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THE ROLE OF PSYCHOLOGICAL STRESS IN CERVICAL AND PROSTATE CARCINOGENESIS

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THE ROLE OF PSYCHOLOGICAL STRESS IN CERVICAL AND PROSTATE CARCINOGENESIS
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my beloved family

致我深爱的家人

ABSTRACT

Psychological stress is common in everyday life, and has been well recognized as one of the major contributors to many mental and physical illnesses, such as mental disorders and cardiovascular diseases. The association between psychological stress and carcinogenesis remains largely inconclusive to date, although possible biological mechanisms, i.e., through dysregulation of immune functions and the neuroendocrine axis, have been proposed. The overall aim of this thesis is to investigate the impact of psychological stress on cancer initiation and progression, using cervical cancer as an example, as well as to investigate the mechanistic contributions of immune and neuroendocrine systems to this link in cervical and prostate cancers, respectively.

In Paper I, we assessed whether loss of a family member due to death, as an extremely stressful life event, increased the risk of cervical cancer. Based on the Swedish National Cervical Screening Register during 1969 to 2011, we conducted two nested case-control studies and found that loss was consistently associated with increased risks of abnormal cytology, *in situ* and invasive cervical cancer. Loss was also positively associated with HPV16 infection, particularly high viral load and recurrent infections, as well as high-risk HPV infections, among 1,696 women who had no cervical cancer.

In Paper II, we estimated the impact of psychological stress, using stress-related mental disorders and stressful life events as proxies, on cervical cancer progression (i.e. cancer-specific survival) in a national cohort of cervical cancer patients diagnosed during 2002-2011 in Sweden. Patients exposed to a stress-related mental disorder or stressful life event had a 31% increased risk of cancer-specific mortality. The association remained statistically significant (25% risk elevation) after adjustment for multiple clinical characteristics.

In Paper III, we examined the signaling of stress-related pathways in tumor tissues of US men with lethal prostate cancer, compared with that of men with nonlethal cancer. Using extreme case-control design, we identified 113 lethal cases and sampled 291 nonlethal cases from the Physicians' Health Study and the Health Professionals Follow-up Study. We found that differential expression of genes within the adrenergic, glucocorticoid, and serotoninergic pathways was significantly associated with the risk of lethal prostate cancer.

In Paper IV, we further evaluated the differential signaling of stress-related pathways between lethal and nonlethal prostate cancers sampled from the Swedish Watchful Waiting Cohort (all with localized disease). The signaling of serotoninergic pathway was significantly associated with the risk of lethal prostate cancer; similar but weaker associations were noted for adrenergic and glucocorticoid pathways. We also explored whether germline genetic variants could explain such differential signaling. We found the variants of rs2296972 (HTR2A) and rs33388 (NR3CI) tended to be associated with lethal prostate cancer, as well as the expression of specific genes of the serotoninergic and glucocorticoid pathways in tumor tissue.

In conclusion, psychological stress is associated with increased risks of cervical cancer initiation, possibly through enhanced oncogenic infection, as well as cervical cancer progression. Differential signaling of stress-related neuroendocrine pathways, including the adrenergic, glucocorticoid, and serotoninergic pathways, in tumor tissue may contribute to prostate cancer progression in both early and relatively advanced prostate cancers; such differential signaling in early cancers may, in part, be explained by genetic predisposition.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV). * Equal contribution.

I. Lu D, Sundström K, Sparén P, Fall K, Sjölander A, Dillner J, Helm NY, Adami HO, Valdimarsdóttir U, Fang F. Bereavement is associated with an increased risk of HPV infection and cervical cancer: an epidemiological study in Sweden. *Cancer Research*, 2016; 76:643-651.

II. Lu D, Andrae B, Valdimarsdóttir U, Fall F, Sparén P, Fang F. Mental disorders, stressful life events, and cervical cancer survival: a nationwide cohort study in Sweden. *Manuscript*.

III. Lu D*, Sinnott JA*, Valdimarsdóttir U, Fang F, Gerke T, Tyekucheva S, Fiorentino M, Lambe M, Sesso HD, Sweeney CJ, Wilson KM, Giovannucci EL, Loda M, Mucci LA, Fall K. Stress-related signaling pathways in lethal and nonlethal prostate cancer. *Clinical Cancer Research*, 2016; 22:765-772.

IV. Lu D, Carlsson J, Penney KL, Davidsson S, Andersson SO, Mucci LA, Valdimarsdottir U, Andrén O, Fang F, Fall K. Genetic expression in neuroendocrine signaling pathways in lethal and nonlethal prostate cancer among men diagnosed with localized disease. *Manuscript submitted.*

RELATED PUBLICATIONS

(not included in this thesis; * equal contribution)

I. Lu D, Fall K, Sparén P, Ye W, Adami HO, Valdimarsdóttir U, Fang F. Suicide and suicide attempt after a cancer diagnosis among young individuals. *Annals of Oncology*, 2013;24(12):3112-7.

II. Zhu J, Lu D, Sveinsson O, Wirdefeldt K, Fall K, Piehl F, Valdimarsdóttir U, Fang F. Is a cancer diagnosis associated with subsequent risk of transient global amnesia? *PLoS One*, 2015;10(4): e0122960.

III. Lu D, Andersson TM, Fall K, Hultman CM, Czene K, Valdimarsdóttir U, Fang F. Clinical diagnosis of mental disorders immediately before and after cancer diagnosis: a nationwide matched cohort study in Sweden. *JAMA Oncology*, 2016;2(9):1188-96.

IV. Shen Q*, Lu D*, Schelin ME, Jöud A, Cao Y, Adami HO, Cnattingius S, Fall K, Valdimarsdóttir U, Fang F. Injuries before and after diagnosis of cancer: nationwide register based study. *British Medical Journal*, 2016; 354:i4218.

V. Bond E*, Lu D*, Herweijer E, Sundström K, Valdimarsdóttir U, Fall K, Arnheim-Dahlström L, Sparén P, Fang F. Sexually transmitted infections after bereavement - a population-based cohort study. *BMC Infectious Diseases*, 2016;16(1):419.

VI. Lu D, Ludvigsson JF, Smedby KE, Fall K, Valdimarsdóttir U, Cnattingius S, Fang F. Maternal cancer during pregnancy and risks of stillbirth and infant mortality. *Journal of Clinical Oncology*, 2017;35(14):1522-1529.

VII. Song H, Fang F, Valdimarsdóttir U, Lu D, Andersson TM, Hultman C, Ye W, Lundell L, Johansson J, Nilsson M, Lindblad M. Waiting time for cancer treatment and mental health among patients with newly diagnosed esophageal or gastric cancer: a nationwide cohort study. *BMC Cancer*, 2017;17(1):2.

VIII. Sundström K, Lu D, Elfström KM, Wang J, Andrae B, Dillner J, Sparén P. Follow-up of women with cervical cytological abnormalities showing atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion: a nationwide cohort study. *American Journal of Obstetrics & Gynecology*, 2017;216(1): 48.e1-48.e15.

CONTENTS

LIST OF ABBREVIATIONS

The following abbreviations have been used in this thesis and in the associated four original publications:

ADRA1D	Adrenergic Receptor Alpha 1D
ADRB2	Adrenergic Receptor Beta 2
CI	Confidence Interval
CIN	Cervical Intraepithelial Neoplasia
CREB	cAMP-Responsive-Element-Binding
DASL	cDNA-mediated annealing, selection, ligation, and extension
eQTL	Quantitative Trait Loci
FFPE	Formalin-Fixed, Paraffin Embedded
FIGO	International Federation of Gynecology and Obstetrics
FISH	Fluorescence in situ Hybridization
GEO	Gene Expression Omnibus
GSEA	Gene Set Enrichment Analysis
HPFS	Health Professionals Follow-up Study
HPV	Human Papillomavirus
HPA	Hypothalamic–Pituitary–Adrenal
HR	Hazard Ratio
HTR	5-Hydroxytryptamine Receptor
ICC	Invasive Cervical Cancer
ICD	International Classification of Disease
IR	Incidence Rate
KEGG	Kyoto Encyclopedia of Genes and Genomes
LISA	Longitudinal Integration Database for Health Insurance and Labour Market Studies
NCSR	National Cervical Screening Register
NKCx	Swedish National Cervical Screening Registry
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PHS	Physician's Health Study

PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate-Specific Antigen
RRR	Relative Risk Ratio
SD	Standard Deviation
SNOMED	The Systematized Nomenclature of Medical Diagnoses
SNP	Single Nucleotide Polymorphisms
TUNEL	Terminal Deoxynucleotide Transferase–Mediated dUTP Nick End Labeling
TURP	Trans-Urethral Resection of the Prostate

1 INTRODUCTION

Psychological stress is common in everyday life, and occurs when we are emotionally challenged by perceived threats. Stress has been well recognized as one of the major contributors to many mental and physical illnesses, such as mental disorders, cardiovascular diseases, metabolic disorders, infection, and also increased mortality.(1) The link between stress and carcinogenesis, however, remains largely inconclusive to date.(2,3) The current evidence lends more support to the role of stress in cancer progression rather than cancer initiation, and highlights its varying effects across cancer sites.(2,3) Over the last decade, groundbreaking experimental studies have provided insights into the multiple biological mechanisms underlying this link.(4,5) For example, stress-induced immune dysfunction may predispose individuals to oncogenic infections, proposing a potential particular link to infection-related cancers including cervical cancer (5) and adrenergic signaling affected by stress has been demonstrated to play a pivotal role in several cancers, including prostate, breast and ovarian cancers.(4)

Human papillomavirus (HPV) infection is a necessary cause for cervical cancer.(6) Most women who contract HPV, however, never develop cervical cancer.(7) It is plausible that psychological stress may predispose women to oncogenic HPV infections and possible subsequent cervical cancer initiation.

Although the findings linking psychological stress to cancer progression are more consistent compared to findings on cancer initiation,(3) very few studies so far have specifically examined the impact of psychological stress on cervical cancer survival(8-10), and none of these studies have had the statistical power to draw a firm conclusion, likely due to the rarity of the disease in developed countries.

Evidence from cell lines and animal studies underscore the roles of several stress-related signaling pathways in the progression of a variety of cancers, including prostate cancer.(4) However, data in human settings are lacking and the four major stress-related pathways have never been examined simultaneously to understand the different weights of their mechanistic contributions to prostate cancer progression.

Emerging findings further imply that stress-related pathways, particularly adrenergic pathway, may exert most pronounced impact on the early stage of cancer progression, in order to initiate dissemination(11), but no human studies have evaluated the role of stress-related pathways specifically in the progression of early-stage prostate cancer. It is also largely unknown whether genetic variation contribute to the mechanisms linking stress-related pathways to prostate cancer progression.

2 BACKGROUND

2.1 PSYCHOLOGICAL STRESS

In 1915, *Walter Cannon* first proposed the "fight-or-flight" theory describing how animals react to threats.(12) In 1936, *Hans Selye* first introduced the "general adaptation syndrome",(13) later renamed to the term "stress", as the non-specific response to any noxious stimulus threatening homeostasis - the maintenance of internal stability against challenges from the external environment.(14) There are two aspects of stress - the perceived stimulus that threatens homeostasis is referred to "stressor", while the "stress response" is defined as the reaction aimed to regain homeostasis.(1) Also, stress is generally classified in two different phases – acute and chronic stress – with different influence on our body.(15) Given the misuse of the broad definition, *Koolhaas* and colleagues proposed that the "stress" terminology may be applied to uncontrollable and/or unpredictable stimuli only.(16) Of note, stress comprises psychological and physical stress; the latter is however out of the scope in this thesis.

Biological Responses

The hypothalamic pituitary adrenocortical (HPA) axis and sympathetic nervous system (SNS) are generally considered as two key players in stress responses. These two master systems coordinate and synchronize the peripheral physiology at multiple levels of cells, tissues and organs, in interaction with the external environment. When the perception of a stressor is processed by the central nervous system, stress hormones are released by the HPA axis and SNS as a part of the biological stress response, leading to fast energy mobilization and redistribution. The HPA axis releases the hormone cortisol from the adrenal glands into the circulation. The SNS secretes catecholamine by nerves and adrenal medulla. Consequently, the blood pressure and heart rate increase to deliver more energy, and the immune system is activated. The homeostasis will then be re-established if the stressor falls within the adaptive capacity. However, the recovery of the physiological action would be compromised if the demands continue to exceed the adaptive capacity (i.e., maladaptiveness). Due to the fundamental impact of stress hormones on our physiological system, a variety of health consequences may accompany the stress response, including changes in neurobiology, immune, circulation, metabolism, even mood and behavior, if the reaction is sustained. For instance, stress may result in sustained higher blood pressure and vascular hypertrophy through chronic SNS stimulation,(17) and altered cytokine profiles and immunity through elevated baseline stress hormones.(18)

Measurement

The most common approach for stress measurement is through different rating scales. In 1967, *Thomas Holmes* and *Richard Rahe* developed the Social Readjustment Rating Scale (SRRS), which assesses the experience of 43 stressful life events during the past six months and use the sum of event-specific scores to predict subsequent risk of illness.(19) Later, the

SRRS was improved to the Life Experience Survey based on 57 life events. It allows the separate assessment on both positive and negative events as well as individualized rating for the impact of each event to better reflect the magnitude of perception and coping to individual event.(20) Both of these original rating scales have rarely been used in large-scale epidemiological and clinical studies, instead it is more common to focus on one life event only,(3) such as loss of a family member, or use modified and shortened rating scales.(21) In addition to life events, a number of other rating scales have been developed and applied to directly measure emotional distress, such as the Center for Epidemiologic Studies-Depression Scale and General Health Questionnaire.(22) Furthermore, several mental disorders with a confirmed link to psychological stress, including depression,(23) anxiety,(24) stress reaction and adjustment disorders,(25) are also commonly used as a proxy for psychological stress, at least in psycho-oncology research.(3)

A comprehensive biological measurement for stress is generally lacking in the present literature of human studies.(2) However, the search for ideal stress biomarkers has not ceased. Compared to the rating scales, stress biomarkers could shed light on the underlying biological mechanisms and be less influenced by reporting bias.(26) The most popular biomarker for stress measurement to date is cortisol. The cortisol levels obtained from saliva, blood and urine profile the circadian rhythm and reflect short-term stress response, whereas cortisol levels obtained from hair or nails illustrate the average cortisol concentrations during a longer period of time and measure the chronic stress.(27) Other primary mediators in stress responses may also serve as stress "biomarkers", including sympathetic activity, neurotransmitters, and inflammatory cytokines.(28) Secondary mediators, which represent the cumulative effects of the primary mediators on a specific target, have also been recommended as possible stress "biomarkers", such as heart rate variability, blood pressure, waist-hip ratio, and glycosylated hemoglobin.(28) Of note, the signature of adrenergic pathways in tumor tissue may also correlate with stress levels, reported for depressive symptoms and low social support, among ovarian cancer patients.(29) Additionally, antibodies to latent viruses and vaccination responses to antigen exposure have been suggested as candidates to reflect the effect of stress on immune system.(30) However, none of these biomarkers is specific to stress response. For example, cortisol levels are also associated with physical activity and obesity.

2.2 CERVICAL CANCER

Cervical cancer is the fourth most common cancer in women, with about half a million new cases diagnosed annually worldwide.(31) The incidence of cervical cancer varies geographically, and is much higher in less developed regions. In 2015, with a total number of 563 new cases and 163 deaths, cervical cancer was ranked as the 13th most common cancer among women in Sweden.(32) However, among women younger than 45, cervical cancer is the most, or the second most, common cancer type across 123 countries.(33)

Typically, it takes years for cervical cancer to develop from precancerous lesions, which creates room for early detection by cervical screening. The conventional cytology (i.e.,

Papanicolaou test, also known as Pap smear) and liquid-based monolayer cytology are still the most popular methods for cervical screening. Cytological abnormality determined by cervical screening generally requires further investigation through colposcopy - the macroscopic examination of the cervix during a gynecological exam, sometimes including a biopsy. The histological diagnosis of the biopsy includes 1) cervical intraepithelial neoplasia grade 1-3 (CIN 1-3), or invasive squamous cervical cancer if the lesion originates from squamous epithelium (constitutes 90% cervical cancer); 2) cervical adenocarcinoma *in situ* or invasive cervical adenocarcinoma, if the lesion derives from glandular cells; or 3) normality. Annually around 20,000 Swedish women receive an abnormal screening result, and around 3,300 women get a diagnosis of cervical cancer *in situ*, meaning that the dysplasia spans more than 2/3 of the full epithelium and is interchangeably used with CIN3 in Sweden.(34)

In early stages, cervical cancer may be asymptomatic. Symptoms, such as abnormal vaginal bleeding, pain during sexual intercourse, or pelvic pain, commonly manifest later in the course of the disease. Treatment for CINs includes excision of the lesion (e.g. cervical conization) and destructive techniques such as laser vaporization. Regarding invasive cervical cancer, the surgical approach includes hysterectomy (removal of the entire uterus and part of the vagina) or trachelectomy (removal of the cervix and part of the vagina, but leaves the rest of the uterus intact) for selected patients. Radiation therapy and chemotherapy are demanded for more advanced cases. During 1990-2001, the 5-year relative survival of cervical cancer was about 95% for screening-detected cervical cancer, and 69% for symptomatic cancers, in Sweden.(35)

Risk Factors

HPV infection has been recognized as a necessary but insufficient cause in cervical carcinogenesis.(6) HPV is a diverse family of viruses with over 170 types, and it is transmitted primarily through sexual activity. Most women (75%-80%) contract HPV at some point during their lives, and about 90% of infections are cleared spontaneously within 1-2 years and only very few develop cervical cancer.(36) Among many HPV types, however, only 13 types are classified as oncogenic types, known as high-risk HPV.(37) Notably, HPV16 and HPV18 account for roughly 70% of all cervical cancer cases.(7) Growing evidence also suggests that persistence of HPV infection plays a key role in the malignant transformation.(7) However, the natural history of HPV infections remains largely unknown to date.(38). For instance, it is unclear if the observed “persistent” infection is due to continuously active HPV infection, reappearance after an undetectable state of viral latency, or by a re-infection. Several other factors have also been suggested to contribute the progression of HPV infection, including high-risk HPV types, viral load, and oncogene integration.(39)

Other risk factors, for example smoking, use of hormonal contraceptives, and high-risk sexual behavior, might also contribute to cervical carcinogenesis. Tobacco smoking is associated with a 60% increased risk of cervical squamous cell carcinoma (both invasive and *in situ*) compared to never-smokers, and the risk elevates further by increasing number of cigarettes

smoked per day.(40) Long-term hormonal contraceptives use (>5 years) has been associated with a double risk for cervical cancer.(41) Although debated, early age at sexual debut (typically before the age of 14) has been suggested to increase the risk of cervical cancer.(42) Women with six or more sexual partners have three times higher risk of cervical cancer, compared with women with only one partner.(42) High parity has been suggested as a risk factor for cervical cancer, mainly in less developed countries. The risk increase is however only pronounced among women with >7 full-term pregnancies.(43)

Of note, many risk factors may interact with HPV infection in modulating the risk of cervical cancer, rather than leading the carcinogenesis themselves. For example, instead of being directly carcinogenic to the cervix, oral contraceptive might make cervical mucosa vulnerable to HPV infection due to hormonal influence and subsequently contribute excess risk for cervical cancer.

