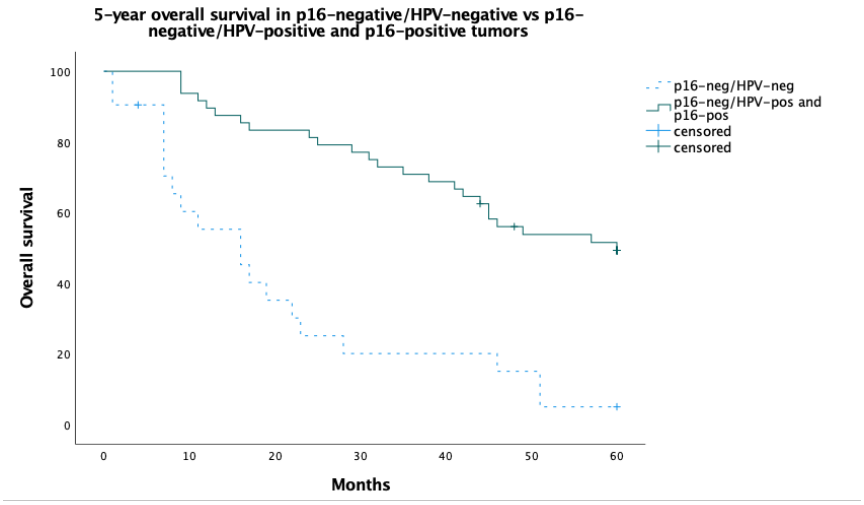



*Figure 11*: Kaplan-Meier curves showing 5-year overall survival in (A) p16-negative/HPV-negative cancers compared with p16-negative/HPV-positive or p16-positive/HPV positive or negative tumors (log rank test <0.001, unadjusted HR 1.645; 95% CI 1.283-2.109) and (B) p16-negative and HPV-negative tumors vs p16 negative and HPV positive or p16-positive tumors regardless of HPV status (log rank p<0.001; adjusted HR 5.125; 95% CI 2.497-10.520).

### 4.2.2 Discussion

The results of *study I* and *study II* provide evidence to support active involvement of HPV in the carcinogenesis of vaginal cancer. Overexpression of p16 in vaginal cancer has been investigated before and our results, with p16 overexpressed in a majority of tumor samples, are in line with previous studies; pooled prevalence of p16 overexpression in vaginal cancer was 72% in a metanalysis by Bertoli *et al.* including six studies (of which one is *study I*) (200).

The results of the two studies differ regarding p16-positivity in HPV-negative tumors, with *study I* showing a higher proportion of p16-positive/HPV-negative tumors. Studies addressing both p16 and HPV prevalence in vaginal cancer are scarce. A study by Alemany *et al.* considered 146 cases of vaginal cancer, in which p16 overexpression was found in 87% of the 110 HPV-positive vaginal cancer cases (21). Only five (14%) of the HPV-negative tumors were p16-positive. In the meta-analysis by Bertoli *et al.* 89% of HPV-positive tumors were also positive for p16, while only 39% of HPV-negative tumors showed p16 expression (200). This raises the issue of whether there was adequate detection of HPV DNA in the tumor samples of our first study, which could also have been subjected to additional testing, such as by real-time E7 and E6 PCR (107).

As previously discussed in the background section of this thesis, immunohistochemical detection of p16 can be regarded as a reliable surrogate marker for HPV-induced transformation, for which reason it is overexpressed in HPV-infected (pre)cancerous tissues of the cervix (56, 57) as well as in vulvar (58, 59) and anal cancer (29). However, it is noteworthy that p16 overexpression is not equivalent to evidence of HPV involvement as an etiological factor, as suggested by our results showing overexpression of p16 in the absence of HPV infection (63). Malignancies of almost every type have displayed mutations or molecular alterations of p16 (205).

The results from *study I* and *study IV* point to p16 expression as a potential prognostic factor for vaginal cancer. *Study I* showed that p16 overexpression was linked to long-term survival in the univariate analysis, while *study IV* showed that p16-positivity correlated to both overall and cancer-specific survival.

The value of immunostaining for p16 as a prognostic biomarker has previously been proposed. A study that included 31 cases of vaginal cancer showed that p16 expression has a significant impact on overall survival across all FIGO stages (74). A similar study that included 32 cases of vaginal cancer treated with radiotherapy, with or without chemotherapy, showed improved overall survival and lower risk of recurrent cancer among p16-positive cases (37). Conversely, in another study that included 57 vaginal cancer patients, p16 was overexpressed in almost all HPV-positive cases, yet was not found to relate to survival, even though HPV positivity considered alone had shown a significant impact on prognosis in early-stage disease (35).

