The Role of Microorganisms in Prostate Cancer Development

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"Du blir aldrig färdig, och det är som det skall"
Tomas Tranströmer
Abstract

Prostate cancer is the most common cancer among Swedish men, but the aetiology of this disease is largely unknown. There is evidence for a linkage between chronic inflammation and prostate cancer. The mechanisms causing prostate inflammation and how this could promote tumour development and progression are however largely unknown. Chronic inflammatory infiltrates are common findings in prostate tissue samples and infection is proposed to be one possible cause for this inflammation. Inflammatory cells release free radicals, cytokines, and growth factors that facilitate increased cell proliferation, DNA damage, mutations, and angiogenesis. However, the present literature on the presence of microbes in prostate tissue and their possible linkage to inflammation and cancer development is limited. Therefore, the aim of this thesis was to investigate if microorganisms are present in prostate tissue and to evaluate their role in inducing prostatitis and prostate epithelial neoplasia.

The presence of microorganisms (virus, bacteria and fungi) was studied in clinical prostate tissue samples to evaluate whether or not the occurrences of microorganisms were different in patients that later developed cancer compared with matched controls that did not. Viruses, bacteria and fungi were found in prostate tissues. Out of eight different viruses investigated, EBV and JC virus were detected, but there were no differences in occurrence in the case group compared to the control group. The fungus Candida albicans was present in a very small proportion of the prostate tissue samples. The predominant bacterium was Propionibacterium acnes and the second most prevalent was Escherichia coli. The presence of Propionibacterium acnes was associated with inflammation and subsequent prostate cancer development. Propionibacterium acnes was further evaluated for its capacity to induce an inflammatory response both in vitro and in vivo. Live Propionibacterium acnes induced a strong immune reaction in prostate epithelial cells in vitro with up-regulation of inflammatory genes and secretion of pro-inflammatory cytokines. Infection with Propionibacterium acnes in rat prostate resulted in a lobe specific inflammation with the most intense inflammation in the dorso-lateral prostate, lasting up to 3 months post-inoculation. Propionibacterium acnes inflammation was also associated with altered epithelial cell morphology, signs of DNA damage and increased cell proliferation.

Taken together, this thesis shows that different viruses and bacteria can be found in prostate tissue. Propionibacterium acnes, the most abundant among the bacteria detected and more prevalent in the cancer than in the control group, exhibits strong prostatitis promoting properties both in vitro
and *in vivo*. In addition, *Propionibacterium acnes* can induce some of the epithelial changes known to occur during prostate neoplasia formation. This thesis therefore suggests that *Propionibacterium acnes* induced chronic prostatitis could promote prostate cancer development. Further studies are needed to elucidate the molecular interplay linking *Propionibacterium acnes* induced inflammation and the formation of a pre-neoplastic state that could evolve into prostate cancer.
Populärvetenskaplig sammanfattning


För vissa cancerformer, som exempelvis cancer i levern, magsäcken, livmoderhalsen och i lymfkörtlarna, är det numera känt att virus, bakterier och kronisk inflammation spelar en viktig roll för sjukdomarnas uppkomst, en kunskap som redan används för att till exempel vaccinera mot cancerframkallande virus. I prostata är kronisk inflammation, d.v.s. prostatit, mycket vanligt och några studier antyder att inflammation skulle kunna orsaka förstadier till cancer. Vilka faktorer som orsakar sådan inflammation och om denna inflammation i sin tur verkligen kan orsaka prostatacancer eller påverka dess förlopp är dock oklart.


För att vidare undersöka om denna bakterie verkligen kan orsaka inflammation i prostata infekterades prostatapitelceller med P. acnes. Resultaten visar att bakterien kan orsaka en stark inflammationsreaktion i körtelceller från prostata, d.v.s. den får prostatakörtelceller att utsända ett flertalet inflammations- och tillväxtstimulerande faktorer.
Mot denna bakgrund; d.v.s. att bakterien ofta förekommer i prostata, möjligen oftare hos män som senare får cancer och att den stimulerar prostataepitelceller att utsändra inflammationsfaktorer, undersökte vi därefter om *P. acnes* kan orsaka prostatit hos försöksdjur, och i så fall, om den bakterie-orsakade inflammationen kan ge upphov till cancer. Injektion av *P. acnes* i prostata hos råttor ledde till kronisk prostatainflammation. Djuren verkade dock så småningom kunna läka infektionen utan tecken på permanent skada. Under den tid prostata var inflammatoriskt hittades skador i körtelceller; cellerna blev omogna, började dela sig och ett enzym som normalt skyddar mot skador i arvsmassan minskade. Inflammationsceller anses bilda faktorer som kan skada arvsmassan och tecken på ökad DNA-skada sågs i prostatakörtelarna hos de infekterade djuren. Resultaten visar alltså att *P. acnes* kan orsaka kronisk prostatainflammation hos råttor och att den bakterieorsakade inflammationen tycks kunna orsaka en del, men inte alla de steg som behövs för att cancer skall kunna utvecklas.