Prognostic Factors

Clinical characteristics, such as tumor stage and pathological features, are the most important indicators for cervical cancer prognosis to date. The stage of cervical cancer is determined clinically at the time of primary diagnosis, mainly based on tumor size and its potential spread to the pelvis. The International Federation of Gynecology and Obstetrics (FIGO) staging system is most widely used. Five-year survival approaches 100% for microinvasive and 70-85% for localized cervical cancer.(44) Surgical staging may help improve the prediction by examining adverse pathological characteristics, including lymph node metastases, depth of cervical invasion, and involvement of lymph-vascular space. Certain histological subtypes, such as adenosquamous carcinoma and small-cell carcinoma with neuroendocrine features, constituting around 5% of cervical cancers, are associated with poorer prognosis.(44)

HPV status and infection activity is a potential predictor for cervical cancer survival. In general, HPV infection indicates a favorable prognosis in patients with cervical cancer, even when controlled for clinical characteristics.(45,46) Conflicting results, however, exist between infections of different HPV types. Several studies reported that HPV18 positive cervical cancer was associated with poorer prognosis after primary treatment compared to HPV16 positive cancer, whereas others indicated HPV16 positivity actually predicted worse outcomes compared to HPV18 positivity.(47,48) It is plausible that viral oncoproteins, encoded by E6 and E7 genes from high-risk HPVs, inactivate the tumor suppressor gene (e.g. p53) and therefore account for the different clinical courses. Although less discussed, overexpression of vascular endothelial growth factor, epidermal growth factor receptor, cyclooxygenase-2 and tumor hypoxia have also been suggested to be potentially able to predict cervical cancer outcomes.(47)

2.3 PROSTATE CANCER

Prostate cancer is the most common cancer among men in Europe and the US, and the second most common cancer among men in the world.(31) In 2012, over one million men were

diagnosed with prostate cancer globally. Almost all prostate cancers occur over the age of 50 (99.9%) (49) and are adenocarcinomas originating from prostatic epithelial cells (95%).(50) Early prostate cancer hardly ever manifests symptoms. Later in the course of disease, early symptoms include frequent urination, difficulty to urinate in a steady stream, painful urination, and blood in the urine. Late symptoms such as bone pain may emerge when the cancer has spread out of the prostate, often to the spine, hips and ribs.

Prostate cancer usually grows slowly and one can live with prostate cancer for years without perceiving any symptom. Men recently diagnosed with prostate cancer are more likely to die from another cause, such as cardiovascular disease, than from prostate cancer.(51) About one third of Swedish patients and 11% of US patients died from their prostate cancer within 5 years after diagnosis from 1961/1973 through 2008.(51) There were 307,000 prostate cancer deaths worldwide in 2012, making prostate cancer the fifth leading cause of cancer-related death in men.(31)

It is of paramount importance to distinguish lethal or aggressive prostate cancer from relatively benign ones at the time of diagnosis, because many low-risk cases could be safely managed with active surveillance or watchful waiting.(52) According to clinical guidelines, physicians evaluate tumor characteristics, predicted prognosis, life expectancy, and individual preferences when tailoring individual treatment plan for prostate cancer.(53) Current treatment options include, but are not limited to, surgery, radiation therapy, hormone therapy, and chemotherapy.

Risk Factors

The etiology of prostate cancer remains enigmatic and poorly understood. Genetics, as well as dietary and lifestyle factors, have been suggested as primary contributors to the risk of prostate cancer. Other established risk factors include age and androgens.

Familial clustering of prostate cancer was first reported in 1956.(54) In 2000, the Nordic twin cohort indicated that genetic factors explained 42% variants in the liability of developing prostate cancer.(55) More recently, the expanded Nordic twin cohort, using novel statistical strategies, suggested even higher genetic contribution to the disease risk (58%).(56) Genome-wide association studies have identified more than 90 common single-nucleotide polymorphisms (SNPs) related to the susceptibility for prostate cancer, which are estimated to explain about 30% of the familial risk for prostate cancer.(57)

The apparent geographic variation in prostate cancer incidence and the remarkably increased prostate cancer risk in migrants moving from low-incidence to high-incidence countries support the contribution of environmental factors to prostate cancer development.(58) A number of dietary agents, such as fat, red and processed meat, vitamin E, have been associated with prostate carcinogenesis.(58) However, few findings have been confirmed in large chemoprevention studies.(58) Higher physical activity is suggested to be associated

with 10% lower risk of prostate cancer,(59) and higher body mass index is related to increased risk of advanced prostate cancer.(60)

Prognostic Factors

Many localized cancers are slow-growing regardless of treatment,(61) whereas others develop metastases despite surgical treatment.(62) Nomogram based on Gleason scores, tumor stage and pretreatment prostate-specific antigen (PSA) levels has been developed to predict the prognosis(63). However, they cannot completely explain the biological heterogeneity of prostate cancer regarding survival. Although the potential roles of variation in genes and gene expressions are much less established in relation to lethal outcome,(64,65) a handful of biomarkers have been identified for prostate cancer progression.

Gleason score

Gleason score is a widely-used system to evaluate prostate tumor grade. It is scored from 1 to 5 according to the histological pattern of differentiation and arrangement of tumor cells in hematoxylin and eosin stained sections. The Gleason patterns 4 and 5 predict poor prognosis. Two most dominant patterns are scored and summed to comprise the Gleason score ranging from 2 to 10. Gleason grade has been demonstrated to have a good correlation with the prostate cancer prognosis and is one of the most powerful predictors.(66)

Tumor stage

The prognostic classification and selection of treatment are highly dependent on tumor stage, which can be determined either by clinical stage or pathological stage. Studies have shown that both clinical and pathological stage are reliable predictors for prostate cancer survival after radical prostatectomy(67), and most of the localized cancers have a relatively benign course even without initial treatment.(61)

Biological markers

PSA, a glycoprotein enzyme released by the epithelial cells of the prostate gland, is present in small amount in the blood of healthy men but often rises in prostate cancer or other prostate diseases. PSA can provide information for screening (under debate), diagnosing, monitoring treatment response and recurrence for prostate cancer.(68) However, whether PSA is predictive for long-term survival independent of tumor grade and stage requires further investigation.(69)

Ki-67 is one of the cell-cycle-regulating proteins and expressed in every phase of the cell cycle, except in resting cells. The Ki-67 index (the percentage of positive nuclei in immunohistochemistry staining) has been used as a reliable biomarker to measure cell proliferation. Consistent evidence has shown that Ki-67 is associated with disease progression and survival in prostate cancer.(69) However, given its relatively low expression in preoperative biopsies, Ki-67 might not be as effective as the other aforementioned classic factors in prognosis prediction.(69)

Angiogenesis plays a pivotal role in carcinogenesis by providing nutrients and oxygen for tumor growth, as well as direct access to vasculature for metastasis. Microvessel density, a measure of the extent of neoangiogenesis, has been suggested to be a predictor for prostate cancer survival by some, but not all, studies.(69) Other morphological features of angiogenesis, e.g. smaller vessel size and greater irregularity of vessel lumen, might also predict prostate cancer mortality.(70)

Apoptosis, the programed cell death, is commonly visualized by using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays to detect DNA fragmentation. The balance between apoptosis and proliferation determines the rate of tumor growth. Decreased apoptosis has been associated with the recurrence of prostate cancer.(71)

Perineural invasion, the presence of cancer cells surrounding nerves fibers, is an adverse pathologic characteristic reported on prostate biopsy specimens. The perineural space is believed to serve as a path of decreased resistance for tumor cells spreading. Convincing evidence has suggested that perineural invasion in prostate biopsies is a promising prognosticator for prostate cancer.(72) However, controversy still exists whether perineural invasion predicts disease recurrence and survival independently from PSA level and Gleason score.(73,74)

The promoter region of transmembrane protease serine 2 (TMPRSS2) gene commonly fuses with the coding region of v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) gene – a member of the erythroblast transformation-specific family of transcription factors – in prostate cancer.(75) TMPRSS2-ERG gene fusion upregulates the expression of ERG, and has been associated with poorer survival among prostate cancer patients in a watchful waiting setting,(76) but not in alternative settings.(77)

2.4 PSYCHOLOGICAL STRESS AND CANCER

Among a number of health consequences potentially related to stress, the potential link of stress and cancer has been elusive for centuries. *Galen*, the ancient Greek physician, observed that women with the “melancholic” temperament was more likely to develop cancer than women with the “sanguine” temperament.(3) The body of literature on the association between stress and cancer has been dramatically growing during the past 40 years. The question, however, remains unanswered.(2) *Bert Garssen* reviewed the existing findings and summarized a few reasons for the lack of conclusion to date. Many studies were small in size and therefore unable to distinguish the presumably mild effect of stress on cancer from null. The type of cancer is likely highly relevant in this regard – cancers that are of immunological origin or with poor prognosis might be more susceptible to stress. Most studies also lacked control for confounders, such as behavioral factors.(2) A true biological link between stress and cancer is however still highly plausible, given the increasingly strong evidence between stress and cancer progression.(3) Pooling available prospective cohort studies, *Chida* and colleagues quantified the effect-size of psychosocial factors on cancer incidence (Hazard ratio [HR] 1.06, 95% confidence interval [CI] 1.02 to 1.11) and cancer progression (HR 1.03, 95%

CI 1.02 to 1.04) in general.(3) Provided with the potential publication bias as noted, the effect size of psychological stress on cancer incidence and progression is presumably small.(3) The associations are however stronger, in well-designed studies, for certain stressors (e.g. life stressor for cancer progression), as well as for certain cancer types (e.g. lung cancer). In parallel with human findings, new evidence has been accumulating from animal studies shedding light on the biological plausibility underlying the stress-cancer link.(78)

Table 1. Prospective studies investigating the link between psychological stress and cervical cancer.

Study	Country/ region	Participants	Follow-up (years)	Stressor	Effect size
Incidence					
Chang 2015	South Korea	601,775	19 (mean)	Major depression Minor depression Chronic depression	0.72 (0.52-0.98) 1.10 (0.91-1.33) 0.52 (0.30-0.91)
Dalton 2008	Denmark	3,218,440	8 (mean)	Divorce Depression	1.90, significant 1.00, non-significant
Fang 2011	Sweden	4,687,073	0-15	Death of child	1.46 (1.17-1.80)
Goldacre 2007	UK	577,546	0-36	Depression Anxiety	0.82 (0.51-1.27) 0.85 (0.53-1.30)
Hung 2014	Taiwan	20,033	9 (mean)	Depression	1.02 (0.55-1.90)
Ikeda 2013	Japan	44,152	12 (mean)	Social support	"no significant association"
Johansen 1997	Denmark	11,231	25 (mean)	Cancer in a child	1.1 (0.9-1.4)
Kennedy 2014	Sweden	4,219,691	0-40	Death of parent, before/ after age 18	1.4 (1.1-1.6) 1.0 (0.9-1.1)
Kvikstad 1994	Norway	51,695	0-24	Death of spouse Divorce	1.04 (0.80-1.35) 1.44 (1.30-1.60)
Kvikstad 1996	Norway	44,419	0-26	Death of child	1.11 (0.92-1.35)
Shen 2013	Taiwan	19,793	2-6	Anxiety	0.93 (0.54-1.50)
Survival					
Kvikstad 1995	Norway	2,336 1,839	6 (mean) 6 (mean)	Divorce Death of spouse	1.25 (0.99-1.57) 1.59 (0.65-3.89)
Kvikstad 1996	Norway	2,674	0-26	Death of child	1.14 (0.72-1.82)
Li 2003	Denmark	513	1-18	Death of child	1.33 (0.60-2.95)

Stress and Cervical Cancer

Several,(79-81) but not all,(82-85) clinical studies have suggested an association between psychological stress and cervical dysplasia. The findings on the incidence of invasive cervical cancer are also inconclusive, particularly for the associations of stress-related mental disorders (Table 1).(8,86-95) However, the limited evidence from stressful life events (e.g. loss of a family member and divorce) tends to be consistent. All of the six prospective studies are from Nordic countries and assessed the effect of a specific life event.(8,87,88,92-94), and none of them controlled for potential behavioral or lifestyle factors. The impact of different life events might vary – for instance, divorce appears to have a greater impact on cervical cancer risk than widowhood.(94) Moreover, the impact might vary by different ages at exposure – for example, loss of a parent was more likely to affect women in their early lives in terms of cervical cancer risk.(93)

Regarding survival, the evidence is rather scarce (Table 1).(8-10) Three prospective studies suggested that life events were likely associated with an unfavorable prognosis in cervical

cancer, although none of them held enough power to draw a firm conclusion. Future studies linking stress to both incidence and survival of cervical cancer are warranted.

Table 2. Prospective studies investigated the link between psychological stress and prostate cancer.

Study	Country/ region	Participants	Follow-up (years)	Stressor	Effect size
Incidence					
Chen 2016	Taiwan	58,603	5	Anxiety	4.67 (3.06-7.12)
Dalton 2008	Denmark	3,218,440	8 (mean)	Divorce	0.97, non-significant
				Depression	0.93, non-significant
Gallo 2000	USA	804	13	Depression	11.8 (1.00-144.3)
Goldacre 2007	UK	577,546	0-36	Depression	0.69 (0.47-0.97)
				Anxiety	0.70 (0.47-0.99)
Gross 2010	USA	3,177	24	Major depression	1.09 (0.14-8.73)
Heikkilä 2013*	Europe	53,773	12(mean)	Job strain	0.86 (0.68-1.09)
Hung 2014	Taiwan	20,033	9 (mean)	Depression	2.88 (1.96-4.26)
Ikeda 2013	Japan	44,152	12 (mean)	Social support	"no significant association"
Johansen 1997	Denmark	11,231	25 (mean)	Cancer in a child	1.0 (0.8-1.2)
Keinan-Boker 2009	Israel	2,249,462	0-22	World War II, before/ after PSA era	0.47 – 0.75 1.02 – 1.34
Kubo 2006	Japan	14,052	8 (mean)	Rotating shift work	3.00 (1.20-7.70)
Lemogne 2013	France	14,203	15 (mean)	Sick leave for depression	1.39 (0.79-2.43)
				Depressive symptoms	0.99 (0.86-1.13)
				Chronic depression	0.60 (0.38-0.96)
Liang 2011	Taiwan	75,771	Unkown	Depression	1.33 (0.79-2.23)
Metcalfe 2007	Scotland	5,743	31-34	Medium daily stress	1.65 (1.20-2.27)
				High daily stress	1.35 (0.87-2.10)
Nielsen 2007	Demark	5,496	19-21	Daily stress	0.99 (0.90-1.09)
				Divorce	0.92 (0.51-1.67)
				Death of spouse	1.09 (0.59-2.03)
Penninx 1998	USA	1,708	3.8 (mean)	Chronic depressive mood	1.47 (0.20-10.8)
Shen 2013	Taiwan	19,793	2-6	Anxiety	2.17 (1.56-2.93)
Survival					
Jan 2016	Sweden	4,105	4.3 (mean)	High daily stress	1.66 (1.05-2.63)
				Often grieving from loss	1.90 (0.93-3.87)
Li 2003	Denmark	116	1-18	Death of child	4.99 (0.72-34.76)
Prasad 2014	USA	103,809	0-6	Depression	1.16-1.86

* A meta-analysis pooled 12 European cohorts from Denmark, Finland, France, Netherlands, Sweden and UK.