S*tudy I* shows that patients with p16-positive and HPV-negative tumors have significantly better survival (p=0.028). In contrast, in *study IV,* the Kaplan-Meier analysis illustrates a substantially worse survival among patients with p16-negative/HPV-negative tumors; when the groups are dichotomized into tumors that are p16-negative/HPV-negative vs all other remaining tumors, the dual negativity pertaining to p16 expression and HPV status emerges as an independent significant negative prognostic marker in vaginal cancer. The reverse has also been demonstrated by studies on HPV-related anogenital and head- and neck cancers, which found that double positivity in regard to p16 expression and HPV status are associated with a superior prognosis (123, 206, 207).

The inconsistency of results between *study I and study IV* regarding the combined analysis of HPV status and p16 expression may possibly be explained by the differences in defining p16 expression, as previously described in the methods section. Another possible explanation may lie in the limited information concerning HPV status in *study I*. Considering that p16 may have served as a surrogate marker for HPV-positivity, it is possible that some of the tumors considered to be HPV-positive were not subjected to HPV analysis, but showed p16 overexpression.

In conclusion, since testing for p16 is less complicated in the clinical setting than HPV analysis, our results emphasize the need for further clarification of the clinical value of p16 analysis. The study results also highlight the prognostic value of combined testing for HPV and p16, since dual negativity of in these two factors is associated with significantly poorer survival.

## 4.3 PROGRESSION MARKER Ki67 (STUDY I)

### 4.3.1 Results

The degree of expression of Ki67 in the 68 tumor samples was as follows: 7 tumors (10.3%) showing <10% positive cells, 38 (55.9%) showing 10%-50% positive cells, and 23 (33.8%) showing >50% positive cells. There was a significant association between Ki67 expression and both tumor size (p=0.047) and degree of differentiation (p<0.001), but no correlation was found with either short- or long-term survival (p=0.46).

### 4.3.2 Discussion

This study assessed Ki67, determined through immunohistochemistry, as a potential prognostic biomarker of interest; it is already clinically accessible and serves as a reliable indicator of cell proliferation. Ki67 was strongly expressed in a majority of cells. These results indicate a higher rate of proliferation in poorly differentiated tumors and are also associated with larger tumor size. Ki67 may possibly be of value for histopathological grading of vaginal cancer since, together with p16, it is an indicator of poorly differentiated tumors.

Ki67 has been shown to be of clinical value in various gynecological cancers. HPV induces cellular proliferative activity, thereby often upregulating Ki67 in HPV-infected cells. Previous studies on squamous cell carcinoma of the lower genital tract show that Ki67 is associated with worse clinical outcome, increased tumor size, and more advanced FIGO stage at diagnosis (203). In cervical adenocarcinoma, increased Ki67 expression in low-grade tumors and in tumors that are higher stage at diagnosis correlated with worse prognosis (208). It has been postulated that expression of Ki67 may be of similar prognostic value in vulvar carcinoma (209). Increased Ki67 expression in vaginal cancer has previously been demonstrated, but not associated with survival, FIGO stage, or tumor size (38, 75, 203).

One exception is a study including 80 patients treated with radiation therapy, where Ki67/MIB1 remained as an independent risk factor associated with better prognosis in the multivariate survival analysis (71). In summary, the results of *study I* indicate that Ki67 expression may lack prognostic relevance in vaginal cancer.

## 4.4 LRIG1, LRIG2 AND LRIG3 (STUDY II)

### 4.4.1 Results

Our study of 70 patients with vaginal cancer found that 83% of the tumors showed high expression of both LRIG1 and LRIG2, while LRIG3 was not expressed at all in 53% of tumors and was not highly expressed in any of the remaining tumor samples.

High expression of LRIG1 showed a positive correlation with HPV (p=0.0047) and a negative correlation with cancer progression and was thereby associated with a higher cure rate (p=0.0004) and better cancer-specific survival rate (log-rank test: 0.011). The improvement in survival was most pronounced in HPV-negative cancers (log-rank test: p=0.027). LRIG1 expression remained as an independent statistically significant factor for survival in the Cox regression multivariable analysis (HR 0.408; 95% CI: 0.18-0.91). The LRIG proteins did not correlate with any clinical or tumor characteristics. Neither LRIG2 nor LRIG3 expression correlated to survival rates.

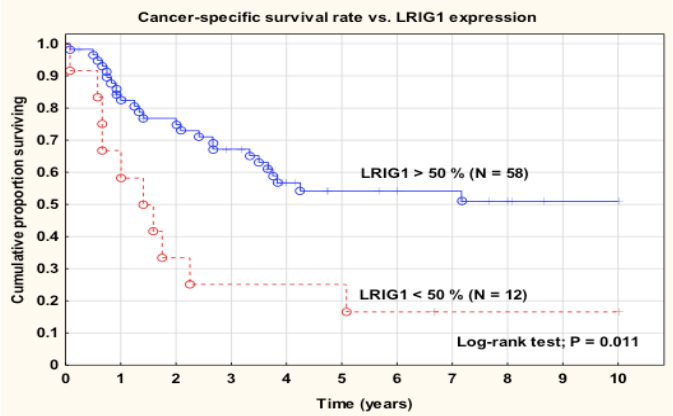



*Figure 12*. Cancer-specific survival depending on LRIG1 expression in immunohistochemical staining (Score 3 vs score 0-2). Log-rank test showed a statistically significant difference (p=0.011).