Original papers

This thesis is based on the following studies, which are referred to in the text by their Roman numerals:


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTH</td>
<td>Dihydrotestosterone</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
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<tr>
<td>GSTP1</td>
<td>Glutathione –s-transferase pi</td>
</tr>
<tr>
<td>IF</td>
<td>Immunoflouresence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphatate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PhIP</td>
<td>2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine</td>
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<tr>
<td>PIA</td>
<td>Proliferative inflammatory atrophy</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostatic specific antigen</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmitted infection</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TURP</td>
<td>Transurethral resection of prostate</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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1. Introduction

Prostate cancer is by far the most common cancer in Swedish men. Its aetiology is largely unknown, but as it is estimated that about 20% of all human cancers are caused by chronic infection, it is possible that microorganisms could play a role also in the pathogenesis of prostate cancer. Prostatitis, caused by infectious agents, but also by other factors, is a very common finding in men of all ages and this inflammation is preferably seen in the parts of the prostate where cancer is detected in elderly men. Already in the early 1950s, sexually transmitted infections (STI) were proposed as a risk factor for prostate cancer development. Since then epidemiological, clinical and experimental studies on both STIs and non-sexually transmitted agents have been investigated in their relation to prostate carcinogenesis. The question whether prostate cancer can be induced by chronic inflammation, and if so, what the causes for this cancer promoting inflammation are, is largely unanswered.

1.1. The Prostate

- A small organ with few essential functions but the centre of many common diseases

1.1.1. Anatomy

The human prostate is a part of the male reproductive system. It is located anterior to the rectum distal to the urinary bladder and wraps around the urethra. Due to its anatomical position, infectious agents can reach the prostate mainly through the urine or as ascending sexually transmitted infections. The normal prostate has the same size and shape as a walnut and it mainly consists of tubuloalveolar glands that empty their secretions into the urethra. In humans the prostate is divided into three different zones; the peripheral, the central and the transitional zone. The peripheral zone, located posterior to the urethra and ejaculatory ducts, comprises the majority (65%) of the prostate volume and is the part of the gland were 70% of all prostate cancer originates. Inflammation is also frequently localized to the peripheral zone. The central zone constitutes approximately 25% of the normal prostatic volume and is rarely affected by carcinoma or inflammation. The transitional zone comprises about 5-10% of the prostate in young adults but as benign prostate hyperplasia (BPH) originates in this part of the prostate the volume generally increases with age.
In contrast to the rather homogenous structure of the human prostate, the prostate in rodents is composed of three (or four) lobes; the ventral, dorso-lateral (also considered as separate dorsal and lateral) and the anterior prostate ⁸. There is no clear-cut homology between specific rodent prostate lobes and human prostatic zones but it has been observed that neoplasms are usually derived from the dorso-lateral lobe in rodents ⁹, ¹⁰ and has therefore been proposed to be corresponding to the peripheral zone ¹¹, ¹². Gene expression analysis also support the idea that the dorso-lateral lobe is most similar to the peripheral zone of the human prostate ¹³.

1.1.2. Histology

The prostate consists of two basic structures; glands and fibromuscular stroma. The glands consist of three fully differentiated cell types; basal epithelial, luminal epithelial and neuroendocrine cells. The basal cells rest on the basement membrane and harbour a small number of stem cells ¹⁴. These stem cells proliferate and give rise to basal, luminal or neuroendocrine cells. The differentiation to luminal cells is believed to occur through an intermediate phenotype, expressing markers specific for both basal and luminal epithelial cells ¹⁵, ¹⁶.

The basal cells are characterized by their expression of cytokeratin (CK) ⁵, CK ¹⁴ and p63. In contrast to the basal cells, the secretory luminal cells are terminally differentiated and have limited capacity to divide. They are characterized by expressing the androgen receptor (AR), CK ⁸, CK ¹⁸ and synthesizes and secrete many of the key proteins found in the ejaculate for example prostate specific antigen (PSA) and prostate acid phospatase ¹⁷-¹⁹. Neuroendocrine cells are rare. They secrete several neuropeptides but their function is not fully elucidated. These cells express markers such as chromogranin A, synaptophysin and are AR negative ²⁰.

The fibromuscular stroma is made up of smooth muscle cells, fibroblasts, blood vessels, nerves, lymphatics and infiltrating immune cells. The stroma gives physical support to the glandular epithelium, enables contraction and mediates release of growth factors that regulate epithelial growth and homeostasis.

1.1.3. Function

The main function of the prostate is to synthesise and secrete the proteins and fluid that, together with contributions from the seminal vesicles, form most of the ejaculate. Although the prostate is involved in fertility, it is not
required for reproduction. The major protein produced by the luminal epithelial cells is PSA. PSA is a protease that helps to liquefy the semen so that the sperms way to the egg is facilitated. PSA is produced in humans but not in rodents. Normally PSA is secreted into the glandular lumina and by muscular contractions in the stroma, pumped into the urethra during ejaculation. During conditions such as prostate cancer, inflammation and benign prostate hyperplasia (BPH), the basal epithelial cell layer and the basement membrane are disrupted and PSA may leak into the surrounding stroma and vasculature. Thereby PSA is elevated in the blood and is used as a diagnostic marker for prostate diseases. The prostate is also known to produce antimicrobial products such as zinc, lysozyme and defensins.

1.1.4. Regulation

The prostate is dependent on androgens for development, growth and function. Testosterone, the main circulating androgen, is produced in the testes by the Leydig cells. In the prostate, testosterone is converted to the considerably more potent androgen dihydrotestosterone (DTH) by the enzyme 5-alpha reductase. DTH (and testosterone) binds to AR in the cytoplasm and is then translocated to the nucleus were it induces expression of androgen–related genes.