Stress and Prostate Cancer

Investigating the true incidence of prostate cancer is practically difficult after the introduction of PSA, because of the variable accessibility to PSA screening. To date there are 17 prospective studies or meta-analyses examining the association between psychosocial factors and prostate cancer incidence (Table 2).(87,89-92,95-106) The findings are conflicting – for example a large pooled meta-analysis showed that work stress was likely associated with a lower risk of prostate cancer,(99) whereas another cohort indicated that shift workers were at 3-fold risk of prostate cancer.(101) Although the findings were not statistically significant, two other studies have shown largely opposite associations of daily stress with the risk of prostate cancer.(104,105) The significant associations were also opposite for depression(87,89,90,102) and anxiety.(89,95,96) The discrepancy of findings before and after PSA era may suggest a potential contribution of screening seeking behaviors to prostate

cancer detection. For example, exposure to severer stressors might entail different health consciousness and willingness to seek for medical counseling, leading to different use of PSA screening and different incidence of prostate cancer.(100)

On the other hand, to the best of our knowledge, only three prospective studies examined the potential role of psychological stress on prostate cancer survival.(10,107,108) Despite limited evidence, the findings are consistent across a range of stressors. However, only one study took into account the impact of competing risks on these findings,(107) as for example psychological stress is rather consistently related to a higher risk of cardiovascular mortality among prostate cancer patients.(109)

Major Biological Mechanisms

Several potential biological mechanisms have been proposed for the link between psychological stress and carcinogenesis.(5) Given the pivotal role of SNS and HPA axis in stress system, two major pathways, i.e. adrenergic and glucocorticoid pathways, have drawn considerable attention. Both SNS and HPV axis exert profound influences on immune regulation, suggesting another important pathway through stress-induced immunosuppression and oncogenic infection. Although less investigated, other neuroendocrine transmitters potentially affected by psychological stress have also been suggested for altered cancer progression. For instance, serotonin promotes tumor cell growth and angiogenesis,(110) whereas dopamine exerts opposite actions.(111)

Adrenergic pathway

Emerging evidence has illustrated that the activation of SNS promotes cancer progression through tumor growth, dissemination, angiogenesis, malignant transformation, macrophage infiltration, and inflammation.(11) β-adrenergic receptors, G-protein-coupled receptors, mediate most of the effects of stress hormone catecholamine released by SNS. Adrenergic receptor beta 2 (ADRB2) is the dominant adrenergic receptor which has been identified on several types of cancer cells, including prostate cancer.(112) β-adrenergic receptors transmit the information from the extracellular environment to the inside of the cell and subsequently increase the intracellular level of cAMP that activates cAMP-dependent protein kinase (PKA). The downstream cAMP-responsive-element-binding (CREB) protein is either directly activated by PKA or through serine/threonine-protein kinase PAK4, and has been linked to tumor cell proliferation, dissemination, angiogenesis, as well as inhibition of apoptosis.(5) Moreover, by inhibiting the ras homology family member A, PKA induces neuroendocrine differentiation in prostate cancer cell lines, although evidence remains scarce *in vivo*.(112) Furthermore, through regulating CREB, the ADRB2/cAMP/PKA signaling pathway stimulates the androgen receptor responsive gene transcription and eventually modulates the activity of androgen receptors,(113) which is an established mechanism specifically involved in prostate cancer development. Lastly, although debated (114), results from two large observational studies suggested that use of β–adrenergic receptor blocking agents may improve cancer-specific survival among prostate cancer patients (115,116).

Glucocorticoid pathway

Glucocorticoid receptor, a member of the nuclear receptor family, is the cognate binding protein to the glucocorticoids released by HPA axis. Its activation regulates a great deal of biological processes, such as metabolism, growth and cellular apoptosis. Accumulating data substantially supports the role of glucocorticoid signaling in the progression of solid tumor through increased cell proliferation, inhibited apoptosis and DNA repair activity.(4,117) Glucocorticoid also has the potential to induce chemotherapy resistance by blocking tumor cell death,(117) although cortisol is routinely administered through chemotherapy for potential nausea and allergic reactions. As a result of chronic stress, flattened diurnal cortisol rhythms and elevated nocturnal cortisol have been commonly noted among cancer patients, particularly patients with advanced stages of disease.(118,119) The altered cortisol patterns may, in part, reflect the distress status,(120) and/or avoidant coping.(121) In addition, the flattened cortisol slope has been linked to compromised survival in several cancer populations, including breast, lung, and renal cell cancers.(118,122,123)

Immunosuppression and viral oncogenesis

The SNS and HPA axis work hand-in-hand in effecting the immune response: both catecholamine and glucocorticoids regulate different aspects of immune function, including antigen presentation, T-cell proliferation, cell-mediated and humoral immunity.(5) For example, chronic stress may induce lymphocyte reduction mediated through increased Fas receptor expression.(124) In general, compromised cellular immunity induced by chronic stress assists tumor initiation and progression.(5) The role of stress-induced immunosuppression is, therefore, possibly substantial in infection-related cancer in particular. That said, impaired cellular immunity might indirectly lead to a higher risk of oncogenic viral infection and DNA damage.(124) In fact, the first experimental demonstration of stress and cancer was based on tumor viruses in mouse models.(125) It has been shown that several human tumor viruses, including HPV16 and 33, are sensitive to the signaling pathways activated by catecholamine and/or glucocorticoids. As a result, stress hormones could reactivate latent tumor viruses, stimulate expression of viral oncogenes, and inhibit antiviral responses in the host. For example, in response to glucocorticoids, high-risk HPVs activate gene expression,(126,127) interact with cellular proto-oncogenes,(128) and evade cellular immune response through down-regulating the expression of class I human leukocyte antigens.(129) Conversely, glucocorticoid antagonists suppressed the HPV activity in cell lines.(130,131)

3 AIMS

The overall aim of this thesis was to investigate the potential impact of psychological stress on cancer initiation and progression, using cervical cancer as an example, as well as to understand the mechanistic contributions of immune and neuroendocrine systems to this link in cervical and prostate cancers.

The specific aims were:

- To assess the potential associations of loss of a family member, as an extremely stressful life event, with the risks of HPV infection, cervical dysplasia, and invasive cervical cancer.
- To estimate the impact of psychological stress, using stress-related mental disorders and stressful life events as proxies, on the cancer-specific survival among patients with cervical cancer.
- To demonstrate the differential signaling of stress-related molecular pathways in tumor tissue of US men with lethal prostate cancer, compared with men with nonlethal disease.
- To further verify the differential expression of genes in stress-related pathways between lethal and nonlethal prostate cancer among Swedish men with localized disease, and to explore whether genetic variants could explain the potential differences.

4 STUDY MATERIALS

4.1 SWEDISH DATA SOURCES

4.1.1 Swedish public and health registers

Sweden is well-known for having nationwide and high-quality registers in public and health data. Every resident in Sweden is assigned a unique personal identification number, which allows the cross-linkage of data through all Swedish public and health registers. In this thesis, we took the advantage of several major registers in Sweden to measure the indicators for psychological stress, cancer incidence and survival, as well as to identify the demographic characteristics through cross-linkages.

The Swedish Multi-Generation Register documents parental information on Swedish residents who were born from 1932 onward and alive in 1961.(132) Information on both biological and adoptive parents are available, and the coverage reaches 97% for mothers and 95% for fathers. With the parental information, the family linkage can be largely extended to other family members, such as children, spouses (confined to those with a registered common child), and siblings, of the index persons.

The Swedish Causes of Death Register comprises of the death certificates of all Swedish residents since 1911. The death certificate is compulsory for all deaths, and filled out by the physicians in charge during the last hospitalization, or by a family physician or medical examiners if death occurs outside of the hospital. The underlying cause of death is recorded in the register, and is considered as a valid source of information for studies of many diseases, including cancer.(133)

The Swedish Patient Register has collected records of hospital discharge diagnosis since 1964/1965, and covered 60% of the entire country in 1969, 85% in 1983, and 100% from 1987 onward.(134) The primary and secondary diagnoses are coded according to the Swedish versions of International Classification of Disease (ICD) system (ICD-7, before 1969; ICD-8, 1969-1986; ICD-9, 1987-1996; and ICD-10, from 1997). From 2001, the collection is expanded to hospital-based outpatient visits with a coverage of >80%. The register has been validated for many diseases and regarded as a reliable source for clinical diagnosis in general.(134)

The Swedish Cancer Register was established in 1958 to collect all malignancies and certain benign tumors diagnosed in Sweden.(135) Health care providers are requested to report any newly detected cancer to the register by law. The completeness is therefore virtually 100%. Besides cancer site, information on histology and tumor stage is also largely available from 2004 in the register.

The Longitudinal Integration Database for Health Insurance and Labor Market Studies (LISA by Swedish acronym) includes all Swedish residents aged 16 years and older since 1990. It

integrates information on marital status, employment, income, education level, etc., from the social and educational sectors as well as labor markets on a yearly basis.

Other nationwide public registers were also linked to identify demographic information, for instance country of birth through the Total Population Register, and, to determine the end of follow-up, for example on the date of emigration through the Migration Register.

4.1.2 Swedish National Cervical Screening Registry (NKCx by Swedish acronym)

Cervical screening was introduced to Sweden in the mid-1960s and the organized screening program was initiated in 1970s. Initially, women aged 30 to 49 (later 25 to 49) years were invited to screening every 3 to 4 years. From 1998, all women aged 23 to 50 are invited to screening in every third year and those aged 51 to 60 in every fifth year.(136) The NKCx has collected cervical screening records through both organized and opportunistic screening from 1969, and became nationwide since 1995.(137)

4.1.3 Two population-based studies of HPV infection and cervical cancer

The National Cervical Screening Register (NCSR) Study aimed to investigate the HPV infection in relation to cervical cancer risk, based on 1,431,723 women whose first registered smear was classified as cytologically normal in six Swedish counties during 1969-2002.(138,139) It included 1,360 women diagnosed with *in situ* or invasive cervical cancer (i.e. cases) and 1,360 controls randomly selected using incidence density sampling and matched to the case women on county of residence, age and calendar date of first normal smear registered.

With a similar purpose, the Uppsala Study was based on 146,889 women who lived in Uppsala county and participated in the cervical screening at least once during 1969-1995.(140) Among women with a first registered smear of clear result, 478 women diagnosed with *in situ* cervical cancer were enrolled as cases and 608 controls matched to the cases on age and calendar date of the first normal smear were randomly selected from the study base using incidence density sampling.

For both studies, all available smears during the study period, from included case and control women, were obtained from the county laboratories and tested for HPV infection. In the NCSR study, DNA was extracted from the archival smears using a validated method described elsewhere(141) and amplified by polymerase chain reaction (PCR).(142) The 23 types of HPV including HPV16 were then determined through a PCR enzyme immunosorbent assay and reverse dot blot hybridization procedure(143), or a multiplex fluorescent bead-based assay.(142) Among HPV16 positive samples, the absolute number of viral copies of the E7 gene per microliter were quantified as the viral load according to the Taqman real-time quantitative PCR.(144) All analyses were performed by the WHO HPV LabNet Global Reference Laboratory, Malmö, Sweden and blinded to the sample information. For the Uppsala Study, DNA extraction was done using the same method and HPV16 was

detected using a specific fluorescent hybridization probe based on the Taqman real-time quantitative PCR.(145) A certain number of PCR cycles were required to reach the threshold of accumulating fluorescence beyond the background level. The threshold cycle value was therefore calculated in HPV16 positive smears as an estimate of viral load, which is inversely correlated with the initial number of viral copies in the sample (i.e., the lower the threshold cycle value, the higher the viral load). All HPV16 analyses were done at the Department of Medical Genetics in Uppsala and blinded to clinical information.

For the Uppsala Study, information on lifestyle factors including smoking, sexual behaviors, use of oral contraceptives, parity, and abortion, was collected for 90% of participants using a comprehensive questionnaire through telephone interview.(146)

4.1.4 National Cervical Cancer Audit

The National Cervical Cancer Audit included all newly diagnosed cervical cancer cases in Sweden from 2002 through 2011. Originally, 4,646 women with an invasive cervical or unspecified uterine cancer(147) were identified from the Swedish Cancer Register. After reviewing the archived histological specimens, 4,269 women were included in this cohort. Clinical information on tumor stage, histology, mode of detection, primary treatment, and adjuvant therapy was collected through reviewing corresponding medical records.

4.1.5 Swedish Watchful Waiting Cohort

The Swedish Watchful Waiting Cohort consisted of 1,367 men who underwent trans-urethral resection of the prostate (TURP) for symptomatic benign prostatic hyperplasia and were incidentally diagnosed with localized prostate cancer in the Örebro and South East Health Care Regions in Sweden during 1977-1998.(65) The population-based prostate cancer quality registers operating in these regions were used to identify eligible cases. The specimens of tumor tissue were available for 1,256 men, and these were re-reviewed for cancer confirmation. According to the standard treatment plan at that time, all men were followed expectantly without initial treatment until disease progress, namely watchful waiting.

4.2 US DATA SOURCE

The Physician's Health Study (PHS) was a randomized control trial involving 29,067 US male physicians from 1982, originally aimed to prevent cardiovascular disease and cancer.(148) The Health Professionals Follow-up Study (HPFS) is an ongoing prospective cohort initiated in 1986, which is comprised of 51,529 US male health professionals. Its primary purpose is to identify the causes of heart disease and cancer.(149) The PHS and HPFS Prostate Tumor Tissue Cohort was nested within PHS and HPFS. As a result, 3,108 men with histologically confirmed prostate cancer were eligible for inclusion. Tumor specimens from prostatectomy and TURP were available for 2,200 participants, whereas the specimens through biopsy were not included.

5 STUDY DESIGN AND METHODS

5.1 BEREAVEMENT, HPV INFECTION, AND CERVICAL CARCINOGENESIS (PAPER I)

We included 2,466,107 women who were born in Sweden from 1932 onward and were registered for at least one Pap smear in the NKCx between 1969 and 2011 (Figure 1). We followed all women from January 1st 1969 or birth, whichever came later, until a first diagnosis of *in situ* or invasive cervical cancer, total hysterectomy, death, emigration, or December 31st, 2011, whichever occurred first.

During the follow-up, 14,011,269 registered smears were identified. Based on the age at smear and elapsed time from the latest smear, we calculated the degree of screening participation as previously described.(150) In brief, we divided the actual participation years by the sum of years scheduled for screening, until the first registered abnormal cytology. Subsequently we classified the screening adherence into five categories, namely the quartiles of the calculated degree of participation as well as the "unscreened" (i.e., only registered with opportunistic smears through the entire study period).

We carried out three parallel analyses to examine the associations of loss of a family member – one of the extremely stressful life events – with HPV infection, first abnormal cytology, *in situ* and invasive cancers.

5.1.1 HPV infection

In the NCSR Study and the Uppsala Study, the control women were randomly selected from the cervical screening participating population in different Swedish counties and had no *in situ* or invasive cervical cancer during respective study periods. We linked these control women to our study base, namely the NKCx, and identified 1,770 women with 4,987 smears that were eligible for inclusion (Figure 1). After excluding smears with poor cellularity (N=515) and repeated smears of the same workup (N=439), we finally included 1,696 women with 4,033 smears for the analysis of HPV16 infection. The repeated smears were defined as consecutive smears with elapsed time from the first smear of less than one year, assuming that the following smears were possibly a part of the same diagnostic workup. Among 4,033 smears tested for HPV16, there were 3,830 HPV16 negative and 203 positive smears, of which 160 were further measured for HPV16 viral load(138,140) and were subdivided into high or low level according to the median viral load value. Due to the observed batch effect, the cut-off values were estimated both per calendar period of sampling (1969-1984, 1985-1989, 1990-1994, or 1995-2005) and per calendar period of test (2005-2007 or 2008-2009) in the NCSR Study. HPV16 positive and negative smears were treated as case and control smears in this analysis.

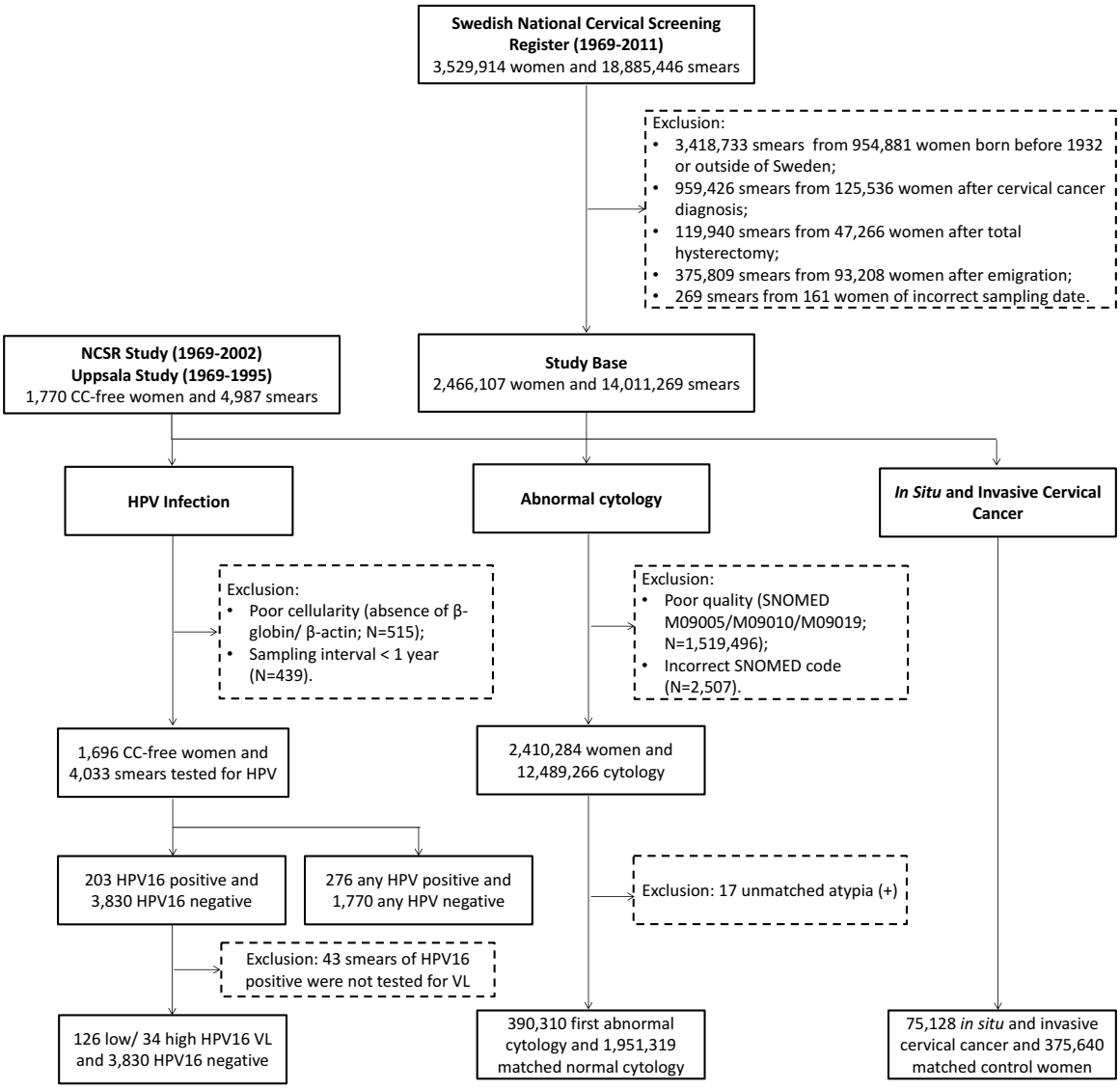

Figure 1. The flow chart of Paper I. CC, cervical cancer; HPV, Human papillomavirus; NCSR, National Cervical Screening Register; SNOMED, the systematized nomenclature of medical diagnoses defined by Swedish Association for Clinical Cytology; VL, viral load.