### 4.4.2 Discussion

*Study III*, to our knowledge the first study performed on the LRIG proteins in vaginal cancer, shows that LRIG1 expression was associated with improved cancer-specific survival. The result of this study expands knowledge from previous studies investigating the importance of the LRIG proteins as potential prognostic factors in cancers where HPV infection is an important etiological factor.

LRIG1 correlated significantly with HPV positivity, but not with any other clinical parameter or tumor characteristic in our cohort. The association of LRIG1 with positive HPV status is consistent with findings from studies of oropharyngeal cancer, where LRIG1 expression correlated with HPV positivity, while an inverse correlation was found between LRIG2 and HPV status (153). Our results found no association between HPV status and either LRIG2 or LRIG3. In contrast, high expression of LRIG3 has been shown to correlate with HPV positivity in both normal cervical epithelium and in precancerous cervical lesions (210).

High LRIG1 expression was shown in the multivariate analysis to be an independent factor indicative of superior patient survival when considering risk factors such as patient age, tumor stage, tumor size, and HPV status. The difference in survival rate in regard to LRIG1 expression was even more pronounced in HPV-negative tumors.

Our study indicates that LRIG1 holds prognostic value as a biomarker, while immunohistochemical assessment of LRIG2 and LRIG3 showed no association with clinical parameters, HPV status, or survival. Prior research has aimed to explore the association between the LRIG proteins and cancers in which HPV infection plays an etiological role. HPV-positive oropharyngeal tumors with high LRIG1 expression was shown to correlate with very good prognosis (153). LRIG1 expression correlates with HPV positivity, while the opposite holds true concerning the association between LRIG2 and HPV status. High expression of LRIG3 correlated with HPV positivity in both normal cervical epithelium and in precancerous cervical lesions (210). However, these results conflict with those from a study on vulvar squamous cell carcinoma, in which high LRIG2 expression, but not LRIG1 expression, was shown to be a significant independent prognostic factor for overall survival. In contrast to our results, absence of or weak LRIG1 staining was associated with better overall survival in HPV-negative tumors (211).

In cervical cancer, the LRIG proteins have been proposed as useful prognostic markers when considering different stages and histological types of disease. Briefly summarized, LRIG1 has been associated with improved survival in both early-stage squamous cervical cancer (212, 213) and in cervical adenocarcinoma (214). LRIG2, however, was found to be associated with worse prognosis in early-stage squamous cell cervical cancer. Interestingly, the combination of high LRIG2 and low LRIG1 was found to be a predictor of very poor prognosis in these women (212). Finally, LRIG3 expression did not correlate with 10-year survival in squamous cell cervical cancer. In contrast, a high expression of LRIG3 in cervical adenocarcinoma was shown to be a statistically significant independent predictor of improved patient survival (214).
In conclusion, the value of LRIG proteins as prognostic factors in gynecological cancers has not yet been fully established, for which reason future studies will contribute to improved understanding.

As previously described in this thesis, much more is known about the function of the LRIG1 protein than its LRIG2 and LRIG3 counterparts. LRIG1 is currently considered to be a tumor suppressor, acting as a regulator of growth factor signaling (140, 141), while LRIG2 is thought to be a tumor promoter (160, 212, 215), and LRIG3 a tumor suppressor (210, 216). In view of the elusive functions of LRIG2 and LRIG3 it is difficult to draw any conclusions from the fact that we could not report any significant results regarding LRIG2 and LRIG3. On the other hand, in light of the small population size in this study, it is noteworthy that LRIG1 emerges as a significant predictor of survival, which emphasizes its potential use as a prognostic factor. In conclusion, prior studies differ as to the prognostic value of LRIG proteins in gynecological cancers, but future studies should increase our understanding. The question of whether possible mechanistic connections exist between the LRIG proteins and HPV infection requires further investigation.

## 4.5 DYSKERIN (STUDY III)

### 4.5.1 Results

The majority of dyskerin expression was reflected by weak cell staining intensity in 54 (79.4%) tumor samples, while moderate staining was seen in 5 (7.4%) and strong intensity in 8 (11.7%). Expression was seen in either the nuclear bodies, or in nucleoplasm, or in both in the case of 39 (57.3%) tumor samples. Only one sample was negative for dyskerin.

Analysis regarding possible associations between dyskerin expression and tumor and patient characteristics showed that higher expression correlates with a high degree of differentiation (p=0.032). Expression was more commonly found in basaloid and keratinizing tumors than in other histological types. Otherwise, no significant association was found between dyskerin expression and any other tumor or patient characteristic. Although not rising to statistical significance, it should be mentioned that dyskerin expression was found in 81% of HPV-negative tumors, compared with just 66% in HPV-positive tumors.