Prostate growth control can be studied by androgen withdrawal (castration). When the supply of androgens is lost, the prostate luminal epithelial cells will undergo apoptosis, resulting in involution of the prostate gland. However, both stromal and basal epithelial cells are maintained during castration. AR is also expressed in the fibromuscular stroma and androgens regulate epithelial growth and regression through paracrine factors (called andromedins) released by the stroma. The exact nature of these andromedins is not fully established but several members of the insulin-growth factor (IGF), fibroblast growth factor (FGF) and wnt families are involved. Studies in transplanted tissue recombinants and in animals where AR is selectively knocked-out in the epithelium and in the stroma, shows that stromal AR is necessary and that epithelial ARs are not required for castration induced glandular involution. The main function of AR in the luminal epithelium is to maintain differentiation (such as PSA secretion) and suppress proliferation of these cells.
1.2. Prostate disorders

Three different and very common diseases affect the prostate: prostatitis, benign prostatic hyperplasia and prostate cancer.

1.2.1. Prostatitis

Prostatitis is a very common and multifaceted disease. It affects men of all ages and it is estimated that about 50% of all men will experience symptoms of prostatitis at some time during their lives 37. It is the most common urological disorder in men under the age of 50 and the third most common urological disorder, after benign prostatic hyperplasia and prostate cancer, in men older than 50 38. Reported rates of prostatitis are similar in North America, Europe, and Asia 39. Common symptoms include pain (genitourinary, pelvic, or rectal), voiding symptoms and sexual dysfunction. According to the National Institutes of Health (NIH), prostatitis is classified into the following four categories: I: Acute bacterial prostatitis, II: Chronic bacterial prostatitis, III: Chronic prostatitis/Chronic pelvic pain syndrome (CPPS) IV: Asymptomatic inflammatory prostatitis 40.

Bacterial prostatitis (category I and II) is estimated to account for 10% of all prostatitis cases, with the most commonly implicated microorganisms being Escherichia coli, and Enterococcus spp 41, 42. Other common organisms include Proteus mirabilis, Pseudomonas aeruginosa and Klebsiella. Diagnosis is based on symptoms, physical examination, urine culture and/or urine culture after prostatic massage. Bacterial prostatitis is treated with antibiotics, preferably a fluoroquinolone, because of their good prostate penetration and activity against most usual bacterial pathogens 43.

A majority of men searching medical care for typical prostatitis symptoms are classified as having chronic prostatitis/CPPS (category III). The aetiology of chronic prostatitis/CPPS is unknown but several hypotheses have been suggested including presence of antibiotic-resistant non-culturable microorganisms, urethral obstruction, autoimmunity, neuropathic pain and psychological dysfunction 44, 45. Microorganisms that have been suggested to be the causes of chronic prostatitis/CPPS include; Chlamydia trachomatis 46, Trichomonas vaginalis 47, Uroplasma urealyticum 48, Mycoplasma genitalium 48, fungi 49 and several viruses 50, 51.

The therapies commonly used for chronic prostatitis/CPPS are empirical treatment with antibiotics, anti-inflammatory drugs and alpha-adrenergic blockers 52.
Asymptomatic inflammatory prostatitis (category IV) is the most common form of prostatic inflammation. The inflammation is often found in biopsies taken from men evaluated for possible prostate cancer, in trans-urethral resection tissue samples from men with BPH or in autopsied prostates 53-57.

1.2.2. Benign prostatic hyperplasia (BPH)

Benign prostatic hyperplasia is a common disorder in elderly men and is characterized by enlargement of the prostate gland caused by proliferation of epithelial and stromal cells in the transition zone. The clinical symptoms result from compression of the prostatic urethra and consequent obstruction of the bladder outlet.

The aetiology of BPH is unknown. Androgens are known to play a permissive role in BPH. This means that androgens have to be present for BPH to occur, but do not necessarily directly cause the condition. Men who are castrated prior to puberty or have a 5α-reductase-type 2 deficiency do not develop BPH 58 and a 5α-reductase inhibitor significantly decrease prostate volume 59. An inflammatory origin of BPH have been proposed because of the very common finding of inflammatory infiltrates in BPH lesions 55, 60 and that these inflammatory cells release cytokines and growth factors that stimulate the stroma and epithelial cells to hyperproliferation 61, 62 63. Men that have suffered from prostatitis have a greater risk to later develop benign prostatic hyperplasia64, 65. Many studies have demonstrated the presence of heterogeneous bacterial strains in BPH specimens 55 but if infectious agents can induce the inflammation that triggers BPH development has not been extensively examined.

Screening and diagnosis procedures for BPH are similar to those used for prostate cancer; voiding problems lead to digital rectal examination, PSA-testing and ultrasound examination of the prostate and urinary tract. Treatment is medical or surgical. The medical treatments commonly used include α-blockers, to relax stroma smooth muscles and/or 5α-reductase inhibitors, which inhibit DTH production. Surgery is performed when medical treatment fails. The golden standard surgery treatment is transurethral resection of the prostate (TURP) were parts of the prostate is removed by electrocautery or sharp dissection through the urethra.
1.2.3. *Prostate cancer*

1.2.3.1. *Epidemiology*

Prostate cancer is the most common cancer in Sweden with an incidence of 9697 per year (2010) (The National Board of Health and Welfare). The incidence of prostate cancer has increased dramatically over the last 20 years probably caused by the introduction of PSA testing, which has lead to an increased number of diagnoses in asymptomatic men. In the last 5 years, the incidence has slightly decreased indicating that the peak is reached. Prostate cancer mortality is almost unchanged during these years with 2460 deaths (2010) annually. Approximately 5% of Swedish men die from prostate cancer. Men diagnosed with prostate cancer are on average 70 years old, while the majority of prostate cancer deaths occur in men over 79 years. Interestingly, the number of men diagnosed with advanced, metastatic disease, have declined the last years indicating that more men are potentially curable.