In addition to HPV16, smears from the NCSR Study were also tested for 21 additional HPV types, including15 high-risk and six low-risk types (Figure 1). 2,046 of 2,464 (83%) smears were thereby classified to any positive or all negative smears in terms of all HPV types tested, and used as case and control smears in this analysis, respectively. We further defined smears with a positive detection of any low-risk or high-risk HPV type as low-risk or high-risk HPV infection, respectively. In some smears, low-risk and high-risk HPV types were concurrent.

5.1.2 Abnormal cytology

Cytological diagnosis on smears was available in the NKCx and coded in the systematized nomenclature of medical diagnoses (SNOMED) by the Swedish Society of Clinical Cytology. We, therefore, defined the abnormal cytology as a smear with a cytological

diagnosis of atypical, low-grade, or high-grade abnormality (Table 3). We identified the first abnormal smear (if any), i.e. the case smear, during the study period for each woman, and the follow-up was censored afterwards. Using incidence density sampling, we randomly selected five cytologically normal smears from other women per case smear, which were individually matched to the case smear by birth year and screening adherence. In total, we included 390,310 first abnormal and 1,951,319 control smears (Figure 1).

Table 3. Swedish standard cytology nomenclature.

Classification	Description	SNOMED
Sample quality	Inadequate	M09005, M09010
	Endocervical cells lacking	M09019
Normal	Normal	M00110
Atypical	Atypical squamous cells of undetermined significance	M69710
	Atypical glandular cells of undetermined significance	M69720
Low-grade	CIN 1	M74006
	Koilocytosis	M76700
High-grade	CIN 2	M74007
	CIN 3	M80702
	Squamous cell carcinoma	M80703
	Adenocarcinoma or adenocarcinoma *in situ*	M81403
	Atypia in cells of uncertain origin	M69700
	Atypical squamous cells – cannot exclude high-grade dysplasia	M69719
	Malignant neoplasm, unspecified	M80003
	Malignant neoplasm of uncertain origin	M80009

5.1.3 *In situ* and invasive cervical cancers

Information on diagnosis of *in situ* and invasive cervical cancer was available through the linkage to the Swedish Cancer Register. *In situ* cancer comprised both carcinoma *in situ* (equivalent to CIN 3) and adenocarcinoma *in situ.* We identified the first diagnosis of histopathologically confirmed *in situ* or invasive cervical cancer during the follow-up for each woman (if any) and thereby defined the case women. Using incidence density sampling, we randomly selected five women without any cervical cancer per case woman, and they were individually matched to the case woman by birth year and screening adherence. In total, we obtained 69,674 *in situ* and 5,454 invasive cancers as well as 375,640 control women for this analysis (Figure 1). In the analysis of invasive cancer, we did not include the 300 invasive cancers that were developed from the *in situ* cancer, because the follow-up for these women was censored at the diagnosis of *in situ* cancer.

5.1.4 Bereavement

Through the Swedish Multi-Generation Register, we identified the family members (i.e., parents, children, spouse, and siblings) for all women in the study base. Bereavement, namely loss of a family member due to death, was identified by linking all family members to the Swedish Causes of Death Register from 1961 to 2011. Any loss before the reference date, namely the date of smear for the analyses of HPV infection and abnormal cytology while the matched date for the analysis of *in situ* or invasive cervical cancer, was regarded as the exposure. We further classified the loss events into loss of a parent or loss of a child, spouse, or sibling, as well as loss due to natural or unnatural (namely self-harm or other injuries)

cause of death. In addition, we divided the time since loss (namely the elapsed time from the date of latest loss to the reference date) into 1 year, 2 to 4 years, or ≥5 years.

5.1.5 Lifestyle factors

Lifestyle factors, such as smoking, high-risk sexual behavior, use of oral contraceptives, and high parity, may influence the associations of loss of a family member with risks of HPV infection and cervical cancer development. 469 control women in the Uppsala Study were invited to participate in a questionnaire interview, and 345 women completed the interview. We included these women with available information on such lifestyle factors, and compared the potential difference of lifestyle factors between women with and without loss using Chi-square for categorical variables or Mann-Whitney U tests for continuous variables.

5.1.6 Statistical analyses

Analyses of HPV Infection

We estimated the odds ratio (OR) of HPV16 infection and the relative risk ratios (RRRs) of HPV16 viral load levels using unconditional logistic regression and multinomial logistic regression, respectively. To specifically address the impact of loss of a family member on recurrent HPV infection, we stratified the analyses by the HPV16 status in the preceding smears. In addition, for smears tested for 32 HPV types, we derived the ORs of any positive as well as high-risk and low-risk HPV infections using unconditional logistic regression. All analyses were controlled for age at smear and within-subject correlation using robust variance estimates.

Analyses of abnormal cytology

We calculated the OR of the first abnormal cytology using conditional logistic regression. We further stratified the analysis by screening adherence. We divided the high and low screening adherence according to the median degree of participation among all screened women. Because abnormal cytologies require further workup, some may lead up to diagnosis of *in situ* or invasive cervical cancer. To address the concern of such driving force, in a sensitivity analysis, we excluded abnormal cytologies with a subsequent diagnosis of either *in situ* or invasive cervical cancer within the following six months.

Analyses of in situ and invasive cervical cancer

Similar to the analyses of abnormal cytology, we first derived the ORs of *in situ* and invasive cervical cancer from conditional logistic regression, and then stratified the analyses by screening adherence. Additionally, to evaluate the potential effect modification,(88,93) we stratified the analyses by age at the reference date, and plotted ORs using locally weighted scatter-plot smoothing.(151)

5.2 MENTAL DISORDERS, STRESSFUL LIFE EVENTS, AND CERVICAL CANCER SURVIVAL (PAPER II)

Based on the national Cervical Cancer Audit, we included 4,269 women with cervical cancer and followed them from the date of cancer diagnosis until death, emigration, or December 31st, 2013, whichever came first. Through the Causes of Death Register, we identified any death of the patients during the follow-up and the underlying cause. Information on demographic characteristics (including country of birth and highest educational level) and comorbidity in Charlson index(152) at the time of diagnosis was obtained through cross-linkage to Swedish registers.

The cancer-specific mortality was our primary outcome, while overall mortality was regarded as the secondary outcome. We defined the cancer-specific mortality if the death was recorded due to cervical cancer (ICD-10: C53) or unspecified uterine cancers (C55).(35) We assessed stress-related mental disorders and stressful life events as two separate indicators reflecting severe psychological distress experienced by women with cervical cancer.

5.2.1 Stress-related mental disorders

We used newly diagnosed mental disorders (i.e., close to cancer diagnosis and thereafter) as a proxy for the severe psychological distress experienced as a result of the diagnosis, treatment and progression of cervical cancer.(153) Several mental disorders were common among cancer patients and potentially related to psychological distress,(153-155) for instance stress reaction or adjustment disorders (ICD-10: F43), depression (F32-F33), and anxiety disorder (F40-F41). We therefore studied these three disorders, and defined the exposure as the first inpatient or outpatient visit for these disorders as the underlying reasons (i.e., the primary diagnosis) from one year before cancer diagnosis until the end of follow-up. A new diagnosis of such mental disorders was assured if no hospital visit regarding the same mental disorders was observed during the year preceding the defined diagnosis date of these mental disorders.

5.2.2 Stressful life events

Stressful life events are other common stress sources for cancer patients in addition to the malignant disease. We studied four life events that are both major and relatively common,(19) including loss or severe illness of a family member, divorce, and job loss. Because recent life events might better reflect the emotional burden,(156) we ascertained any first occurrence of these life events from one year before cancer diagnosis until the end of follow-up. Similar to the definition of newly diagnosed mental disorders, for the severe illness of a family member, we only included newly diagnosed illnesses in the family members.

Using the same methods as in Study I, we identified the family members (i.e., parents, children, spouse, and siblings) of all women and ascertained any loss of a family member due to death. By linking all family members to the Patient and Cancer Registers, we also ascertained any severe illness of the family members which was defined *a priori* as illness with a disability weight over 0.40 in the Global Disease Burden 2004(157) and relatively

common in Sweden (Table 4). The change of marital status from "married" to "unmarried" in LISA was regarded as divorce. Job loss was assessed when one was registered as "actively seeking for a job" in LISA. Because patients may discontinue work on their own choice due to a poor health, we did not consider the recorded "no employment" alone as job loss.

Table 4. Definition of severe illnesses of the family members, based on the disability weight in Global Disease Burden 2004.

Disease	**ICD-10**	**Disability weight***
First-ever stroke	I61-I64	0.920
Cretinism	E00	0.804
Severe depressive episodes	F322, F323, F332, F333	0.760
Metastatic and terminal cancer†	C77-C79, or tumor stage as M1	0.75-0.81
Injured spinal cord	T093	0.725
Alzheimer and other dementias	F00-F03, F051, G30, G311	0.666
Tetanus	A33-A35	0.638
Japanese encephalitis	A830	0.616
Bacterial meningitis	G00, G03	0.615
Poisoning	X60-X69	0.608-0.611
Dengue hemorrhagic fever	A91	0.545
Chronic obstructive pulmonary disease	J44	0.530
Schizophrenia	F20-F29	0.528
Burns 20%-60%	T312-T316	0.441
Burns >60%	T317-T319	0.441
Acute myocardial infarction (treated)	I21, I22	0.439
Fractured skull	S02	0.431
Rectovaginal fistula	N823	0.430
Multiple sclerosis	G35	0.411
	Reasons	**Disability weight***
Excluded:		
Major congenital malformations	Unlikely to be newly diagnosed for family members of cervical patients.	0.593-0.850
Gonorrhea/Onchocerciasis/Trachoma – blindness	Sequela of these infections is not identifiable in registers and they are extremely rare in Sweden.	0.581-0.600
Chlamydia – ectopic pregnancy	The ditto	0.549
Tubo-ovarian abscess	Unlikely to be "severe" in Sweden.	0.548
AIDS not on ART (untreated)	The universal coverage of healthcare in Sweden guarantees equal access, and untreated AIDS is rare.	0.505
Malaria – neurological sequelae	Sequela of this infection is not identifiable in registers and it is extremely rare in Sweden.	0.471
Ascariasis – cognitive impairment	The ditto	0.463
Appendicitis	Unlikely to be "severe" in Sweden.	0.463
Meningitis – mental retardation	Sequela of this infection is not identifiable in registers and it is extremely rare in Sweden.	0.459
Pertussis – encephalopathy	The ditto	0.452

* Disability weights represent the magnitude of health loss associated with the outcome on a scale from 0 to 1. 0 implies a condition equivalent to full health, while 1 means a state equivalent to death.

† In addition to the Patient Register, the Cancer Register was used to complement the diagnoses of metastatic or terminal cancer using the same ICD-10 codes and tumor stage as M1. The Cancer Register is virtually complete for newly-diagnosed malignancies, and information on tumor stage is largely available from 2004 onward.

5.2.3 Statistical analysis

We treated the exposure to stress-related mental disorders and stressful life events in a time-varying manner. Namely, women were defined as exposed from the date of cancer diagnosis onward if they encountered mental disorders or life events in the year preceding cancer diagnosis. For women who were exposed to mental disorder or life events after cancer diagnosis, they were first classified as unexposed and then exposed from the date of occurrence of the mental disorder or life event. Women were defined as unexposed throughout if they did not experience any mental disorders or life events from one year before cancer diagnosis until end of follow-up.

We first compared differences of demographic and clinical characteristics between exposed and unexposed women using t test or chi-square test. We then estimated the hazard ratios (HRs) of overall mortality among exposed women, compared to unexposed women, from the Cox proportional hazards model. In this analysis, we adjusted for age at diagnosis, calendar year at diagnosis (2002-2004, 2005-2007, or 2008-2011), highest education level (primary school or unknown, high school, or college and beyond), country of birth (Nordic or non-Nordic), and number of family members at diagnosis.

For cancer-specific mortality, we examined the associations of mental disorders and life events collectively and individually. As the primary analysis, we first adjusted for the aforementioned covariates as a simple model; and we, in a full model, additionally controlled for FIGO stage (IA, IB, II, or ≥III), histology (squamous cell or others), mode of detection (due to screening or symptoms), primary treatment (operation, palliative care, or others), adjuvant therapy (yes or no), and Charlson comorbidity (yes or no). To understand the potentially different impact of characteristics of the life events, we further assessed bereavement by cause of death (i.e., natural or unnatural cause of death) and severity of the illness of a family member (i.e., the disability weights ≥0.5 or 0.4-0.5).

To shed light on the temporal pattern, we estimated the HRs of cancer-specific mortality in different time windows after mental disorders or life events, i.e., within first six months, six months to <two years, or two years and beyond, since the exposure. The analysis during the first six months after exposure was to illustrate the potential reverse causality (i.e., mental disorders or life events were the consequences, rather than the causes, when one was dying of cancer). Provided with the potentially varying effect of psychological stress through the cancer continuum,(4,153) we also examined the risk of cancer-specific mortality by the timing of the occurrence of mental disorders and life events, i.e., from one year before to one year after cancer diagnosis and thereafter.

5.3 STRESS-RELATED SIGNALING PATHWAYS AND LETHAL PROSTATE CANCER (PAPERS III & IV)

Within the PHS and HPFS Prostate Tumor Tissue Cohort, we sampled 404 men using the extreme-case design. Namely, among men with sufficient usable tumor tissue, we included all men (N=113) who died of prostate cancer or developed distant metastases during follow-

up as lethal cases, and randomly sampled 291 men who lived eight years after cancer diagnosis and who neither developed metastases nor died of prostate cancer through 2012 as nonlethal cases. The archival formalin-fixed, paraffin embedded (FFPE) tumor specimens were retrieved for all included men. 404 tumor tissues and 202 normal adjacent tissues (whenever available) were profiled for the gene expression.

Within the Swedish Watchful Waiting Cohort, using the same design, we first sampled 262 men to profile the gene expression in tumor tissue; and, in a second sampling, we included 396 men to genotype candidate SNPs in adjacent normal tissue. For the analysis of gene expression, we included 141 men who died of prostate cancer during follow-up as lethal cases and 121 men who lived seven years after cancer diagnosis as nonlethal cases, among men with high-quality tumor tissue. For the analysis of genetic variants, we enrolled 126 lethal and 270 nonlethal cases from men with available normal tissue. Altogether, 186 lethal and 325 nonlethal cases were included from the Swedish data.

We carried out the analysis of gene expression in stress-related pathways for both of the US and Swedish data, and additionally performed the analysis of candidate SNPs in these pathways in the Swedish data. We examined the lethal cancer as the primary outcome, and assessed the biomarkers and histopathological characteristics as the secondary outcomes.

5.3.1 Candidate genes in the stress-related pathway

We studied four stress-related pathways with a confirmed link to psychological stress, namely the adrenergic, glucocorticoid, serotoninergic, and dopaminergic pathways. We used the Kyoto Encyclopedia of Genes and Genomes (KEGG), Pathway Maps (Thomson Reuters) and previous literature to identify the candidate genes in each pathway. To concentrate on the tumor-specific impact, we excluded genes in the branches of signaling cascade leading to cardiovascular and neuronal functions from the adrenergic signaling pathway. In total, 234 genes from the studied four pathways were included for the analysis of gene expression. Of note, an individual gene could contribute to multiple pathways.

To conservatively assess the independent effect of the pathway without crosstalk, for the US data, the analysis was further restricted to the 46 exclusive genes prior to all data analyses. The exclusive genes were defined as the upstream genes preceding to any common signaling transduction among four pathways. The majority is therefore the receptor genes except for the glucocorticoid pathway. Regarding the Swedish data, 234 genes whenever available (N=163) were used for the analysis of gene expression. In an alternative approach, we restricted the analysis to 116 specific genes which are only present in one pathway, in order to control for the crosstalk between pathways as much as possible.

5.3.2 RNA extraction, profiling and data preprocessing

For the US samples, we first extracted RNA from the tumor and adjacent normal tissues on the Biomek FxP automated platform using the Agencourt FormaPure FFPE kit (Beckman), and then amplified it using the WT-Ovation FFPE System V2 (Nugen). After the whole

transcriptome amplification, the cDNA was hybridized to the GeneChip Human Exon 1.0 ST microarray (Affymetrics), which profiles expression of >28,000 genes with an average of 26 probes per gene. We have conducted a pilot study to validate the gene expression quantification from FFPE tissues on this platform (158). The Affymetrix data was normalized using the Robust Multi-array Average method according to samples and batches,(159) and was mapped to 20,254 unique named genes using the NetAffx annotations. The expression data have been submitted to the Gene Expression Omnibus (accession number: GSE62872).

For the Swedish samples, we extracted RNA from the tumor tissue in a 96-well format using the CyBi-Well liquid handling system (CyBio AG), and quantified it using the NanoDrop spectrophotometer (NanoDrop Technologies). Using the four cDNA-mediated annealing, selection, ligation, and extension (DASL) assay panels, we then profiled the expression data of 6100 genes.(160) The expression data were normalized by using a cubic spline algorithm and submitted to the GEO (accession number: GSE16560).

5.3.3 Genotyping

For the Swedish samples (396 men with normal tissue available), we additionally genotyped 36 SNPs across the main receptor genes (N=14) in the four pathways. The set of SNPs were selected based on previous literature suggesting a link to prostate or other cancers. We extracted the DNA from normal tissue using the Sequenom iPLEX platform assay at the Genotyping Core Facility at Children's Hospital, Boston.(161) After excluding SNPs with a call rate <95% (N=4), with a minor allele frequency <5% (N=1), and with significant deviation from Hardy-Weinberg equilibrium (N=1), 30 SNPs were included for the analysis. We also excluded eight men due to low genotyping quality of the sample (genotyped <85% working SNPs).