Dyskerin expression in vaginal cancer as it relates to disease progression and survival showed significant differences between the dichotomized groups. Tumors with high dyskerin expression were associated with significantly worse cancer-specific survival compared with those showing low expression (log rank: p=0.009) (Figure 13). Dyskerin expression was found in the Cox multivariate proportional hazards analysis to be a statistically significant independent risk factor in this regard (HR 3.701; 95% CI 1.094-12.517) Mean survival was 50 months in patients with dyskerin overexpression, compared with 92 months in patients with

low expression (p=0.017). All but one of the 14 patients who suffered recurrent disease with distant metastases demonstrated high expression in their tumor samples (p= 0.0001). Moreover, a lower proportion of patients demonstrating high dyskerin expression achieved primary cure, compared with those demonstrating low expression (81% vs 94%), while recurrence rate was higher in this group (40% vs 19%), but neither of these findings were statistically significant.

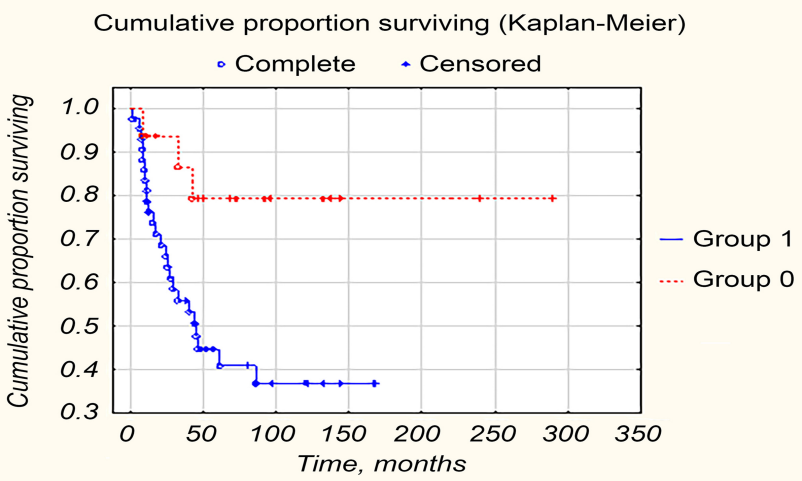



*Figure 13:* Cancer-specific survival rates depending on immunohistochemical staining intensity of dyskerin staining. Group 0=negative or 1+ staining. Group 1= 2+ or 3+ staining (log rank test; p=0.009).

### 4.5.2 Discussion

In this study dyskerin proved to be of interest as a potential prognostic factor in vaginal cancer. In our cohort of tumor samples from vaginal cancer patients, a significant association was found between high dyskerin expression and worse cancer-specific survival. Moreover, high dyskerin expression was more frequently observed in tumor samples from poorly differentiated cancers and among patients with shorter survival times, lower primary cure rates, higher overall recurrence rates, and in those with recurrent disease with distant metastasis, which suggests that tumors demonstrating high expression are more prone to be aggressive and to progress. These finding are well in line with the knowledge that altered dyskerin expression is associated with tumor formation and with worse survival in a variety of cancers (217-219).

We have hypothesized that dyskerin upregulation may stimulate formation of the telomerase holoenzyme complex in vaginal cancer. Along with E6 activation of hTERT, the telomerase holoenzyme complex plays an important role in development and progression of HPV-induced cancer. Since dyskerin expression was found in a lower proportion of HPV-positive tumors, it has been suggested that dyskerin expression is essential for telomerase activity in these tumors.

Dyskerin overexpression, as it relates to a seemingly more aggressive tumor phenotype, has also been linked to activation of several other oncogenic pathways, independent of the telomerase complex and cell immortalization mechanism (171). For example, dyskerin expression has been found to play an oncogenic role in MYC (217), in relation to the transcription factor Hypoxia-inducible factor 1 (*HIF-1*) (218), and to an increase in specific rRNA modifications involved in the translational processes in ribosomes (171). However, the functional mechanisms of dyskerin in relation to HPV and vaginal cancer remain to be elucidated.

## 4.6 WRAP53ß (STUDY III)

### 4.6.1 Results

In *study III*, 61 of 68 samples could be assessed for WRAP53ß staining intensity, fraction of stained cells, and location of this protein within the cells. The majority of tumor samples expressed WRAP53ß. Although it could be found in either nuclear bodies or in the nucleoplasm, in most samples it was found in both compartments. Only two samples showed diffuse staining. No correlation was found between either expression or location of this marker and tumor or patient characteristics, nor with survival.