Today approximately 75 000 men in Sweden are living with a prostate cancer diagnosis, but autopsy studies indicate that asymptomatic prostate cancer is actually present in more than 50% of men in their 5th decade or older. Worldwide, the highest incidence of prostate cancer is seen in Western countries and in some African regions and the lowest incidence is seen in Asian countries. The highest incidence of prostate cancer death is seen in African-Americans and the second highest risk of dying in prostate cancer is seen in Sweden. The explanation to variations in both incidence and mortality in prostate cancer around the world is not known, although genetic, environmental and socioeconomic factors could all be of importance.

1.2.3.2. *Diagnosis and therapy*

Localised prostate cancers do not often cause any symptoms and if they do these are similar to those of BPH i.e. voiding problems. A patient searching hospital care for voiding problems will have his PSA value measured in a blood test and the prostate can be examined by rectal palpation. The PSA test is taken to assess the risk of prostate cancer. In Sweden, a PSA value < 3ng/ml is considered normal and a PSA value > 3ng/ml indicates that some of the conditions prostate cancer, BPH or prostatitis exist. At substantially elevated PSA values, prostate cancer is often the cause.
Ultrasound guided needle biopsies are taken from the prostate in patients with elevated PSA levels and if a biopsy contains cancer it is graded according to the Gleason system 69. Their differentiation pattern, ranging from 1 to 5, were 5 represents the lowest differentiated and most aggressive tumour pattern, grade the tumour glands. The most common and the second most common grade is summarised into the Gleason score (2-10). Tumours with Gleason score <6 have a good prognosis whereas tumours with Gleason score >8 are associated with an unfavourable outcome. In patients with Gleason score 6 and 7, which constitute about 75% of cases diagnosed outcome is highly variable and at present largely unpredictable 70.

Localised prostate cancer is treated with radical prostatectomy or radiotherapy. If the life expectancy of the patient is short and the tumour is at an early stage it is common that the patient is only subjected to watchful waiting (no treatment until symptoms of metastases) or active monitoring (treatment when signs of tumour progression are detected). Advanced prostate cancer is characterized by dissemination of prostate cancer cells, typically to lymph nodes and bone. Bone scintigraphy, computed tomography and PSA level in serum are used to determine how advanced the tumour is. For advanced and metastatic prostate cancer there is no cure and the therapy is palliative with surgical or chemical castration. Castration initially reduces proliferation and increases apoptosis in prostate tumour cells because of androgen depletion 71. However, after some time (about 1-3 years) the tumour relapses and start to grow in a castration resistant manner 72.

1.2.3.3. Histopathology

Almost all prostate cancers originate in the glandular epithelium. Prostate cancer is generally a multifocal disease, i.e. each patient has several different cancers simultaneously in the prostate. Some of these tumours are highly differentiated and probably clinically insignificant but others can be poorly differentiated and highly malignant. Most cancers are however of intermediate grade (score). Some intermediate grade cancers are clinically insignificant whereas others are potentially lethal. Unfortunately current diagnostic methods, i.e. histological Gleason grading of the tumours in needle biopsies, do not provide sufficient prognostic information.

Although the aetiology and pathogenesis of prostate cancer is largely unknown it is fairly well established that prostate cancer originates in precancerous lesions of prostate glands 2, 73 (Figure 1). In the peripheral zone of the prostate, atrophic glands are commonly seen 74. In some of these
glands the epithelial cells show high proliferation, up-regulation of anti-apoptotic factors and cells with genetic alterations such as silencing due to hypermethylation and mutations accumulate. Furthermore there is an increase in epithelial cells that possess a phenotype between basal and mature luminal cells, so called intermediate cells \(^7\). Adjacent to such glands the stroma undergoes morphological changes, inflammatory cells such as macrophages and lymphocytes are common, and there are signs of increased angiogenesis. This type of lesion is called proliferative inflammatory atrophy (PIA) \(^2\). PIA lesions are thought to arise as a consequence of the regenerative proliferation of prostate epithelial cells in response to injury caused by inflammatory oxidants \(^2, 76, 77\). The cause of this inflammation is not known but infections and other mechanisms (see below) have been suggested \(^2\). In some glands adjacent to PIA, prominent precancerous epithelial atypia arises, and such lesions are termed prostate intraepithelial neoplasia (PIN). PIN is characterized by luminal cell hyperplasia, enlargement of nuclei and nucleoli and nuclear atypia \(^78\). Several molecular pathways/genes that are linked to prostate cancer are also altered in PIA and PIN, for example the tumour suppressor genes NKX3.1, CDKN1B and PTEN \(^2, 75, 79\). Silencing of the “care taker” gene Glutathione S-transferase gene pi (GSTP1), due to hypermethylation in the promoter region, is widely seen in both PIN lesions and in prostate cancer and to a lesser extent also in PIA lesions \(^80, 81\). Recently it was detected that approximately 50% of men with prostate cancer harbour a somatic fusion-gene between an androgen regulated gene (TMPRSS2) and genes encoding EST family transcription factors \(^82\). This gene, which is seen already in a subset of PIN cases, function as an oncogene and result in increased androgen driven cell growth. Interestingly the fusion-gene apparently also stimulates prostate tumour growth by stimulating intratumoral prostaglandins and tumour inflammation \(^83\). The question whether inflammation plays any role in the appearance of the fusion-gene, is still unanswered. It is not detected in PIA lesions \(^82\). Fusion–genes in general are however created under conditions of insufficient DNA-repair. In contrast to prostate cancer, basal cell numbers are reduced, but not absent in PIN. Prostate adenocarcinoma can be confirmed by the absence of basal cells using immunostaining for p63 and cytokeratin 5 and 14. Areas with PIA and/or PIN can bee seen merging with overt cancer, suggesting that there may be a continuum of changes from PIA over PIN to cancer \(^84, 85\).
Figure 1. Progression pathway model for human prostate cancer.
1.2.3.4. Aetiology