5.3.4 Biomarkers and histopathological characteristics

We assessed the biomarkers and histopathological characteristics for both US and Swedish data unless indicated. The total sample size varied by biomarkers and histopathological characteristics due to data availability.

Cell proliferation

We measured the expression of Ki-67 on 4 or 5 μm sections of tumor tissue to assess the cell proliferation, using the rabbit polyclonal antibody (Vector Labs).(162,163) After immunohistochemical staining, we quantified the percentage of Ki-67 positive nuclei among all tumor nuclei using the Ariol instrument SL-50 (Applied Imaging).

Apoptosis

We used the TUNEL assay on 4 or 5 μm sections of tumor tissue to estimate the proportion of tumor cells undergoing apoptosis. The Apoptag Peroxidase *In situ* kit (Chemicon International) was used for staining, and the percentage of positively-stained cells over all tumor cells was calculated.(162,164)

Angiogenesis

The angiogenesis was only assessed in the HPFS in the US sample based on the protein expression of endothelial cell marker CD34. After staining by the anti-CD34 mouse monoclonal antibody (QBEnd-10) and peroxidase blocking reagent (Dual Endogenous Enzyme Block, DakoCytomation), semi-automated image analysis (Image ProPlus 4.5 software, Media Cybernetics) was used to quantify the size and architecture.(70) Microvessel density was defined as the number of vascular structures per one high-powered field. Vessel size was calculated as the mean vessel diameter (μm), and area occupied by a vessel (μm^2).

TMPRSS2–ERG fusion

In the Swedish sample, we assessed the ERG rearrangement status by using an ERG break-apart fluorescence in situ hybridization (FISH) assay.(165) In cases not assessed by FISH, qPCR was performed in a portion of the RNA extraction used for DASL.(75) Relative quantification was preformed using the $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ method.(166)

Other biomarkers and histopathological characteristics

Gleason score was re-reviewed through hematoxylin and eosin slides for all patients. Prostate-specific antigen (PSA) levels at diagnosis and tumor stage were obtained from the medical records and pathology reports in the US data, whereas tumor stage was re-reviewed in the slides in the Swedish data. Presence of perineural invasion was also assessed in the slides. Prostatic intraepithelial neoplasia (PIN) was only reviewed in adjacent areas in the Swedish samples.

5.3.5 Statistical analysis

Pathway analysis

In both the US and Swedish samples, we examined the overall associations of differential gene expression in four candidate pathways with the risk of lethal prostate cancer as the primary outcome, as well as with biomarkers and histopathological characteristics as the secondary outcomes. In the US sample, we performed the likelihood ratio test on the logistic regression model to compare the null model (controlled for age at diagnosis and cohort membership) with the full model (additionally including the genes in the candidate pathway), because of small pathway sizes (6-17 exclusive genes). Regarding the Swedish sample, we assessed the associations using the global test, in order to handle larger and different sizes of gene sets(167), and adjusted for age at diagnosis. Because we aimed to describe the potential difference in signaling of these pathways, instead of developing prediction models, we did not adjust for Gleason score or tumor stage in our primary model; we however controlled for and performed subgroup analyses by these variables for both samples in the secondary model.

We conducted different sensitivity analyses for both samples. For the US sample, we carried out the Gene Set Enrichment Analysis (GSEA) (168) to assess the importance of the candidate pathways relative to other unrelated genes in terms of the lethal cancer. GSEA is a

competitive pathway test comparing the differential expressions of the genes in the working pathway with differential expression of other genes. This test does not control for clinical covariates, in order to calculate P-values based on permuting individuals in the study. It does, however, provide a direction for the association, namely whether up-regulation or down-regulation of the gene expression is associated with the lethal cancer. In the Swedish sample, we restricted the pathway analysis to 116 specific genes which were not shared with other candidate pathways, to reduce the crosstalk between candidate pathways.

To understand if the differential signaling of the candidate pathways pertains in the tumor tissue, we also assessed the associations in the adjacent normal prostate tissue in the US sample.

To shed light on the potential biological mechanisms for the noted associations, we further examined the relationships of the candidate pathways with biomarkers and histopathological characteristics for both samples. In the US samples, we classified all outcomes into categorical variables and assessed the pathway significance using likelihood ratio tests on logistic regression (if binary outcome) or proportional odds models. For the Swedish samples, all histopathologic characteristics were used as binary outcomes and all quantitative biomarkers were used as continuous outcomes, examined in the global test.

SNP analysis

We only performed the SNP analysis for the Swedish sample. Using logistic regression model with no further adjustment, we examined the associations of candidate SNPs with the risk of lethal prostate cancer. We first explored different effect models, including additive, recessive, dominant, and over-dominant models; and then estimated the ORs based on the most significant effect model. To shed light on the underlying mechanisms, we also examined the associations with the biomarkers and histopathological characteristics for the significant SNPs found for lethal cancer, using the same effect model.

To further understand the impact of genetic variation on the pathway signaling, we performed the Quantitative Trait Loci (eQTL) analysis using linear regression model(169) to assess the correlation between significant SNPs noted above and the expression of genes in the same pathway. For this analysis, we adjusted for age at diagnosis, calendar period at diagnosis, Gleason score, tumor stage, and lethal cancer in the subgroup of men that were both profiled for gene expression and genotypes (N=154; 82 lethal and 72 nonlethal cases).

Since we were examining four independent pathways, we considered a P value <0.0125 to indicate a statistical significance in the pathway analyses of both samples, as correction for multiple testing.(170) As we took a pathway-based approach to test specific *a priori* hypotheses, we did not further control for multiple testing in the subsequent analyses of SNPs in the Swedish sample.

5.4 ETHICAL CONSIDERATIONS

Papers I & II are largely based on public and health registers in Sweden. Both studies were approved by the Regional Ethical Review Board in Stockholm (EPN 2012/1783-32, 2011/1547-32, and 2015/719-32), and the requirement of informed consent from participants was waived. Data linkage was carried out by the Statistics Sweden and the National Board of Health and Welfare, and the personal identity number was replaced by a study identification number. The host institute secures the data through strict safety guidelines and limited access.

Paper III, including prostate cancer patients from the PHS and the HPFS in the US, was approved by the institutional review boards at the Harvard School of Public Health and Partners Health Care (2009P001231/BWH). Written informed consent was obtained from each participant. The Swedish Watchful Waiting Cohort was originally approved by the Regional Ethical Review Board in Linköping (EPN M58-05), who also decided to waive the requirement of informed consent forms from included patients. Based on this cohort, Paper IV was approved by the Regional Ethical Review Board in Uppsala (EPN 2012/361). Both studies assessed mRNAs, and Paper IV additionally SNPs, using the specimens collected from surgical treatment. Although most peer-reviewed journals require the submission of microarray data to public repositories, the personal information is anonymized and the odds for inappropriate spread of such information are minimal.

6 RESULTS

6.1 BEREAVEMENT, HPV INFECTION, AND CERVICAL CARCINOGENESIS (PAPER I)

HPV Infection

Compared to women without loss, loss of a family member was positively associated with HPV16 infection (Table 5). The association was stronger among women with a history of HPV16 infection (P<0.0001), and the risk elevation tended to be greater among women with high screening adherence (P=0.060). Moreover, stronger and significant association was noted for high viral load infection of HPV16 (RRR 2.84, 95% CI 1.29 to 6.29), rather than low viral load infection (RRR 0.73, 95% CI 0.73 to 2.44). The positive association of loss was further extended to infection of any HPV types tested (Table 5). The stronger and significant association was noted for infection of high-risk HPV types (OR 1.55, 95% CI 1.01 to 2.37) rather than infection of low-risk types (OR 1.13, 95% CI 0.51 to 2.51).

Table 5. Odds ratios (ORs) of HPV infection after bereavement.

	Negative smear N(%)	Positive smear N(%)	OR (95% CI)*
HPV16 infection			
No bereavement	3,038 (79.3)	158 (77.8)	1.0
Bereavement	792 (20.7)	45 (22.2)	1.62 (1.05-2.50)
Stratified by previous HPV16 infection			
No			
No bereavement	2,862 (79.7)	139 (81.3)	1.0
Bereavement	731 (20.4)	32 (18.7)	1.31 (0.86-2.00)
Yes			
No bereavement	176 (74.3)	19 (59.4)	1.0
Bereavement	61 (25.7)	13 (40.6)	3.94 (1.24-12.48)
P for difference†			<0.0001
Stratified by screening adherence			
High			
No bereavement	2,039 (81.3)	90 (75.0)	1.0
Bereavement	468 (18.7)	30 (25.0)	1.95 (1.13- 3.38)
Low/unscreened			
No bereavement	999 (75.5)	68 (81.9)	1.0
Bereavement	324 (24.5)	15 (18.1)	1.15 (0.58-2.28)
P for difference†			0.060
Any HPV§			
No bereavement	1,426 (80.6)	230 (83.3)	1.0
Bereavement	344 (19.4)	46 (16.7)	1.54 (1.03-2.30)

* CI, confidence interval. ORs were estimated using unconditional logistic regression, and were controlled for age at smear and within-subject variance.
† The P values for the interaction terms between bereavement and the stratification factors.
§ Low-risk HPV includes types 6, 7, 11, 42, 43, and 70. High-risk HPV includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82.

Abnormal Cytology

Loss of a family member was associated with a slightly but significantly increased risk of abnormal cytology (OR 1.04, 95% CI 1.03 to 1.05; Table 6). Such associations were consistently observed for atypical (OR 1.04, 95% CI 1.02 to 1.05), low-grade (OR 1.03, 95% CI 1.02 to 1.05), and high-grade abnormality (OR 1.04, 95% CI 1.02 to 1.06). Of note, the dose-response effect, i.e. number of losses, on abnormal cytology was evident (P<0.0001; Table 6). Moreover, the ORs were significantly greater for loss of a child, spouse, or sibling, loss due to unnatural cause of death, and among women with higher screening adherence. The association, however, was not modified by time since loss. The association of loss with the risk of abnormal cytology hardly changed after excluding abnormal cytologies leading to cervical cancer diagnosis in six months (OR 1.03, 95% CI 1.02 to 1.04).

Table 6. Odds ratios (ORs) of abnormal cytology after bereavement.

	Normal cytology N(%)	Abnormal cytology N(%)	OR (95% CI)*
No bereavement	1,389,548 (71.2)	275,830 (70.7)	1.0
Bereavement	561,771 (28.8)	114,480 (29.3)	1.04 (1.03-1.05)
Number of bereavements			
1	401,738 (20.6)	81,260 (20.8)	1.03 (1.02-1.04)
2	136,626 (7.0)	27,844 (7.1)	1.05 (1.04-1.07)
3	20,418 (1.1)	4,578 (1.2)	1.17 (1.13-1.21)
≥4	2,989 (0.2)	798 (0.2)	1.40 (1.29-1.52)
P for trend†			<0.0001
Type of bereavement			
Loss of a parent	474,561 (24.3)	95,323 (24.4)	1.02 (1.01-1.03)
Loss of a child, spouse, or sibling	87,210 (4.5)	19,157 (4.9)	1.12 (1.10-1.14)
P for difference§			<0.0001
Cause of bereavement			
Unnatural cause	66,224 (3.4)	14,602 (3.7)	1.12 (1.09-1.14)
Natural cause	495,547 (25.4)	99,878 (25.6)	1.02 (1.01-1.03)
P for difference§			<0.0001
Time since bereavement			
≤1 year	97,087 (5.0)	19,797 (5.1)	1.04 (1.02-1.05)
2-4 years	118,769 (6.1)	24,546 (6.3)	1.05 (1.04-1.07)
≥5 years	345,915 (17.7)	70,137 (18.0)	1.03 (1.02-1.04)
Stratified by screening adherence			
High			
No bereavement	588,071 (76.6)	116,343 (75.8)	1.0
Bereavement	179,499 (23.4)	37,173 (24.2)	1.06 (1.04-1.07)
Low			
No bereavement	428,556 (58.1)	85,016 (57.6)	1.0
Bereavement	309,433 (41.9)	62,593 (42.4)	1.03 (1.01-1.04)
Unscreened			
No bereavement	372,921 (83.7)	74,471 (83.5)	1.0
Bereavement	72,839 (16.3)	14,714 (16.5)	1.02 (0.99-1.04)
P for difference‡			0.002

* CI, confidence interval. Birth year and screening adherence were matched on and therefore inherently adjusted for in all models.
† Cochran-Armitage test.
§ Wald test.
‡ The P value for the interaction terms between bereavement and screening adherence.

Cervical Cancer

We observed mildly increased risk of *in situ* or invasive cervical cancer after loss of a family member (Table 7). Excess risk was also noted for *in situ* (OR 1.07, 95% CI 1.04 to 1.09) and invasive cancer (OR 1.09, 95% CI 1.02 to 1.17), respectively. The associations of *in situ* or invasive cancer were more pronounced for multiple losses, loss of a child, spouse, or sibling, loss due to unnatural cause of death, and among women with higher screening adherence (Table 7). The risk elevations, however, did not differ by time since loss. Women with loss of a child, spouse or sibling were consistently at higher risk of *in situ* cervical cancer throughout different age bands, whereas loss of a parent was only related to a mildly increased risk in younger ages (Figure 2). Similar patterns were noted for the risk of invasive cervical cancer.

Table 7. Odds ratios (ORs) of *in situ* or invasive cervical cancer after bereavement.

	No cervical cancer N(%)	Cervical cancer N(%)	OR (95% CI)*
No bereavement	267,919 (71.3)	52,797 (70.3)	1.0
Bereavement	107,721 (28.7)	22,331 (29.7)	1.07 (1.05-1.09)
Number of bereavements			
1	80,052 (21.3)	16,414 (21.9)	1.06 (1.03-1.08)
2	23,558 (6.3)	4,868 (6.5)	1.09 (1.05-1.13)
3	3,439 (0.9)	878 (1.2)	1.37 (1.27-1.49)
≥4	672 (0.2)	171 (0.2)	1.40 (1.18-1.66)
P for trend†			<0.0001
Type of bereavement			
Loss of a parent	90,068 (24.0)	18,203 (24.2)	1.04 (1.02-1.06)
Loss of a child, spouse, or sibling	17,653 (4.7)	4,128 (5.5)	1.20 (1.16-1.25)
P for difference§			<0.0001
Cause of bereavement			
Unnatural cause	13,997 (3.7)	3,254 (4.3)	1.19 (1.14-1.23)
Natural cause	93,724 (25.0)	19,077 (25.4)	1.05 (1.02-1.07)
P for difference§			<0.0001
Time since bereavement			
≤1 year	18,145 (4.8)	3,728 (5.0)	1.06 (1.02-1.10)
2-4 years	22,629 (6.0)	4,710 (6.3)	1.07 (1.04-1.11)
≥5 years	66,947 (17.8)	13,893 (18.5)	1.07 (1.05-1.09)
Stratified by screening adherence			
High			
No bereavement	109,281 (77.5)	21,425 (76.0)	1.0
Bereavement	31,769 (22.5)	6,785 (24.1)	1.11 (1.07-1.14)
Low			
No bereavement	96,282 (62.1)	19,037 (61.4)	1.0
Bereavement	58,763 (37.9)	11,972 (38.6)	1.04 (1.01-1.07)
Unscreened			
No bereavement	62,356 (78.4)	12,335 (77.5)	1.0
Bereavement	17,189 (21.6)	3,574 (22.5)	1.07 (1.02-1.12)
P for difference‡			0.018

* CI, confidence interval. Birth year and screening adherence were matched on and therefore inherently adjusted for in all models.

† Cochran-Armitage test.

§ Wald test.

‡ The P value for the interaction terms between bereavement and screening adherence.

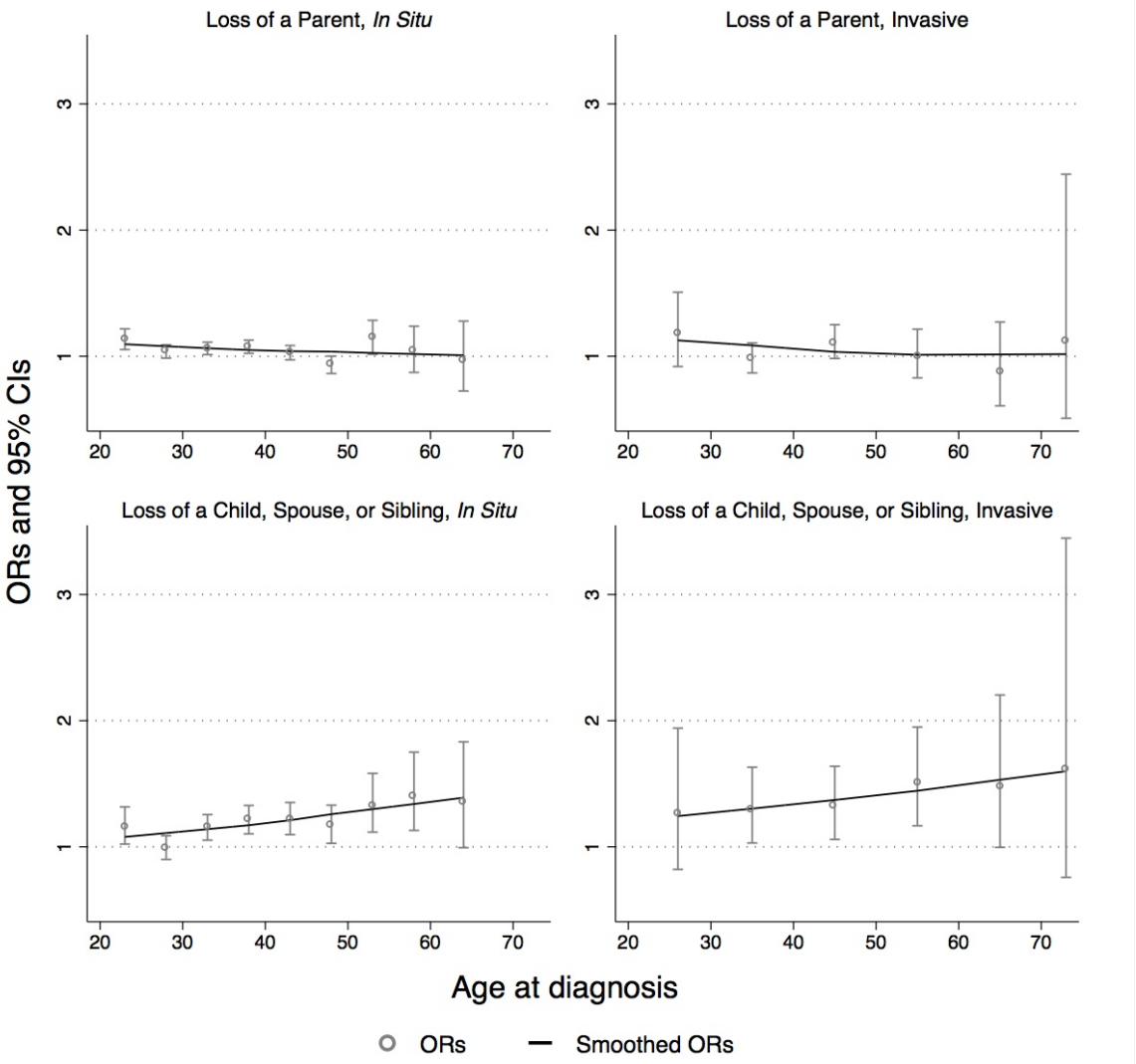

Figure 2. Odds ratios (ORs) of *in situ* and invasive cervical cancer after bereavement, by age at diagnosis. We performed the subgroup analyses on *in situ* cervical cancer by ⩽25 years, every 5 years thereafter, and ⩾61 years and analyses on invasive cervical cancers by ⩽30 years, every 10 years thereafter, and ⩾71 years. We plotted the scatters of odds ratios (ORs) and confidence intervals at the median age of each age group, as well as lines of smoothed ORs which were derived from locally weighted scatterplot smoothing.