### 4.6.2 Discussion

WRAP53ß was not shown to be of any significance for the diagnosis of vaginal cancer. The role of this protein in relation to telomerase transportation and function appears to be unaltered. It was expressed in a majority of tumor samples; in previous findings the level of WRAP53β has been shown to be higher in several cancer cells than in normal cells (220). Among patients with rectal cancer undergoing radiation therapy, overexpression of WRAP53β in metastases was linked with better prognosis (221).The importance of the WRAP53ß location has also been demonstrated in breast cancer, where nuclear location was associated with better outcome than subcellular location (222). Analogous findings were observed in head and neck cancer, where nuclear expression of WRAP53β was associated with better prognosis than expression in the cytoplasm (223). Loss of nuclear expression was associated with enhanced radioresistance. Since WRAP53ß has not been studied in relation to HPV status in head and neck cancer, or in other cancers to our knowledge, no comparisons can be made.

In gynecological malignancies, WRAP53ß has to date only been studied in epithelial ovarian cancer. Again, nuclear expression of both WRAP53ß mRNA and protein were associated with shorter survival. In tumors with low levels of WRAP5ß expression, downregulation of key factors involved in DNA damage response was observed (224).

Although we were unable to demonstrate the value of WRAP53ß as a potential prognostic factor in vaginal cancer, this possibility certainly deserves further investigation to elucidate its role as a potential new biomarker that could contribute to novel treatment strategies and improved survival.

## 4.7 CD4+ AND CD8+ TILs (STUDY IV)

### 4.7.1 Results

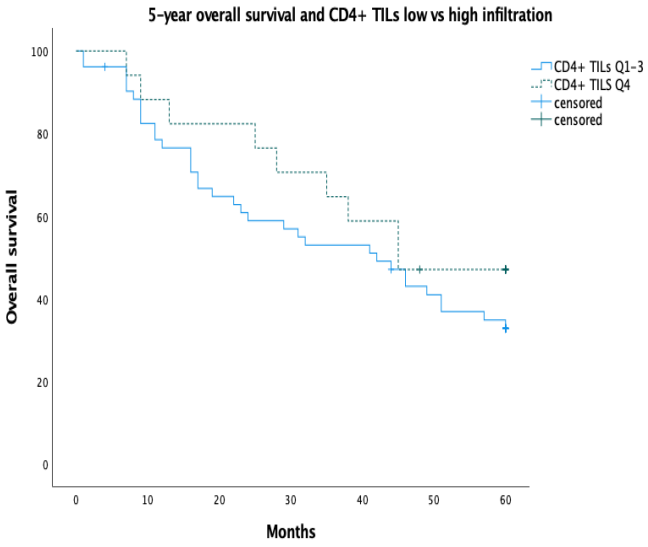
The mean and median CD4+ TIL counts were higher in HPV-positive tumors than in HPV-negative tumors (median value 3.7. vs 3.5; median test p=0.824), though the differences failed to reach significance). The number of CD8+ TILs was also higher in HPV-positive tumors than in HPV-negative tumors, but again failed to reach statistical significance (median value 12.3 vs 5.8; median test, p=0.330).

Concerning p16-positive cancers, the CD4+ and CD8+ TIL counts yielded higher mean values of TILs with wider ranges. The median value of CD8+ TIL counts was significantly higher in p16-positive cancers (median test, p=0.032), but not the median value of CD4+ TIL counts (median test, p=0.336).

Survival analyses were performed using the dichotomized groups, pitting the TILs counts in the first to third quartiles against those in the fourth quartile. Kaplan-Meier curves indicate that a higher density of both CD4+

TILs and CD8+ TILs is associated with a more favorable prognosis, albeit without significance (CD4+ TILs, logrank, p=0.325; CD8+ TILs, log rank, p=0.127) (Figure 14). Similarly, in patients with FIGO stage II-IV tumors and higher density of CD4+ and CD8+ TILs appear to have a better prognosis, albeit without reaching significance (log rank, p= 0.148 and p=0.160, respectively) (data not shown).

**A**




**B**

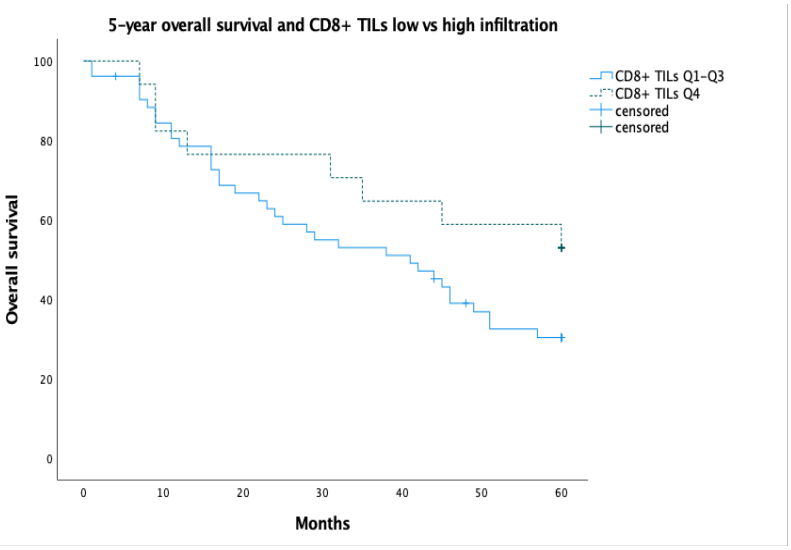



*Figure 14*: Kaplan-Meier curves showing 5-year overall survival in patients relation to density of (A) CD4+ TILs and (B) CD8+ TILs, dichotomized into low and high densities (log rank p=0.325 respectively p=0.127).