Even though prostate cancer is very common, rather little is known about its causes. A number of risk factors have been proposed, where some are well established and others are weak and controversial. The most established risks are age and family history. Diet, hormones and inflammation are examples of factors that may cause prostate cancer but have not been similarly well elucidated. Given the marked heterogeneity in prostate cancer aggressiveness, where some harmless cancers are extremely common, whereas other less common are highly malignant, it is not unlikely that different subgroups of prostate cancer could have different causes.

1.2.3.4.1. Old age

The greatest risk factor for developing prostate cancer is advanced age. As histological foci of asymptomatic cancer are extremely common already in the 5th decade of life and increase with age, it is believed that all men will develop clinically relevant prostate cancer if they only live long enough. The reason why prostate cancer is so common among elderly is probably due to the increased number of genetically altered cells that occur as a natural consequence of increasing age. Signs of chronic inflammation are very common in elderly men and various studies have shown age-dependent gene expression changes, particularly in the stroma, in genes associated with inflammation, oxidative stress, and cell proliferation. 

1.2.3.4.2. Family history

A large proportion of prostate cancer patients report family history of the disease. Twin studies have shown that more than 40% of prostate cancer cases are estimated to be explained by hereditary factors. Moreover, a meta-analysis has shown that having a first-degree relative with prostate cancer increases the risk for disease with a relative risk of 2.24 (95% confidence interval 2.08-2.41). A number of high-risk candidate genes responsible for hereditary cancers have been identified. Nevertheless, these are rare and indicate the familial prostate cancer is a consequence of many affected loci rather than specific susceptibility genes. Interestingly, two of these germ-line susceptibility genes are involved in host response to infection, RNASEL and MSR1. Ribonuclease L (RNASEL) encodes an enzyme that degrades single stranded RNA, leading to apoptosis in virally
infected cells \(^9^1\). The macrophage scavenger receptor (MSR1) encodes a receptor expressed on macrophages and is able to bind lipoproteins on both Gram-positive and Gram–negative bacteria \(^9^2\). Furthermore, areas within the prostate that show evidence of inflammation are often populated by macrophages that express MSR1 \(^7^7\). The question whether prostatitis is more common in families with hereditary prostate cancer has to my knowledge not been examined.

1.2.3.4.3. Diet/life style

Migration studies have shown that Asian men will experience an increased risk of developing prostate cancer when moving to the USA \(^9^3\). The prevalence of small asymptomatic prostate cancers is similar all around the world but clinically symptomatic tumour and prostate cancer death are more common in countries with a western life style \(^9^4\). These differences may be caused by factors in Asian diets that inhibit prostate cancer growth and/or factors in Western diets that stimulate prostate cancer growth. For example, some vegetarian diets contain factors that inhibit prostate cancer growth (like phytoestrogens and omega-3) \(^9^5^\text{-}^9^7\) whereas high consumption of red meat \(^9^8\) and saturated fat \(^9^9\) may promote prostate cancer development. A recent study showed that treatment with phytoestrogens of prostate cancer cell lines resulted in demethylation of the GSTP1 promotor region \(^1^0^0\).

Interestingly, feeding Fisher 344 rats with one factor present in over grilled red meat, 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine (PhIP), first results in chronic prostatitis followed by appearance of PIA-like glandular atrophy precancerous epithelial lesions and later overt cancer \(^1^0^1^\text{-}^1^0^2\). This observation in an experimental model clearly links chronic inflammation with cancer development.

1.2.3.4.4. Hormones

The prostate requires steroid hormones for growth and development, but such hormones are also essential for cancer maintenance and growth. Their role in the initiation of prostate cancer is unknown.

Treatment of rodents both neonatally and in adult life with estrogen or mixes of estrogen and androgens or with environmental chemicals with estrogen-like effects can induce precancerous lesions and in some cases also cancer (see \(^1^0^3^\text{-}^1^0^4\) for review). Interestingly this is preceded by development of chronic prostatitis. Similarly, exposure of humans to elevated levels of
estrogen during fetal life or chronically later in life is associated with an increased risk of prostate cancer\(^{105,106}\).