Lifestyle Factors

Women with loss of a family member were older at the time of the interview and, if ever smoking, had started smoking at a younger age (Table 8). However, loss was not related to self-reported smoking status, use of oral contraceptives, sexual behaviors, parity, or number of abortions (P >0.05).

Table 8. Distribution of lifestyle factors between women with and without bereavement*

Characteristics	No bereavement	Bereavement	*P for difference*†
Number	155	190	-
Age at interview	42 (26-63)	49 (29-63)	<0.0001
Age at bereavement	-	42 (9-57)	-
Smoking			
Ever smoker‡	90 (58.1)	107 (56.3)	0.741
Current smoker	37 (23.9)	52 (27.4)	0.460
Age at smoking debut	16 (12-35)	17 (12-37)	0.013
Oral contraceptive use§			
Ever user	130 (84.4)	154 (81.1)	0.414
Current user	19 (12.3)	12 (6.3)	0.055
Sexual behavior			
Age at sexual debut	17 (12-30)	17 (11-30)	0.520
No. of sexual partners			
Maximum	5 (1-53)	4 (1-35)	0.236
At age 20-25	2 (0-42)	2 (0-15)	0.921
Parity	2 (0-6)	2 (0-6)	0.086
Number of abortions	0 (0-4)	0 (0-2)	0.978

* Among 345 women included for this analysis, one woman had missing data on use of oral contraceptive; two women had missing data on age at sexual debut; and four women had missing data on number of sexual partners. Number (percentage) or median (range) was presented.
† Chi-square test was used to test the different distributions by categorical variables (smoking and oral contraceptive use); and Mann–Whitney U test for continuous variables (age at interview, age at smoking debut, age at sexual debut, number of sexual partners, parity and abortion).
‡ Ever smoker was defined as women who smoked at least six months.
§ Combined estrogen-progestin compounds only.

6.2 MENTAL DISORDERS, STRESSFUL LIFE EVENTS, AND CERVICAL CANCER SURVIVAL (PAPER II)

We identified 1,839 (43%) patients with cervical cancer that experienced a stress-related mental disorder or stressful life event from the year before cancer diagnosis until end of follow-up (Table 9). 388 (21.1%) patients encountered a mental disorder, while 1,634 (88.9) patients had a life event. Compared to the unexposed patients, the exposed patients were younger at diagnosis, and were more likely diagnosed in earlier calendar years and free of comorbidities at diagnosis. Their cancers were also more likely below stage III, from squamous cell origin, detected by screening, and treated surgically.

In the average follow-up of 4.3 years, 1,396 (32.7%) patients died and 1,009 (72.3%) of these had cervical cancer-specific death. Patients with either a mental disorder or stressful life event from one year before cancer diagnosis onward had a significantly increased risk of overall mortality (HR 1.23, 95% CI 1.08 to 1.40), irrespective of age, calendar year, family size at diagnosis, education level, or country of birth, compared to the unexposed patients.

Table 9. Demographic and clinical characteristics between cervical cancer patients with and without exposure to any stress-related mental disorders or stressful life event.

	Patients without exposure N(%) or mean ± SD	Patients with exposure N(%) or mean ± SD	P for difference
Total number	2,430	1,839	
Age at diagnosis, years	56.7±19.4	50.2±15.4	<0.001
Calendar year at diagnosis			<0.001
2002-2004	638 (26.3)	645 (35.1)	
2005-2007	674 (27.7)	605 (32.9)	
2008-2011	1,118 (46.0)	589 (32.0)	
FIGO stage			<0.001
IA	448 (18.4)	421 (22.9)	
IB	875 (36.0)	816 (44.4)	
II	470 (19.3)	348 (18.9)	
III+	637 (26.2)	254 (13.8)	
Histology			0.047
Unknown	2 (0.1)	0 (0.0)	
Squamous cell cancer	1,721 (70.8)	1,334 (72.5)	
Adenocarcinoma	527 (21.7)	391 (21.3)	
Adenosquamous cell cancer	82 (3.4)	68 (3.7)	
Others	98 (4.0)	46 (2.5)	
Mode of detection			<0.001
Screening	626 (25.8)	624 (33.9)	
Symptom	1,804 (74.2)	1,215 (66.1)	
Operation			<0.001
No	1,232 (50.7)	629 (34.2)	
Conization	180 (7.4)	150 (8.2)	
Trachelectomy	56 (2.3)	36 (2.0)	
Hysterectomy	179 (7.4)	179 (9.7)	
Radical hysterectomy	783 (32.2)	845 (45.9)	
Chemotherapy			0.476
No	1,670 (68.7)	1,245 (67.7)	
Yes	760 (31.3)	594 (32.3)	
Radiation therapy			0.090
No	1,160 (47.7)	926 (50.4)	
Yes	1,270 (52.3)	913 (49.6)	
Palliative care			<0.001
No	2,153 (88.6)	1,788 (97.2)	
Yes	277 (11.4)	51 (2.8)	
Comorbidity at diagnosis			<0.001
No	1,700 (70.0)	1,404 (76.3)	
Yes	730 (30.0)	435 (23.7)	

FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation.

Exposure to a stress-related mental disorder or a stressful life event was associated with a 31% increased risk of cancer-specific mortality (95% CI 1.13 to 1.52; Table 10). After additional adjustment for multiple clinical characteristics, the association became slightly weaker but remained statistically significant (HR 1.25, 95% CI 1.07 to 1.44). Although some results were not statistically significant, an increased risk of cancer-specific mortality was found for all individual mental disorders and life events. Of note, the association of loss of a family member due to unnatural causes of death tended to be stronger, compared to loss due to natural causes. A similar pattern was observed for having a family member with more severe illness (i.e., of disability weight ≥0.5), compared to having a family member with less severe illness.

Table 10. Hazard ratios (HRs) of cancer-specific mortality after stress-related mental disorders or stressful life events.

	N (IR)	HR (95% CI)*	HR (95% CI)†
Reference	733 (62.0)	1.0	1.0
Stress-related mental disorders or stressful life events	276 (39.8)	1.31 (1.13-1.52)	1.25 (1.07-1.44)
By individual disorders or events			
Mental disorders	69 (52.3)	1.83 (1.42-2.35)	1.55 (1.21-2.00)
Stress reaction or adjustment disorder	15 (44.5)	1.80 (1.08-3.02)	1.79 (1.07-2.99)
Depression	27 (47.1)	1.60 (1.08-2.35)	1.21 (0.82-1.78)
Anxiety	27 (66.1)	2.16 (1.46-3.18)	1.97 (1.34-2.91)
Life events	226 (36.5)	1.22 (1.04-1.43)	1.19 (1.02-1.40)
Loss of a family member	104 (39.7)	1.23 (0.99-1.53)	1.23 (0.99-1.53)
Due to natural cause	98 (39.2)	1.23 (0.99-1.53)	1.23 (0.98-1.53)
Due to unnatural cause	10 (54.0)	1.56 (0.83-2.92)	1.81 (0.96-3.41)
Severe illness of a family member	136 (38.8)	1.29 (1.06-1.56)	1.19 (0.98-1.45)
Disability weight ≥0.5	97 (39.3)	1.37 (1.10-1.71)	1.24 (1.00-1.55)
Disability weight 0.4-0.5	44 (34.5)	1.13 (0.83-1.54)	1.07 (0.78-1.46)
Divorce	35 (36.5)	1.21 (0.86-1.70)	1.08 (0.77-1.52)
Job loss	22 (20.2)	1.35 (0.88-2.09)	1.40 (0.90-2.16)

CI, confidence interval; HR, hazard ratio; IR, incidence rate, per 1,000 person-years.
* HRs were adjusted for age at diagnosis, calendar year at diagnosis (2002-2004, 2005-2007, or 2008-2011), education level (primary school or unknown, high school, or college+), country of birth (Nordic or non-Nordic), and family size at diagnosis.
† HRs were additionally adjusted for FIGO stage (IA, IB, II, or III+), histology (squamous cell cancer or others), mode of detection (screening or symptom), primary treatment (operation, palliative care, or others), adjuvant therapy (yes or no), and Charlson comorbidity (yes or no).

Significantly increased risks of cancer-specific mortality were noted both within six months and ≥2 years after exposure to a mental disorder (Table 11). By contrast, the significant association with cancer-specific mortality was only noted ≥2 years after exposure to a stressful life event.

Table 11. Hazard ratios (HRs) of cancer-specific mortality after stress-related mental disorders or stressful life events, by time since exposure.

	N (IR)	HR (95% CI)*	HR (95% CI)†
Mental disorders			
0 to <6 months	23 (143.2)	2.53 (1.67-3.85)	2.13 (1.40-3.23)
6 months to <2 years	28 (64.1)	1.51 (1.03-2.22)	1.24 (0.85-1.82)
≥2 years	18 (25.0)	1.77 (1.09-2.88)	1.62 (1.00-2.64)
Life events			
0 to <6 months	30 (48.5)	0.94 (0.65-1.35)	0.89 (0.61-1.28)
6 months to <2 years	109 (57.1)	1.23 (1.00-1.52)	1.17 (0.96-1.45)
≥2 years	87 (23.8)	1.42 (1.10-1.83)	1.42 (1.10-1.83)

CI, confidence interval; HR, hazard ratio; IR, incidence rate, per 1,000 person-years.
* HRs were adjusted for age at diagnosis, calendar year at diagnosis (2002-2004, 2005-2007, or 2008-2011), education level (primary school or unknown, high school, or college+), country of birth (Nordic or non-Nordic), and family size at diagnosis.
† HRs were additionally adjusted family size at diagnosis, FIGO stage (IA, IB, II, or III+), histology (squamous cell cancer or others), mode of detection (screening or symptom), primary treatment (operation, palliative care, or others), adjuvant therapy (yes or no), and Charlson comorbidity (yes or no).

The excess risk of cancer-specific mortality was greater for stress-related mental disorders occurring more than one year after cancer diagnosis, compared to mental disorders occurring during the year before or the year after cancer diagnosis (Table 12). By contrast, significant associations with cancer-specific mortality were only noted for stressful life events that took place from one year before to one year after cancer diagnosis.

Table 12. Hazard ratios (HRs) of cancer-specific mortality after stress-related mental disorders or stressful life events, by timing of the occurrence of such mental disorders or life events.

	N (IR)	HR (95% CI)*	HR (95% CI)†
Mental disorders			
1 year before or after diagnosis	48 (62.8)	1.59 (1.19-2.14)	1.37 (1.02-1.84)
>1 year after diagnosis	21 (37.9)	2.68 (1.72-4.18)	2.38 (1.52-3.73)
Life events			
1 year before or after diagnosis	175 (54.7)	1.31 (1.10-1.55)	1.28 (1.08-1.52)
>1 year after diagnosis	51 (17.1)	0.96 (0.71-1.30)	0.93 (0.68-1.26)

CI, confidence interval; HR, hazard ratio; IR, incidence rate, per 1,000 person-years.
* HRs were adjusted for age at diagnosis, calendar year at diagnosis (2002-2004, 2005-2007, or 2008-2011), education level (primary school or unknown, high school, or college+), country of birth (Nordic or non-Nordic), and family size at diagnosis.
† HRs were additionally adjusted for FIGO stage (IA, IB, II, or III+), histology (squamous cell cancer or others), mode of detection (screening or symptom), primary treatment (operation, palliative care, or others), adjuvant therapy (yes or no), and Charlson comorbidity (yes or no).

6.3 STRESS-RELATED SIGNALING PATHWAYS AND LETHAL PROSTATE CANCER IN US MEN (PAPER III)

In the US sample, we included tumor issue from 150 men in PHS and 254 men in HPFS (Table 13). Men from HPFS were more likely to have a Gleason score≥7, and therefore had more lethal cases. Normal tissue was available from 202 of these men.

We found that differential expression of genes in the adrenergic, glucocorticoid, and serotoninergic pathways in prostate tumor tissue was significantly associated with the risk of lethal cancer (Table 14). The associations remained statistically significant for the glucocorticoid and serotoninergic pathways after adjusting for Gleason score and tumor stage. The association of differential signaling of the glucocorticoid pathway was also significant in tumors of both lower and higher stages, as well as in tumors of Gleason scores between 8 and 10. In the competitive pathway test (GSEA), we observed that the differential expression of genes in the adrenergic pathway was significant in relation to the risk of lethal cancer (P=0.001), compared to the differential expression of other unrelated genes. The adrenergic pathway tended to be down-regulated in the tumor tissue of lethal cancers. Of note, no pathway was differentially expressed in the adjacent normal tissue of the lethal cancers as compared to the nonlethal ones.

Table 13. Clinical characteristics of men with prostate cancer in Paper III.

	PHS	HPFS
Total number	150	254
With normal tissue, N(%)	82 (54.7)	120 (47.2)
Calendar year of diagnosis, range	1982-2005	1986-2004
Age at diagnosis (years), mean±SD	66±6.5	65±6.4
Lethal cases, N(%)	30 (20.0)	83 (32.7)
Gleason score, N(%)		
5-6	33 (22.0)	24 (9.4)
7 (3+4)	48 (32.0)	91 (35.8)
7 (4+3)	28 (18.7)	74 (29.1)
8-10	41 (27.3)	65 (25.6)
Tumor stage, N(%)*		
T1/T2, N0/Nx	89 (59.3)	150 (59.1)
T3, N0/Nx	49 (32.7)	83 (32.7)
T4, N1, M1	12 (8.0)	21 (8.3)
PSA at diagnosis, N (%)		
0-3.9 ng/ml	15 (10.0)	18 (7.1)
4-9.9 ng/ml	79 (52.7)	119 (46.9)
10+ ng/ml	35 (23.3)	75 (29.5)
Unknown	21 (14.0)	42 (16.5)

Physician's Health Study (PHS) and Health Professional Follow-up Study (HPFS)

* In the PHS, 18 men were missing pathological stage but had information on clinical stage, and therefore we used this information as an approximation for tumor stage: 12 were T1 or T2, 1 was T3, and 5 were T4 or N1 or M1. In this HPFS, 17 men were missing this information, and we used their clinical stage: 9 were T1 or T2, 1 was T3, 7 were T4 or N1 or M1.

Table 14. P-values of pathways analyses in terms of risk of lethal prostate cancer in the PHS and HPFS Prostate Tumor Tissue Cohort.

	N	Adrenergic pathway	Glucocorticoid pathway	Serotoninergic pathway	Dopaminergic pathway
Number of genes		11	12	17	6
In tumor tissue					
Primary model*	404	0.0010	<0.0001	0.0019	0.053
Adjusting for Gleason	404	0.058	0.0012	0.013	0.39
Adjusting for Gleason and stage	404	0.043	0.0006	0.0062	0.31
Stratified by Gleason†					
Gleason 7	241	0.063	0.098	0.017	0.51
Gleason 8-10	106	0.39	0.0002	0.062	0.16
Stratified by stage					
T1/T2, N0/Nx	239	0.071	<0.0001	0.13	0.12
T3/T4, N1, M1	165	0.034	0.0004	0.088	0.31
GSEA test‡	404	0.001 (↓)	0.31 (↑)	0.58 (↑)	0.47 (↓)
In normal tissue					
Primary model*	202	0.31	0.29	0.37	0.58

* In the primary model, we performed the likelihood ratio test to compare the null model (adjusting for age at diagnosis and cohort membership) with the full model (additionally including the genes in the candidate pathway) in logistic regression model.

† The analysis was not performed for men with Gleason 5-6 because of no lethal case.

‡ No covariate was controlled for in the gene set enrichment analysis (GSEA), which provides an indication of whether the candidate pathway is up-regulated (↑) or down-regulated (↓) in the tumor tissue of lethal prostate cancer.

The differential signaling of the adrenergic pathway was associated with increased cell proliferation and higher Gleason score (Table 15), and tended to be related to increased angiogenesis (i.e., vessel area and diameter of blood vessels) and perineural invasion. The significant associations were noted for the glucocorticoid signaling in terms of cell proliferation, angiogenesis (except for the microvessel density), perineural invasion, Gleason score, and tumor stage. The differential signaling of the serotoninergic pathway was only significant in tumor tissue with perineural invasion.

Table 15. P-values of pathways analyses in terms of biomarkers and histopathological characteristics in the PHS and HPFS Prostate Tumor Tissue Cohort.