Finally, tumors with both p16-positivity and high-density infiltration of CD8+ TILs showed significantly better survival (log rank, p= 0.003) (Figure 15).

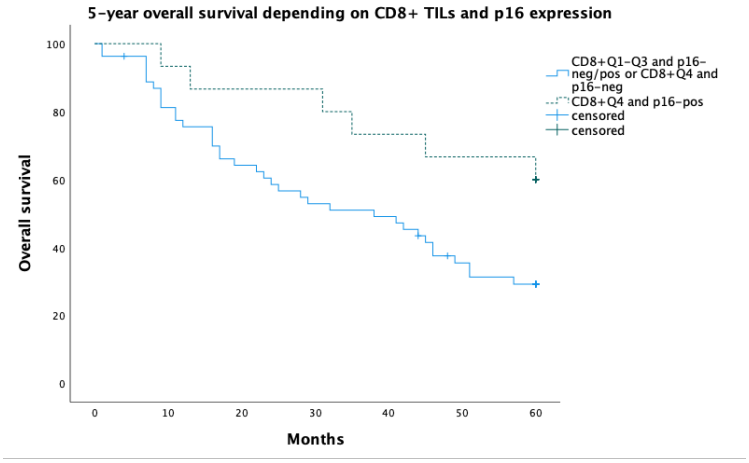



Figure 15: Kaplan-Meier curves demonstrating significantly improved survival among patients whose tumors demonstrated both p16-positivity and high-density infiltration of CD8+ TILs (log rank, p= 0.003).

### 4.7.2 Discussion

In summary, the results of this study indicate that high infiltration density of both CD4+ and CD8+ TILs in HPV-positive tumors appears to be associated with better survival. However, since the data favoring this trend failed to reach significance, the results require confirmation in a larger study. Only those tumors characterized by high-density infiltration of CD8+ TILs in combination with p16-positivity were shown to have a significantly better prognosis.

The rationale behind examining the infiltration density of CD4+ and CD8+TILs in relation to HPV status, p16 expression, and to prognosis is rooted in the hypothesis that the improvement in prognosis conferred by positive HPV status may relate to enhanced immune surveillance within the tumor microenvironment (225). Studies on HPV-driven anogenital and oropharyngeal cancers provide evidence of an immunological difference between HPV-negative and HPV-positive tumors: higher numbers of infiltrating CD4+ and CD8+ TILs were observed in HPV-positive cancers (192, 198, 226). In our tumors samples, we found that HPV-positive tumors had a higher median and mean number of CD4+ and CD8+TILs with a wider range in counts than did HPV-negative tumors. The results are in line with previous findings, but without reaching statistical significance, possibly due to an underpowered study design, for which reason further investigation is warranted.

The 5-year overall survival curves that take CD4+ and CD8+TIL infiltration into account are in line with previously published studies of cervical cancer and oropharyngeal cancers in which an active immune response, especially as reflected by higher CD8+ TIL infiltration, correlates with better survival among HPV-positive cancer patients (197, 198, 227, 228). Tumors that were positive for p16 and demonstrated high CD8+ TIL infiltration were associated with significantly better survival. However, the reasons for this finding remain to be clarified and further insight may be provided by studies that consider expression of cytokines and immune cell infiltration of specific subgroups of cytotoxic T-cells, as discussed more in paper IV. In conclusion, the results from this exploratory study will hopefully provide initial motivation to further investigate the role of the tumor microenvironment in relation to tumor infiltrating lymphocytes in HPV-positive and HPV-negative vaginal cancer.

## 4.8 CONCLUDING REMARKS

A few issues concerning the research and studies included in this thesis deserve additional reflection. Primary vaginal carcinoma is a rare cancer of the female genital tract. Research into this rare cancer is sparse due to its low incidence (229). RARECARE, the leading network on rare cancers in Europe, defines a rare cancer as one in which less than 6 cases per 100 000 individuals occurs per year (230). According to this definition, the aggregate incidence of all rare cancers amounts to 22% of all cancers (231). Rare cancers are associated with a number of concerns regarding inequities in diagnosis and treatment, which may result in suboptimal clinical outcomes for such patients (232), as reflected in survival statistics, which are worse for rare cancers than for common cancers (Figure 16) (230).