### 1.2.3.4.5. Inflammation

Already in 1863 the German pathologist Virchow suggested that cancer was induced by chronic inflammation. His hypothesis was that some classes of “irritants” caused tissue injury, inflammation and increased cell proliferation\(^{107}\). Today 25\% of all cancers are estimated to be induced by chronic inflammation or infection\(^{108}\). For example, gastric cancer can be caused by chronic infection with the bacterium *Helicobacter pylori*, liver cancer by chronic infection with hepatitis viruses, Burkitt’s-lymphoma by chronic infection with Epstein-Barr virus, and cervical cancer by human papilloma virus infection\(^1,109,110\) (Table 1). It should however be noted that only a small portion of cases infected with these agents develop cancer, suggesting that individual susceptibility is of importance. Moreover there are also examples where chronic inflammation apparently does not cause malignancy, for example rheumatoid arthritis is apparently not associated with tumours in affected joints. Notably, the relationship between cancer and inflammation is also bidirectional. Activation of oncogenes often result in the secretion of cytokines and chemokines and premalignant and malignant cells therefore secrete factors attracting inflammatory cells and thus cause chronic inflammation in and around tumours without the involvement of infectious agents\(^{111}\). Cancer-induced inflammation generally promotes tumour growth and spread\(^{112,113}\).

The mechanisms by which chronic inflammation may cause cancer are not fully defined but are usually explained by the tissue injury and enhanced cell proliferation caused by the persistent inflammation. Furthermore, inflammatory cells release factors that can promote cancer development. For example, in order to kill infectious agents inflammatory cells produce reactive oxygen and nitrogen oxide species (ROS and RNOS) that cause damage to DNA. Cells that proliferate in such a milieu are at an increased risk for accumulation of mutations leading to increased expression of oncogenes and/or inactivation of tumour suppressor genes. Inflammatory cells (macrophages, neutrophils, eosinophils, dendritic cells, mast cells and lymphocytes) also secrete cytokines, chemokines and growth factors that can promote cell proliferation, inhibit apoptosis and stimulate angiogenesis\(^{109}\), all necessary steps in the development of a tumour\(^{113}\).
Table 1. Tumours Linked to Infections

<table>
<thead>
<tr>
<th>Infection agent</th>
<th>Tumour type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Gastric cancer, gastric lymphoma</td>
</tr>
<tr>
<td>Human papilloma virus</td>
<td>Cancer of the cervix, anal and genital cancers</td>
</tr>
<tr>
<td>Hepatitis B and C virus</td>
<td>Hepatocellular carcinomas</td>
</tr>
<tr>
<td>Epstein-Barr virus</td>
<td>B-cell and Burkitts lymphoma, nasopharyngeal cancer, gastric cancer</td>
</tr>
<tr>
<td>Human herpes virus 8</td>
<td>Kaposi sarcoma</td>
</tr>
<tr>
<td>Human lymphotropic retrovirus 1</td>
<td>Adult T-cell leukemia</td>
</tr>
<tr>
<td><em>Schistosoma heamatobioum</em></td>
<td>Bladder cancer</td>
</tr>
<tr>
<td><em>Opisthorchis viverrini</em></td>
<td>Cholangiosarcoma</td>
</tr>
</tbody>
</table>

Modified from 1, 114

For example, TNF-α, released by macrophages and T-lymphocytes, enhance the formation of NOS and thereby increase the risk for DNA damage and mutation in epithelial and stromal cells 115. Cytokines and chemokines may also result in epigenetic changes altering gene expressions in ways promoting cancer development 116. IL-6 is another pro-inflammatory cytokine that have been shown to act as an autocrine or paracrine growth factor in some malignancies 117 and IL-8 is known to promote angiogenesis 118. In addition, an imbalance of pro- and anti-inflammatory cytokines may prevent the self-limiting process of the inflammatory response, leading to a chronic inflammation 119. It is also likely that factors produced by a particular infectious agent can promote cancer development 110. For example some *H. pylori* strains harbour the cag pathogenicity island (PAI) that encodes several virulence factors. One of the most important is the cytotoxic associated antigen (Cag A), which is “injected” into the epithelial cells through a type IV secretion system. Once inside the cytoplasm the bacterial protein interacts with several cellular pathways which in turn result in the promotion of proliferation, apoptosis, motility and induction of inflammatory gene expression 109, 120. Strains having the cag PIA are more likely to be associated with gastric adenocarcinoma than those lacking it 120.
Is there a support for a relation between inflammation and prostate cancer?

In the light of the observations that so many tumours are linked to infectious events or inflammatory states and the fact that histological inflammation is common in prostate it would not be surprising if also prostate cancer could be related to chronic infection. Indeed, several lines of evidence do indicate that chronic inflammation (of different causes) may be a key component in the pathogenesis of prostate cancer. Chronic, mainly asymptomatic inflammation is, as mentioned above, very common in the prostate and prostatitis and cancer both arises in the same part of the prostate, the peripheral zone 2. Histopathological studies show that intraepithelial neoplasia (PIN) and cancer develops in glands previously altered and atrophic as a result of chronic inflammation, so called PIA 76, 84, 121. Epidemiological studies show an increased risk of prostate cancer in men with a history of prostatitis 64, 122-124. A meta-analysis of 11 case-control studies found an increased risk of prostate cancer odds ratio (OR): 1.6 in men with a history of prostatitis, particularly in population-based case-control studies (OR: 1.8) 122. Although, some studies have reported negative results 125. Also, men with a history of a sexually transmitted infection (STI) have been shown to have a higher risk for developing prostate cancer (OR: 1.4) 126, 127, 2.3 for a history of syphilis and 1.4 for a history for gonorrhoea 126. Indication that STI infect the prostate and contribute to prostatic inflammation stems from studies that show that PSA is elevated in men with a confirmed diagnosis of a STI 128. In addition, long-term treatment with anti-inflammatory drugs appears to reduce the risk of developing prostate cancer 129, 130.