	N	Adrenergic pathway	Glucocorticoid pathway	Serotoninergic pathway	Dopaminergic pathway
Cell proliferation*	314	0.0061	<0.0001	0.49	0.096
Apoptosis*	255	0.24	0.29	0.50	0.51
Angiogenesis*					
Microvessel density	174	0.13	0.21	0.31	0.19
Vessel area	174	0.031	0.0001	0.58	0.051
Diameter of vessels	174	0.025	0.0001	0.79	0.10
Perineural invasion	132	0.025	0.0002	0.0097	0.032
Gleason score†	404	<0.0001	<0.0001	0.54	0.078
Tumor stage‡	404	0.070	0.003	0.66	0.52
PSA at diagnosis§	341	0.25	0.06	0.42	0.37

* The biomarkers were classified into four levels according to quartiles.
† Gleason score was classified into 5-6, 3+4, 4+3, and 8-10.
‡ Tumor stage was classified into T1/T2, N0/Nx, M0; T3, N0/Nx, M0; and T4, N1, M1.
§ PSA (prostate-specific antigen) levels at cancer diagnosis were classified into 0 to 3.9 ng/ml, 4 to 9.9 ng/ml, and ≥10 ng/ml.

6.4 STRESS-RELATED SIGNALING PATHWAYS AND LETHAL PROSTATE CANCER IN SWEDISH MEN WITH LOCALIZED DISEASE (PAPER IV)

In the Swedish sample, men with lethal cancer were older at cancer diagnosis and more likely to have Gleason score≥7 and stage T1b, compared to men with nonlethal cancers (Table 16).

Table 16. Clinical characteristics of men with localized prostate cancer in Paper IV.

	Lethal cancer	Nonlethal cancer
Total number	186	325
Age at diagnosis, years±SD	74.1±6.6	71.5±6.6
Gleason score, N(%)		
4-6	39 (21.0)	179 (55.1)
7	64 (34.4)	95 (29.2)
8-10	62 (33.3)	26 (8.0)
Unknown	21 (11.3)	25 (7.7)
Tumor stage, N(%)*		
T1a	43 (23.1)	168 (51.7)
T1b	143 (76.9)	157 (48.3)

Pathway analysis

We found that the differential signaling of the serotoninergic pathway was significantly associated with the risk of lethal prostate cancer (P=0.007; Table 17). Differential expression of adrenergic and glucocorticoid pathways was also suggested between lethal and nonlethal prostate cancers (P=0.014 and P=0.020, respectively). However, no association remained statistically significant when adjusting for, or stratified by, tumor stage or Gleason score. In the sensitivity analysis, we restricted the analyses to the specific genes in each pathway, and differential expressions were suggested for the serotoninergic, adrenergic, and glucocorticoid pathways.

The association of differential signaling of the serotoninergic pathway was statistically significant for increased cell proliferation and TMPRSS2-ERG fusion, as well as suggested for inhibited apoptosis (Table 17). Significant associations with cell proliferation and TMPRSS2-ERG fusion were also noted for the adrenergic pathway. Genes in the glucocorticoid pathway were differentially expressed in tumors with a Gleason score of 8-10 or with the TMPRSS2-ERG fusion.

Table 17. P-values of pathway analyses in terms of lethal prostate cancer as well as biomarkers and histopathological characteristics in the Swedish Watchful Waiting Cohort.

	N	Adrenergic pathway	Glucocorticoid pathway	Serotoninergic pathway	Dopaminergic pathway
Number of genes		95	49	48	31
Lethal cancer					
Primary model*	262	0.014	0.020	0.007	0.092
Adjusting for stage	262	0.013	0.029	0.008	0.063
Adjusting for stage and Gleason	236	0.093	0.346	0.211	0.161
Stratified by Gleason					
Gleason 4-6	89	0.797	0.623	0.528	0.791
Gleason 7	95	0.462	0.151	0.795	0.335
Gleason 8-10	52	0.585	0.880	0.994	0.673
Stratified by stage					
T1a	79	0.119	0.445	0.047	0.958
T1b	183	0.112	0.067	0.089	0.059
The sensitivity analysis†	262	0.017	0.006	0.026	0.203
Secondary outcomes*					
Cell proliferation	217	0.0001	0.090	<0.0001	0.001
Apoptosis	250	0.161	0.222	0.016	0.047
TMPRSS2-ERG fusion	178	0.005	0.012	0.001	0.043
Gleason 8-10	236	0.182	0.0001	0.152	0.138
Tumor stage T1b	262	0.224	0.211	0.443	0.846
Perineural invasion	257	0.107	0.144	0.539	0.231
PIN	257	0.502	0.164	0.379	0.127

* In the primary model, we performed the global test based on logistic regression model for the analyses of lethal cancer, TMPSS2-ERG fusion, Gleason8-10, tumor stage T1b, perineural invasion, and prostatic intraepithelial neoplasia (PIN), whereas the test based on linear regression model was used for the analyses of cell proliferation and apoptosis. All primary models were adjusted for age at diagnosis.

† In the sensitivity analysis, we restricted the genes in the candidate pathways to a set of specific genes, which were not shared with other three pathways. The number of genes was 56 for adrenergic, 34 for glucocorticoid, 18 for serotoninergic, and 8 for dopaminergic pathway.

SNP analysis

Among the 30 candidate SNPs, we only found three that were, or tended to be, associated with the risk of lethal prostate cancer (Table 18). Based on the over-dominant model, the genetic variation of rs2296972 in HTR2A (in the serotoninergic pathway) carried a decreased risk of lethal cancer (OR 0.49, 95% CI 0.31-0.76, P=0.002), whereas the risk of lethal cancer tended to be higher among men with homozygous rs33388 in NR3C1 (in the glucocorticoid pathway) and rs6277 in DRD2 (in the dopaminergic pathway). No association of genetic variants of these SNPs were noted for biomarkers and histopathological characteristics, except for the variation of rs6277 that tended to be associated with stage T1b.

Further analyses showed that the variation in rs2296972 in HTR2A was associated with the expression of GNAI1 and GNAI2 in the serotoninergic pathway (P=0.035 and P=0.023, respectively), whereas the variation of rs6277 in DRD2 was correlated with the expression of FOS and SRC in the dopaminergic pathway (P=0.021 and P=0.032, respectively). The genetic variation in rs33388 in NR3C1 was associated with expression of genes SMAD4, MAPK14, SLC22A1, POU2F1, MMP13, and NFKB1 in the glucocorticoid pathway (P=0.010 for SMAD4 and P-values from 0.0125 to <0.05 for the others).

Table 18. P-values of SNP analyses in terms of lethal prostate cancer as well as biomarkers and histopathological characteristics in the Swedish Watchful Waiting Cohort[*]

	rs2296972	rs33388	rs6277
Gene	HTR2A	NR3C1	DRD2
Lethal cancer			
Additive model	0.490	0.761	0.198
Recessive model	0.075	0.099	0.014
Dominant model	0.043	0.361	0.802
Over-dominant model	0.002	0.035	0.022
- OR (95% CI)	0.49 (0.31-0.76)	1.59 (1.03-2.44)	1.66 (1.08-2.55)
Secondary outcomes[†]			
Cell proliferation	0.817	0.190	0.528
Apoptosis	0.930	0.413	0.388
TMPRSS2-ERG fusion	0.211	0.256	0.933
Gleason 8-10	0.421	0.918	0.403
Tumor stage T1b	0.986	0.654	0.027
Perineural invasion	0.477	0.493	0.267
PIN	0.141	0.920	0.262

CI, confidence interval; OR, odds ratio; PIN, prostatic intraepithelial neoplasia.

[*] We performed the logistic regression model for the analyses of lethal cancer, TMPSS2-ERG fusion, Gleason8-10, tumor stage T1b, perineural invasion, and PIN, whereas the linear regression model was used for the analyses of cell proliferation and apoptosis. No covariate was controlled for in the models.

[†] Only three SNPs associated with lethal prostate cancer were further assessed in associations with secondary outcomes. Given the over-dominant model fitting the SNP analysis best for lethal prostate cancer, the associations with secondary outcomes were therefore all based on the over-dominant model.

7 DISCUSSION

7.1 STUDY DESIGN, BIAS AND CONFOUNDING

7.1.1 Cohort and nested case-control studies

Many analytic studies about the impact of psychological stress on cancer incidence were carried out using the case-control design,(3) comparing different aspects of psychological stress among cancer patients with cancer-free individuals. Commonly, the selection of controls was not population-based and the assessment of stress was retrospective. On the other hand, a well-designed cohort study holds the unique advantages of minimal selection and recall bias. The nested case-control design that we employed for the analyses of cervical dysplasia and cancer in Paper I is computationally less demanding and equivalent to the full cohort analysis in validity.(171) Cohort studies could be very costly, whereas only a portion of the biological samples from the entire cohort are tested, for example the HPV status, if using the nested case-control design. This is one of the major reasons that the NCSR and Uppsala Studies we used for analysis of HPV infection in Paper I were originally conducted using the nested case-control design. Although we adopted the case-control design for this analysis, the included control women (i.e. without *in situ* or invasive cervical cancer) were randomly selected from the screening participating population in several Swedish counties. Moreover, the loss of a family member was identified from the prospectively collected information in national registers, which is less prone to recall bias. Cohort studies can be very time-consuming, for instance when studying cervical cancer survival. Given the unique personal identification number in Sweden, we were, however, able to follow all individuals in the cervical cancer cohort in Paper II through cross-linkage to national public and health registers, such as the Causes of Death and Migration Registers. As a result, the register-based cohort study, for example Paper II, enables efficient data collection and maintains the large-scale and virtually complete follow-up.

The most straightforward approach to study psychological stress is to measure the perceived stress level individually. Such information is unfortunately not available in registers. Instead, we assessed the loss of a family member as a proxy for psychological stress in Paper I. Similarly, we examined psychological stress using the stress-related mental disorders and several major stressful life events (including loss of a family member) in Paper II. Although these proxies are highly correlated to psychological stress, the magnitude of perceived stress levels are also influenced by individuals' coping strategies and levels of social support.(172) That said, the misclassification of exposure exists to some extent in our analyses. For instance, it is plausible that divorce is not very stressful for some individuals (Paper II). However, such misclassification (i.e., not stressed individuals were classified to the exposed group) would theoretically dilute the risk in the exposed group and lead to an underestimated relative risk. Indeed, we observed weaker associations of divorce with cancer-specific mortality among patients with cervical cancer. To further allay such concern, we also performed analyses on types and numbers of stress indicators to shed light on the magnitude of stress level that the individuals actually perceived. It is possible that loss of a family

member due to unnatural cause of death (i.e. suicide or accident) is in general more stressful compared with loss due to natural death (e.g. chronic disease). Reassuringly, in both Papers I and II, we noted that the association of loss due to unnatural causes of death was consistently stronger in terms of the risks of cervical cancer incidence and mortality. We also showed a clear dose-response effect of loss of a family member on the risks of cervical dysplasia and cancer: the more losses experienced, the higher the risk of cervical carcinogenesis.

In both Papers I and II, we obtained the information on cancer incidence and mortality from the Cancer and Causes of Death Registers, which were evaluated as complete and valid sources in general.(133,135) We also measured the HPV status and viral load using standard and validated methods.(138,140,173,174) Minimal information and misclassification biases in the ascertainment of outcomes were therefore assured for both studies. However, in Paper I, some cervical dysplasia might have been misclassified as normal cytology (i.e. the controls), because cervical cytology is well-recognized for its high specificity but moderate sensitivity.(7) Such potential misclassification, if any, should be non-differential in terms of women's status of loss of a family member, which would have only attenuated the true association of loss with the risk of abnormal cytology. Studies addressing cervical dysplasia using validated histological diagnosis are, however, warranted in future.

One of the most apparent limitations of the register-based cohort studies is perhaps the lack of information on potential confounders. For example, socioeconomic status may be associated with both loss of a family member and the risk of cervical cancer. However, we carefully adjusted the analyses for, and stratified the analyses by, screening adherence, whenever possible, in Paper I. It is well-recognized that screening adherence is highly correlated to socioeconomic status(175), as well as the risks of cervical dysplasia and cancer. In Paper II, we have also thoroughly adjusted for educational level at the time of cancer diagnosis as a proxy for socioeconomic status in all analyses. Lifestyle factors are potential confounders for the association of stress with the risk of cervical cancer incidence; and, to some extent, are correlated to the socioeconomic status and screening adherence as well. However, in Paper I, we showed that women with loss of a family member did not significantly differ from women without loss in terms of several known lifestyle risk factors for cervical cancer, such as smoking, use of oral contraceptives, and sexual behaviors.(40-42) This analysis was performed in a small subset of women who were randomly selected from the screening participating population in Sweden and were interviewed (participation rate >90%).(146) Moreover, the stronger association observed for the infection of high-risk HPV types, compared with low-risk types, refutes the possibility of a complete explanation by lifestyle factors for our findings, presuming that the lifestyle factors alone would have introduced equally increased risks for both types. Most importantly, lifestyle factors are more likely mediators, representing one mechanism linking together bereavement and higher risk of cervical cancer, and should therefore not be adjusted for. For instance, it is plausible that loss of a family member entails potential change of lifestyle, and subsequently alters the risk of cervical cancer. Similarly, in Paper II, the tumor stage and cancer treatment could also serve as both confounders and mediators. It is plausible that receiving a cancer diagnosis with

advanced stage is more stressful and also more likely to lead to death from the cancer disease (i.e., acting as a confounder). It is also possible that individuals with mental disorders attended cervical screening less frequently and therefore are more likely to have advanced stage at diagnosis, and thereby higher mortality (i.e., acting as a mediator). Although we found robust associations either with or without adjustment for these covariates, the true estimates of the total effect of psychological stress on cancer-specific mortality might lie somewhere in between. Finally, due to the nature of observational studies, we cannot rule out the impact of residual confounding (if any) in our findings.

It is also plausible that the onset of mental disorders shortly before death is secondary to the terminal disease of patients that were dying of cervical cancer. But, we observed a significant association with cancer-specific mortality excluding the first two years of follow-up after the onset of mental disorders, which largely alleviates the concern of reverse causality.

7.1.2 Extreme case-control studies

The growth of large-scale cohort studies, such as PHS and HPFS, has produced large repositories of clinical data and biological samples. For disease like prostate cancer, many have a relatively benign course (i.e. nonlethal disease). Measuring biomarkers in a large portion of nonlethal disease is costly and not efficient to identify potential biological signatures for prognosis. For optimal use of available information in the data, the extreme case-control design has been implemented. In this design, all lethal diseases (defined as patients who died of prostate cancer or developed distant metastasis during follow-up in Paper III; and patients who died of prostate cancer during follow-up in Paper IV) were included as cases and nonlethal diseases were randomly sampled from the sub-cohort, in which patients did not die of prostate cancer (and did not develop metastasis) during certain follow-up, as controls. Any potential difference in exposure (i.e. gene expression and genetic variants) between cases and controls would be expected to be the most evident in these "extreme" groups. It has been shown that the extreme case-control design gains in statistical power when assessing the quantitative phenotype, compared to the standard cohort approach.(176). Regarding the sampling of nonlethal diseases in Paper III, we oversampled men with available blood samples, but their clinical characteristics were comparable to all men with nonlethal disease in the entire cohort.

Gene expression in Papers III and IV was measured based on 0.6 mm biopsy cores taken from tumor-enriched areas (>90% tumor cells) in the dominant nodules or the highest Gleason pattern in FFPE tissue blocks. Adjacent normal tissues were also used for genotyping in Paper IV. We used standard and validated methods to measure the gene expression and genetic variants in both studies.(158,177,178) It is plausible that the mRNA differences we observed in both studies may in part be attributable to the tumor microenvironment (such as peripheral neurons or stromal cells). Reassuringly, gene expression in adjacent cells are highly correlated to the tumor cells, because of the field effect where tumor and adjacent cells tend to be alike in many biological aspects,(179) and is unlikely to be the driving force for the noted associations. Compared to the whole-genome

gene expression in Paper III, fewer genes were profiled in Paper IV (i.e., 18-37% genes in pathways were not tested for expression). However, the global test confined every gene to have a small contributory effect on the overall pathway effect, which alleviated concern about the bias of selected genes in our findings. Possible technical biases, such as batch effect and varying sample quality, have been addressed in data pre-processing.

Potential confounders, such as age at diagnosis and cohort membership, were adjusted for throughout the analyses for gene expression. Tumor stage and Gleason score were primarily considered as mediators linking differential gene expression to lethal cancer instead of confounders, and were only adjusted for in a secondary model. For instance, we found the differential signaling of the glucocorticoid pathway was related to higher Gleason scores in both studies, and was significantly associated with lethal cancer even when adjusting for tumor stage and Gleason score in Paper III. Such results lend some support to the possible contribution of the glucocorticoid pathway to the risk of lethal prostate cancer mediated through tumor differentiation. The interaction between different stress-related pathways, namely the crosstalk, may potentially drive the differential signaling of individual pathways in the tumor tissues of lethal cancer. The individual contribution of the glucocorticoid pathways was further strengthened by the remaining associations when we restricted the analyses to genes exclusive or specific to the studied pathways in Papers III and IV.

We corrected for multiple testing by using $P<0.0125$ as statistical significance for all pathway analyses. In the SNP analyses, we still focused on the same four pathways and therefore made no further corrections for multiple comparisons, which might have resulted in some false positive findings. However, we emphasize the fact that the consistent results(180) for both pathway-based gene expression and genetic variants, particularly in serotoninergic pathway, largely refutes the possibility of pure explanation by chance finding.

Finally, we only assessed gene expression at a single time point for each man, and therefore we only captured a biological snapshot to examine the potential differences of stress-related pathways between lethal and nonlethal cancers. This approach does not allow us to make any temporal or functional claims, because it is unknown when the signaling started to alter and how they would have further interacted with cancer treatment. Reassuringly, we corroborated the findings of the US study with the results of the Swedish study in which the lethal outcome largely reflects the natural course of localized disease without any initial treatment.