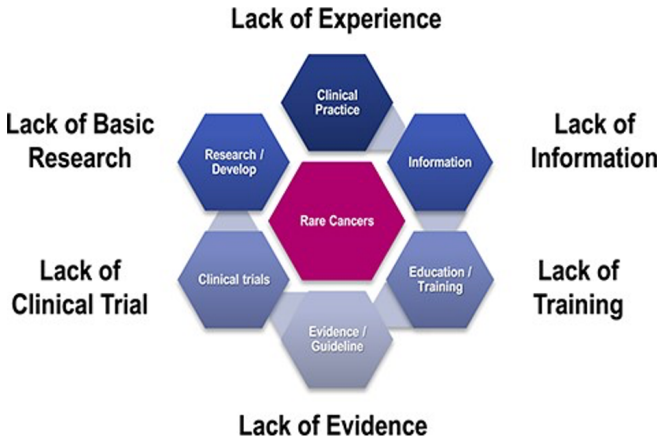



Figure 16. The lack in rare cancers. Reprinted with permission from Oxford University Press; Kawai A, *et al*. Rare cancers in Japan: definition, clinical features and future perspectives. Jpn J Clin Oncol. 2020 Sep 5;50(9):970-975.

Importantly, clinical studies on rare cancers pose distinct challenges. Methodological recommendations for clinical studies of rare cancers have been formulated to encourage research, with the ultimate goal of avoiding discrimination against these patients due to the rarity of their disease (233). The strategy includes observational clinical studies on selected patient subgroups, especially pertaining to natural history and clinical characteristics. Moreover, clinical studies, which in the best of cases may result in great benefit, are justifiable, even though the target population is small. Although associated with a higher degree of uncertainty due to the paucity of eligible patients, an alternative statistical approach using Bayesian analysis is worth consideration (233, 234). In the Bayesian approach, concerns over a statistically significant p-value are replaced by an alternative statistical assessment. This methodology relies on probabilities as they relate to statistical inference, which may allow the data to approach clinical significance, rather than relying on frequentist hypothesis testing, in which the p-value determines statistical significance.

The past few decades have seen the emergence of new opportunities concerning methodologies capable of overcoming challenges and facilitating research on rare cancers. Examples include social media patient communities, genome characterization, studies on cell lines and patient-derived xenografts, as well as small-molecule screening (235). Translational cancer medicine has made enormous technological advances in the fields of various “omics.” However, immunohistochemistry, unlike high throughput quantitative techniques, allows for both qualitative and quantitative assessment of molecular markers in tumor tissue and thereby retains some pragmatic value since such markers are often routinely required for tumor analysis (236).

The weaknesses that are common to the four studies included in this thesis should also be addressed. The study population and biological materials are dated, which may render results with poor external validity. Although retrospective cohort studies provide a useful, practical design for study of a rare disease while allowing for reliable follow-up data, the study cohort should be updated since treatment and survival trends improve over time.

We have conducted four studies based on well-established immunohistochemistry methods. Since immunohistochemical detection of proteins in formalin-fixed, paraffin embedded samples is still the foundation of current routine clinical pathology, it is important to recognize the challenges and inherent limitations of this method. The repeatability, reproducibility, sensitivity, and quantification of results from immunohistochemical assays may be called into question. While standardized protocols for tissue handling, immunohistochemical methodology, and scoring may help to improve the quality of results, they are not always available, as was the case in our exploratory studies of novel biomarkers. This point can also be illustrated by the inconsistency in defining p16 expression in study I and study IV, which made comparisons more difficult. Moreover, there was a lack of strict adherence to the REMARK recommendations discussed in the introduction.

Immunohistochemistry is a semi-quantitative, rather than a quantitative, method, with recognized issues pertaining to subjective interpretation (237). Intraobserver and interobserver variation have been demonstrated in multiple studies. Measures could have been taken to improve the reliability of the results from immunohistochemical analysis; percent agreement and kappa are examples of metrics that are often used to evaluate interobserver variability. On one hand, we need to question the clinical validity and applicability of conclusions drawn from studies showing prognostic differences that different pathologists may have assessed differently. On the other hand, it is important to remember that immunohistochemistry provides us with low-cost feasible methodology and, even more importantly, valuable morphological information to aid in the assessment of the distribution of molecular markers in tumor tissue. As always, larger populations and sample sizes are preferable in the quest to further define and confirm observations arising from the studies.

This thesis presents four studies aimed at investigating both novel and established biomarkers with a focus on patient outcome and survival in primary vaginal cancer. These studies, possessing both exploratory and validating intent, apply clinically feasible methodology to investigate various aspects of the carcinogenic process at both the molecular and tissue levels. In summary, we found that p16 may be of interest as a prognostic marker, especially when combined with assessment of HPV status. Moreover, the LRIG1 and dyskerin molecular markers emerge as potential novel prognostic marker of interest. CD8+ TIL infiltration may also be of interest as a prognostic factor, when considered together with HPV status and p16 expression. The underlying biological mechanisms in HPV-negative vaginal cancers should be further investigated and additional studies with larger cohorts performed to clarify the prognostic impact of HPV in vaginal cancer. Further studies are needed to identify and validate biomarkers and to pursue their usefulness for clinical prognostication at time of diagnosis in order to improve clinical outcome and survival in women with vaginal cancer.