In humans the risk of developing hereditary (see above) or sporadic prostate cancer is apparently associated with alterations in genes involved in inflammatory pathways. Hypermethylation of CpG island sequences in the promoter of the GSTP1 gene, which encodes an enzyme that inactivates electrophilic carcinogens, is an extremely common early epigenetic event in the progression of prostate cancer 131, 132. High levels of GSTP1 expression are typically seen at sites of prostatic inflammation, and loss of GSTP1 expression is commonly seen in PIN and prostatic carcinoma 81. This suggests that GSTp1 serves as a “caretaker” gene and that the decreased expression might render prostate cells more vulnerable to malignant progression. Furthermore several sequence variants in genes involved in inflammatory pathways have been linked to prostate cancer 133.
Single nucleotide polymorphism (SNP) in sequences encoding the pathogen recognition receptors TLR4 and the TLR-1-6-10 gene cluster are associated with prostate cancer risk. The Toll-like receptors (TLR) are key players in innate immunity as they recognize different ligands expressed by bacteria and viruses, such as lipopolysaccharide (LPS), lipoprotein and viral RNA. The activation of TLRs results in nuclear translocation of the transcription factor nuclear factor-kappaB (NF-κB), which then induces the expression of various pro-inflammatory cytokines, chemokines, and effector molecules.

A number of different studies have investigated the correlation of interaction of SNPs in different cytokines and increased risk of prostate cancer. One study found that combinations in variations of IL-1B, IL-10 and TNF was associated with an increased risk for developing prostate cancer. Another study reported that SNPs in IL-4, IL-6, PTGS2 and STAT3 were associated with prostate cancer susceptibility.

Another cytokine that have been evaluated for association with prostate cancer is macrophage inhibitory cytokine (MIC1). MIC1 is believed to have an important role in regulating macrophage activity and sequence variants of MIC1 have been linked to prostate cancer. Together these studies underline that variants in genes associated with inflammation affect prostate cancer risk.

**Microorganisms found in prostate tissue in relation to prostate cancer**

Prostatic inflammation can be induced by various different sources including infection, urine reflux, dietary factors, autoimmune reactions, hormones and corpora amylaceae or a combination of two or more of these factors. Several different infectious agents have been investigated in relation to prostate cancer development. In clinical specimens, tissue based methods (PCR, IHC and ISH) that have investigated the presence of various microorganisms in human prostatic tissues or serological assays (ELISA or IF) that have evaluated antibody titers against different infectious agents have been used.

HPV is the most investigated pathogen in relation to prostate cancer because of its well-established role in genital cancer development. The results from these studies are contradictory and in a recently published meta-analysis no association between HPV infection and prostate cancer could be found. Other viruses that have been investigated include; cytomegalovirus, Epstein-Barr virus, herpes simplex virus.
human herpes virus 8, polyoma viruses JC and BK and the novel γ-retrovirus XMRV. Xenotropic murine leukaemia related virus (XMRV) was first indentified in 2006 by Urisman et al. in prostate tumours from patients that were homozygous for a reduced activity germline variant of the RNASEL gene. After this finding several other studies have reported XMRV in prostate cancer tissues, whereas others have failed to detect the virus in prostate cancer samples. However, recent evidence suggest that XMRV originated from a recombination event between two endogenous murine retroviruses during passage of prostate cancer xenografts in mice, and the presence of XMRV in prostate cancer tissue might results from contamination of mice DNA.

Also different bacteria have been evaluated regarding their role in prostate carcinogenesis, where Chlamydia trachomatis, Mycoplasma sp. or Ureaplasma sp. or Propionibacterium acnes (see below) and E. coli are the most investigated pathogens. The majority of studies that have investigated the presence of bacteria in prostate tissues have been generated in studies from men with chronic prostatitis. Common bacterial species found in these studies are; Enterobacter sp., Enterococcus sp., Pseudomonas sp. (bacteria associated with UTI) or Actinomyces sp., Streptococcus sp. To the best of my knowledge eight independent studies have evaluated the presence of bacteria in prostate cancer tissues, either by 16S rDNA PCR or/and by bacterial culture (Table 2). Seven out of these eight studies reported positive bacterial findings, with frequencies varying from 20 to 89%, and in some of these studies bacterial findings were correlated to histological inflammation. This indicates that bacteria are frequently present in cancerous prostate tissues. Concordance between inflammation and positive bacterial findings suggest that bacteria might have a role in histological (asymptomatic) inflammatory prostatitis.