7.2 GENERAL DISCUSSION

7.2.1 Psychological stress and cervical carcinogenesis

Cervical cancer initiation

In Paper I, we systematically demonstrated that the loss of a family member, as an extremely stressful life event, is consistently associated with increased risk of every known step of cervical cancer initiation, including oncogenic infection of HPV, cervical dysplasia, and invasive cervical cancer.

Psychological stress has been linked to increased risk of infection of several oncogenic viruses in cell lines and animals.(5) We, for the first time, showed that psychological stress, using loss of a family member as a proxy, was positively associated with HPV infection in humans, particularly oncogenic infection for cervical carcinogenesis. The oncogenic infection is well-recognized for infection of high-risk HPV types, especially for persistent infection and high viral load infection of HPV16.(7,138) We found a stronger association with repeated infections, suggesting that psychological stress may compromise host immunosurveillance and consequently increase the risk of persistent or recurrent HPV infection.(5) It has been shown that psychological stress reduces T-cell proliferation in response to HPV16 in women with cervical dysplasia(181), and stress hormones have also been shown to inhibit the immune response by negatively regulating antigen presentation of major histocompatibility complex class I.(129) In addition, we noted a stronger association for high viral load infection of HPV16, lending further support to the potential role of psychological stress in the upregulated expression and malignant transformation of HPV oncogenes, possibly through the dysregulation of HPA axis.(5,127,128)

We further showed that the loss of a family member was consistently associated with elevated risk of cervical precancerous lesions and invasive malignancy. Our previous works have indicated the increased risk of invasive cervical cancer after loss of a parent in childhood or after loss of a child in adulthood.(88,93) Here, we extended the knowledge to the earlier steps of cervical cancer development, namely cervical dysplasia and *in situ* cervical cancer. Moreover, we extensively assessed a range of loss events (i.e., loss of a child, spouse, sibling, or parent), whereas in earlier studies only one specific type of loss was examined.(8,88,93,94) Furthermore, we evaluated whether age modified the risk of cervical cancer after different types of loss events. Of note, loss of child, sibling, or spouse was consistently associated with a greater risk of *in situ* and invasive cervical cancer throughout the life span, whereas the excess risk of these outcomes after loss of a parent was only noted in early life. Lastly, although based on a small subgroup of the study participants, our findings were less likely to be entirely explained by the differential lifestyle factors between women with and without loss of a family member, such as smoking, use of oral contraceptives, and sexual behavior.

Together with the apparent dose-response relationship by number and type of losses, our consistent findings throughout all steps of cervical cancer initiation lend strong support to the notion that psychological stress contributes to cervical cancer initiation, likely through enhanced oncogenic infections of HPV.

Cervical cancer progression

In Paper II, we found that 43% of the cervical cancer patients experienced at least one stress-related mental disorder or stressful life event from one year before cancer diagnosis until end of follow-up (average 4.3 years after diagnosis). The cancer-specific survival was significantly shortened for the exposed patients, regardless of other prognostic indicators including tumor characteristics and cancer treatment.

Except for depression, the impact of anxiety, stress reactions and adjustment disorders on the risk of cancer-specific mortality have not been extensively studied among patients with cancer.(182) The limited evidence on preexisting mental disorders and mortality in any cancer has suggested heterogeneous associations across these disorders.(182,183) However, we found that among patients with cervical cancer, depression, anxiety, stress reaction, and adjustment disorders, either collectively or individually, were consistently associated with a 60%-116% risk elevation of cancer-specific mortality. The consistent findings in our data, compared to the conflicting results in earlier studies, might to some extent be explained by the different cancer type studied. It is highly plausible that, due to the known social stigma(184) and a drastically reduced quality of life(185) among patients with cervical cancer, the mental disorders diagnosed immediately before or after the diagnosis of cervical cancer largely reflect patients' severe stress response toward a potential maladjustment of the cancer diagnosis and treatment. That said, the recent onset of stress-related mental disorders might help to identify a subgroup of emotionally vulnerable patients. It was reported that gynecologic cancer patients with preexisting mental disorders had increased cancer-specific mortality in Australia, due to lower rate of surgery and radiotherapy.(182) In our data, even though we controlled for primary treatment including surgery and adjuvant therapy, we still noted an increased risk of cancer-specific mortality after a recent onset of stress-related mental disorder.

The association of specific stressful life events, such as loss of a family member and divorce,(9,10) with cervical cancer survival has been assessed in a few previous studies. Here, we showed that four major life events, including loss of a family member, severe illness of a family member, divorce, and job loss, either collectively or individually, were consistently associated with a 21%-35% increased risk of cancer-specific mortality, although some associations were not statistically significant. Our findings on loss of a family member and divorce are in general in line with other Nordic studies.(8-10) Another merit of our study is that we extended the knowledge base by showing that other life events, i.e., severe illness of a family member and unemployment, may have similar impact on cancer-specific survival among patients with cervical cancer.

Experimental studies have convincingly demonstrated that psychological stress might modulate tumor growth and progression through the dysregulation of the HPA axis and SNS, regarded as the "direct" effect.(4) Additionally, psychological stress may also play a role in cancer prognosis through potentially delayed diagnosis or altered treatment plan. The so-called "indirect" effect has been suggested among individuals with mental disorders and unmarried individuals.(182,186) Studies have rarely been able to separate the contributions of differential tumor stage and cancer treatment from the overall effect of psychological stress on cancer-specific mortality.(182,187) Indeed, tumor characteristics and cancer treatment may to some extent contribute to the observed associations, because in Paper II most associations attenuated somewhat, although never disappeared, after exhaustive adjustments. Regardless, the overall associations remained robust for both mental disorders and life events,

independent of aforementioned clinical characteristics and cancer treatment, lending strong support to a biological "direct" effect of psychological stress on cervical cancer progression.

Together with our findings in cervical cancer initiation (Paper I), the findings in Paper II highlight a salient role of psychological stress in cervical carcinogenesis, from cancer initiation to progression. This consistency may lend further support to the same biological theory that also links stress to cervical cancer progression, namely psychological stress modulates cervical cancer progression potentially through compromised immunosurveillance. Future studies are however needed to confirm or refute such hypothesis.

7.2.2 Psychological stress and prostate cancer progression

In Papers III and IV, instead of assessing psychological stress through stressful life events, we examined the signaling of four neuroendocrine pathways, with a confirmed link to psychological stress, in the tumor tissue of prostate cancer. We found that, in both US and Swedish samples, the expression of genes from these stress-related pathways in the tumors of men with lethal prostate cancer significantly differed from men with nonlethal cancer. The effects of adrenergic and glucocorticoid pathways were more pronounced in the US sample where more patients had relatively advanced tumor stage and Gleason score, whereas the serotoninergic pathway appeared to play a more important role in the Swedish sample where more men had localized disease. The potential underlying mechanisms may include cell proliferation, tumor differentiation, perineural invasion, and TMPRSS2-ERG fusion. In the Swedish sample, we also found that genetic variation of receptor genes, particularly of HTRs, may explain in part the differential signaling of pathways, at least in the tumors of men who developed lethal cancer from localized disease.

Adrenergic pathway

Our findings suggest that the dysregulation of the adrenergic pathway is associated with lethal prostate cancer, although the role is less prominent in the localized disease. This is in line with previous experimental studies showing that adrenaline and activation of ADRB2 contribute to the development and local invasion of prostate cancer.(188,189) The GSEA test in the US sample indicated that the signaling of the adrenergic pathway was down-regulated in the tumors of lethal cancer at the transcription level. Interestingly, the overexpression of adrenergic receptor at the protein level has been suggested in metastatic prostate cancer, compared to localized cancer.(190) On transcription level, the expression of adrenergic receptors was, however, shown to be lower in metastatic than in localized prostate cancer.(191) The paradox between transcription and protein levels of adrenergic signaling may simply suggest a possible negative feedback loop due to long exposure to an agonist, such as chronic stress.(192) Alternatively, the down-regulated signaling of the adrenergic pathway may be engaged with de-differentiation and epithelial-mesenchymal transition so that the tumor cells gain more potential to invade and migrate.(112) In addition, we found that the differential signaling of the adrenergic pathway was associated with increased cell proliferation in both studies, suggesting a potential mechanism underlying the link between

adrenergic pathway and lethal prostate cancer. It has also been shown that the enhanced signaling of the adrenergic pathway facilitated tumor proliferation in prostate and ovarian cancers in animal models.(188,193) However, we did not find any evidence to support the association between genetic variation of adrenergic receptor genes and cancer progression in localized disease.

Glucocorticoid pathway

We found a broadly consistent association of differential signaling of the glucocorticoid pathway with risk of lethal prostate cancer in both the US and the Swedish studies, although the association was relatively weaker in the Swedish study of men with localized disease. The fact that the differential expression of the glucocorticoid pathway was not statistically significant in the GSEA test in Paper III was likely because two driving genes – PTGES3 and SMAD4 – in the pathway had opposite associations with lethal cancer, and GSEA is more powerful when testing pathways with genes demonstrating effects in the same direction. It is plausible that the glucocorticoid pathway may assist the transition of prostate cancer cells from androgen- to glucocorticoid-dependence growth. While androgens supports tumor cell growth throughout the early stage of prostate cancer, glucocorticoids could directly orchestrate the growth of prostate cancer through mutated androgen receptors and acquire androgen-independent growth in a later stage.(194) Of note, in Paper IV, the downstream cascade of the glucocorticoid pathway subsequent to SMAD4 is possibly bypassed by the TGFβ/SMAD4 signaling axis, which is well-recognized for its role in prostate cancer development.(195,196) That said, the downstream genes included in the glucocorticoid pathway might be less relevant to cancer progression in localized disease. Indeed, in Paper IV, we observed stronger associations in sensitivity analysis where those genes were excluded. We have also noted consistent relationships between differential glucocorticoid signaling and increased cell proliferation in both studies. Although glucocorticoids have the potential to suppress the androgen synthesis and subsequently inhibit tumor growth in some prostate cancer,(197) the differential signaling of glucocorticoid pathway may transform tumor cells to androgen-independent prostate cancer and further accelerate cell proliferation.(198) In addition, the genetic variant of NR3C1 (rs33388) may partly explain the increased lethal cancer risk through the correlation with expression of a number of genes (including SMAD4) in the glucocorticoid pathway.

Serotoninergic pathway

We observed consistent association of differential signaling of the serotoninergic pathway with the risk of lethal prostate cancer in both the US and the Swedish studies. Interestingly, the role of serotoninergic signaling, compared to other stress-related pathways, is more prominent among men with localized disease. Genes in the serotoninergic pathway have previously been linked to prostate cancer differentiation: over-expression of HTR1, HTR2, and HTR4 in high-grade cells and over-expression of HTR2 in low-grade cells of prostate cancer.(199,200) It has been suggested that the downstream regulatory pathway through MAP kinase and PI3K/Akt signaling plays a critical part in prostate cancer progression.(201)

However, the pharmacologic effects of serotonin on cell lines remain inconclusive.(199,200,202,203) The serotoninergic signaling seemed to exert an influence on cell proliferation and apoptosis, though such associations were only noted in the Swedish sample. It is in line with *in vitro* studies showing that activated serotonergic receptors facilitate cell proliferation in prostate cancer, and that blocking the receptors induce apoptosis.(110)

Moreover, we showed a protective effect of the genetic variant of HTR2A (rs2296972) on the risk of lethal prostate cancer in localized disease. The interpretation of the results from the over-dominance model is not clear, but it is not inconceivable that the heterozygote carries a more influential effect on the phenotype than the homozygote (i.e. heterosis).(204) Heterozygote advantage in various health outcomes has been documented for multiple neuroendocrine genes, including HTR2A.(204) It is plausible that a third factor – psychological stress in our case – may also interact with the molecular expression independent of the expected additive effect of genetic polymorphism.(204) This SNP was further correlated to the expression of G protein subunits coupling with serotoninergic receptors in the serotoninergic pathway (i.e., GNAI1/2). Together with the findings at translational level, we propose that the genetic predisposition of HTR2A may contribute to the risk of lethal prostate cancer through serotoninergic signaling in localized disease. As a first possible piece of evidence, we however cannot rule out alternative contributory effects of, for example, somatic mutations in tumor cells or post-translational modifications, in the differential signaling of serotoninergic pathway.

Of note, in the US sample, none of the above pathways was differentially expressed in adjacent normal tissue obtained from the same lethal cases, compared to the normal tissue from nonlethal cases. It is plausible that tumor cells and normal cells respond to stress differently. Our data from both studies did not support a prominent role of dopaminergic signaling in prostate cancer progression. In the Swedish sample, the differential signaling of the four stress-related pathways was consistently associated with the presence of TMPRSS2-ERG fusion, which lends further support to the suggested role of TMPRSS2-ERG in neuroendocrine transformation of prostate tumor cells.(205) Although not noted in the Swedish sample, the consistent associations of differential signaling of all pathways with the presence of perineural invasion were found in the US sample. This finding requires further investigation, though it may in part reflect the high correlation with lethal outcome.(72,206)

In Papers III and IV, we demonstrated the molecular differences in the stress-related pathways, namely the adrenergic, glucocorticoid, and serotoninergic pathways, in tumors of lethal prostate cancer, compared to nonlethal cancer. Such differential signaling may also be involved in tumors with different histopathological features, including cell proliferation, tumor differentiation, perineural invasion, and TMPRSS2-ERG fusion. Furthermore, genetic predisposition may contribute to prostate cancer progression in localized disease, through differential signaling in particular of the serotoninergic pathway. Altogether, our findings provide considerable support to the neuroendocrine link between psychological stress and prostate cancer progression.

8 CONCLUSIONS

- Loss of a family member is consistently associated with all known steps of cervical cancer initiation, from cervical dysplasia to invasive cancer, potentially through enhanced oncogenic infections of HPV. Our findings shed light on the possible modulating effect of psycholocial stress on malignant transformation after a primary HPV infection.
- Stress-related mental disorders and stressful life events are associated with compromised cancer-specific survival, independent of tumor characteristics and cancer treatment. Our results provide further support to the contribution of psycholocial stress on cervical cancer progression, in addition to initiation.
- Signaling of stress-related pathways, particularly adrnergic and glucocorticoid pathways, may be dysregulated in tumors of US men with lethal prostate cancer, compared to nonlethal cancer. These results indicate potential roles of neuroendocrine pathways in prostate cancer progression.
- Differential expression of stress-related pathways, particuarly the serotoninergic pathway, may be involved in cancer development from localized prostate cancer to lethal cancer among Swedish men. Such difference may to some extent be explained by genetic predisposition.

9 FUTURE PERSPECTIVES

A growing body of evidence from both experimental and epidemiological studies supports a role of psychological stress in carcinogenesis. However, challenges remain in order to yield more consistent findings in epidemiological studies.

First, consistent and feasible measurements of psychological stress should be established for epidemiological studies. The diverse definition of stress in the current literature, ranging from poor quality of life to emotional liability to depression, may largely explain the highly heterogeneous results.(3) More consistent findings are, however, noted when "extreme" stress (e.g. extremely stressful life event such as loss of a family member) is examined. Although a range of valid rating scales are available for stress measurement, developing a register-based stressor matrix to individually assess stress level would greatly benefit future epidemiological studies, especially in countries with available public and health registers. The methods used in Paper II stand as a good exercise to better capture the emotional burden from different stress sources among cancer patients.

Secondly, multiple biological mechanisms exist underlying the link between stress and cancer, and therefore the effects of stress may vary across cancer types. This may also, in part, explain the conflicting associations observed in previous studies that were possibly not assessing the most relevant cancer types.(3) Future research may focus on certain cancer types according to specific biological mechanisms. For example, as shown in Paper I, cervical cancer may be of particular interest to pursue, given the specific mechanism of stress-induced immunosuppression and oncogenic infection. Studies in other infection-related cancers, especially virus-related, are warranted in future. In addition, the behavior-related cancers, such as lung and liver cancers, may also be highlighted by possible behavioral pathways, because stress has been show to increase the risk of smoking and alcohol consumption.(207,208)

Thirdly, cancer-specific survival should be examined to estimate the impact of stress on cancer progression. Receiving a cancer diagnosis, as well as living with cancer, is by nature stressful, and have been associated with elevated risks of suicide and cardiovascular deaths.(209) It is therefore plausible that the estimates based on overall cancer survival are to some extent driven by these conditions with a clear and immediate link to stress. Previously, none of the three studies in cervical cancer (Table 1), and only one study in prostate cancer (Table 2), specifically assessed the cancer-specific survival. As we did in Papers II-IV, using the cancer-specific survival as the primary outcome may help capture the "net" effect of stress on cancer progression in future studies.

Another challenge is having large enough sample size. It is plausible that the effect of stress on the risks of cancer initiation and progression is modest. To detect a precise and statistically significant association, very large sample sizes would then be required. This possibly explains the varying findings in many studies with smaller size. Merging registers in Nordic countries could form a unique and powerful resource and provide potential clues on this

puzzle by comprehensively exploring individual stressor, cancer type, and cancer-specific mortality.

Last but not least, a great deal of research is required to translate the findings on stress and carcinogenesis to cancer prevention and care. Although the prevalence of psychological distress is estimated to be high in the general population,(210) no psychological intervention studies so far aims to prevent cancer, likely due to the unconvincing link between stress and cancer initiation. On the other hand, many trials have shown that psychological intervention may reduce stress and improve mood among cancer patients, although very few trials have examined whether psychological intervention could benefit cancer survival.(4) Although the current findings are inconclusive,(211-216) a "second wave" of trials have been called for to target the vulnerable cancer patients(4) and to intervene in early-stage cancers in which the metastatic capacity remains physiologically modifiable.(11) Indeed, we have observed different weights of stress-related pathways in the progression of early-stage prostate cancer (Paper IV), compared to more advanced cancer (Paper III). In addition to psychological intervention, novel means of chemical intervention, for instance targeting the adrenergic pathway, may also open up new strategies for cancer treatment. Several observational studies have revealed that use of beta-blocker – a β-adrenergic antagonist – before cancer diagnosis is associated with prolonged survival in prostate, breast, lung, pancreatic cancers and malignant melanoma.(115,217-220) Randomized controlled trials are, however, needed in future to help draw a causal conclusion.

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