# 5 CONCLUSIONS

*Study I:*

- HPV was present in 43% of the analyzed tumor samples. HPV only correlated with low histopathological grade, and not with survival.
- Ki67 was expressed in 10%-50% of cells in 56% of the analyzed tumors and expressed in >50% of cells in 34% of the tumors. Ki67 correlated with histopathological grade and tumor size, but not with survival.
- p16 overexpression was associated with poorly differentiated tumors and with presence of HPV. P16 overexpression was also associated with long-term survival in vaginal cancer. However, it was not an independent prognostic factor.

*Study II:*

- LRIG1 and LRIG 2 expression were both high in 83% of tumors, while LRIG3 expression was absent or low.
- LRIG1 expression correlated with HPV positivity and with cancer-specific survival. LRIG2 and LRIG3 did not correlate with clinical manifestations, tumor characteristics, or survival.
- LRIG1 was associated with improved survival and may thus potentially serve as a prognostic factor in vaginal cancer.

*Study III:*

- Dyskerin was expressed in all but one tumor sample; the majority of tumors showed weak staining intensity.
- WRAP53ß was also expressed in the majority of tumor samples, but without significant association to clinical variables or survival.
- Dyskerin, but not WRAP53ß, was associated with improved survival and may thus potentially serve as a prognostic factor in vaginal cancer.

*Study IV:*

- Higher infiltration of CD4+ and CD8+ TILs in the tumor microenvironment was observed in HPV-positive and in p16 positive tumors.
- Higher infiltration of CD4+ and CD8+ TILs suggests improved survival, but these results failed to reach significance with the exception of higher infiltration of CD8+ TILs in tumors overexpressing p16. These results indicate that CD4+ and CD8+ TILs infiltration of the tumor microenvironment may hold biological significance and be of interest for study in a larger cohort to achieve better power.
- Overexpression of p16 in tumor samples was associated with superior survival in vaginal cancer.
- The dual negativity of HPV status and p16 overexpression was associated with inferior overall survival.

- The value of HPV as a prognostic factor remains uncertain, but analysis of HPV status in addition to other biomarkers, such as LRIG1, dyskerin, and p16, may hold more value for prognostication.

# 6 POINTS OF PERSPECTIVE

The scope of HPV related disease will see rapid change in years to come. The World Health Organization has launched a global strategy as a call for action to eliminate HPV and cervical cancer as a public health problem. This strategy targets 90% HPV vaccination coverage in girls, along with intensified screening and surveillance of individuals at risk. Increased HPV vaccination coverage in women will reduce the burden of precancerous and cancerous lesions not only in the cervix, but at all anogenital and oropharyngeal sites. However, measures aimed at primary prevention using HPV vaccination will not impact the prevalence of vaginal cancer for decades and in the meantime, it is possible that the number of vaginal cancers will increase as the population ages.

Concerning secondary prevention, learning how to identify women at risk of developing vaginal cancer in the future is important. Studying the prior history of HPV infection in patients who were later diagnosed with vaginal cancer may provide additional knowledge regarding the natural history of the disease. A study is planned to investigate the prevalence of previous HPV infections in screening cytologies. This may also provide knowledge to formulate recommendations for surveillance of HPV-positive women with vaginal lesions. Close monitoring of the impact of the recently introduced screening program on vaginal precancerous and cancerous lesions is also warranted.

Recently, promising studies have been performed on methylation signatures for early detection of cervical cancer and for identification of subtypes in an effort to stratify prognosis in cervical cancer patients. By extension, plans for studies of methylation markers in vaginal cancer are underway as we update our database on vaginal cancers to cover the years 2003 and forward.

Future research on tertiary prevention of vaginal cancer should take into account HPV status when assessing different interventions, which could culminate in changes in clinical practice. We have performed hypothesis-generating studies on potential novel molecular markers relating to HPV status, where we found LRIG1 and dyskerin to be the most interesting for further investigation. Future *in vitro* studies on cell lines using RNA sequencing analysis may clarify the molecular mechanisms for up- and downregulation of these markers.

Based on the results of the fourth study of this thesis, future studies should investigate T-cell subpopulations, especially regulatory T cells, in order to further deepen understanding of the immunological processes in vaginal cancer. Combined analysis of the prognostic factors p16 and HPV status in vaginal cancer emerges from this thesis as the finding with the most relevant clinical application. This study in particular should be performed on a larger, updated study cohort since it could provide a prognostic classification tool that could easily be integrated into clinical practice.

Because clinical studies have generally focused on more common gynecological cancers, such as cervical cancer, treatment strategies for these cancers are based on a broader body of knowledge, but similar or identical treatment strategies could potentially be used in vaginal cancer provided that they share the same etiology and prognostic factors. By taking etiology and prognosis into account, individualized treatment strategies can be devised which eliminate over- and undertreatment. Regardless, both HPV-positive and HPV-negative vaginal cancers, although rare, deserve further attention in the future.

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