Surprisingly very few studies have apparently tried to detect individual infectious agents in PIA and PIN lesions. However, Samanta et al. and Grinstein et al. reported CMV and EBV in human PIN lesions, respectively and Fassi Fehri et al. reported P. acnes to be present in human PIN lesions. Experimental support for an association between infection and prostate cancer development also exists. In a mouse model of chronic bacterial prostatitis, E. coli induced chronic inflammation that lead to preneoplastic tissue alterations similar to PIN. Benign prostate cells infected with Mycoplasma sp. underwent karyotypical changes and inoculation of infected cells in male nude mice initiated tumour growth.
**Table 2. Bacteria in prostate cancer tissues**

<table>
<thead>
<tr>
<th>Material</th>
<th>Detection method</th>
<th>Bacteria Positive samples (%)</th>
<th>Most common organisms</th>
<th>Inflammation</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooper et al. 1988*</td>
<td>Transurethral resection or retropubic prostatectomy</td>
<td>Culture</td>
<td>6 (66%) of 9</td>
<td>Anaerobic cocci, Bacteroides distasonis, Clostridium perfringens</td>
<td>Not reported</td>
</tr>
<tr>
<td>Gorelick et al. 1988</td>
<td>Transurethral resection or suprapubic prostatectomy</td>
<td>Culture</td>
<td>44 (21%) of 209</td>
<td>Escherichia coli, Streptococcus faecalis, Staphylococcus epidermis</td>
<td>Not associated</td>
</tr>
<tr>
<td>Keay et al. 1999</td>
<td>Transperineal biopsies</td>
<td>16S PCR</td>
<td>8 (89%) of 9</td>
<td>Escherichia coli, Bacteroides</td>
<td>Not reported</td>
</tr>
<tr>
<td>Hochreiter et al. 2000</td>
<td>Radical prostatectomy samples</td>
<td>16S PCR</td>
<td>6 (86%) of 7</td>
<td>Not reported</td>
<td>Associated with inflammation</td>
</tr>
<tr>
<td>Krieger et al. 2000</td>
<td>Radical prostatectomy samples</td>
<td>16S PCR</td>
<td>21 (20%) of 107</td>
<td>Escherichia coli, Ureaplasma urealyticum</td>
<td>Not reported</td>
</tr>
<tr>
<td>Leskinen et al. 2003</td>
<td>Radical prostatectomy biopsies</td>
<td>16S PCR</td>
<td>0 (0%) of 10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cohen et al. 2005</td>
<td>Radical prostatectomy samples</td>
<td>Culture</td>
<td>19 (58%) of 34</td>
<td>Propionibacterium acnes, Staphylococcus epidermis</td>
<td>22.85% had focal chronic inflammation, More inflammation in P. acnes positive samples</td>
</tr>
<tr>
<td>Sfanos et al. 2008</td>
<td>Radical prostatectomy samples</td>
<td>16S PCR: 16s PCR:</td>
<td>16S PCR:</td>
<td>Acinetobacter spp., Escherichia spp., Pseudomonas spp.</td>
<td>16S PCR:</td>
</tr>
<tr>
<td></td>
<td>Culture:</td>
<td>26 (8%) of 30</td>
<td>Culture:</td>
<td>Propionibacterium acnes, Staphylococcus</td>
<td>Culture:</td>
</tr>
<tr>
<td></td>
<td>Specific PCR:</td>
<td>P. acnes PCR: 10 (4%) of 200</td>
<td>Specific PCR:</td>
<td>Not reported</td>
<td>Specific PCR:</td>
</tr>
<tr>
<td></td>
<td>Culture:</td>
<td>11 (33%) of 30</td>
<td>Specific PCR:</td>
<td>C. trachomatis PCR: 1 (0.5%) of 200</td>
<td>Specific PCR:</td>
</tr>
<tr>
<td>* This study only reported anaerobes (Copper et al. 1988)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.3.4.6. Propionibacterium acnes

-a novel cause for prostatitis and prostate cancer?

The facultative anaerobic Gram-positive bacterium, *Propionibacterium acnes* (*P. acnes*), is part of the normal flora in the skin 170, oral cavity 171, large intestine 172, the conjunctiva 171 and the external ear canal 170. Generally, *P. acnes* have a positive effect on the human health by preventing colonization of severe pathogens, but when the host becomes compromised (trauma, injury or alterations in immune status) it can display a pathogenic potential 173. *P. acnes* is mostly associated with the skin disease *acne vulgaris* 173, 174, but it is also associated with a variety of inflammatory diseases such as prosthetic joint infections 175, shunt associated central nervous system infections 176, endocarditis 177, sarcoidosis 178 and the SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) syndrome 179.

On the skin, *P. acnes* resides in the hair follicles where the oxygen level is optimal. Sebum-rich areas of the skin support the growth of *P. acnes* since the bacterium utilizes free-fatty acids and glycerol as energy sources. The main end products of the metabolism are propionic acid, thereby the name *Propionibacterium acnes*. In *acne vulgaris*, *P. acnes* is believed to induce and maintain an inflammatory response in the pilosebaceous unit. There are several mechanisms by which *P. acnes* may induce an inflammatory reaction; *P. acnes* produce chemotactic factors that attract neutrophils 180. The neutrophils then ingest the bacteria, resulting in release of hydrolases that is responsible for disruption of the follicular epithelium 181. Moreover, *P. acnes* releases lipases, proteases and hyaluronidases that further contribute to the tissue injury 182, 183. The host response to *P. acnes* is characterized by production of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin 1-α (IL-1α) and interleukin 8 (IL-8) 184. *P. acnes* have been shown to induce these cytokines in both macrophages and keratinocytes through the TLR2 and TLR4 signalling pathways 185-187.

Recently, and thus during the course of my PhD studies, *P. acnes* has been reported to be frequently prevalent in both benign and malignant prostate tissues 158, 169, 188, 189. In 2005, Cohen et al. detected *P. acnes* as the most prevalent microorganism in prostate cancer tissues, identified by bacterial culture in 35% of prostate cancer specimens removed by prostatectomy 158. Furthermore, *P. acnes* was positively associated with prostatic inflammation and this raised the question if *P. acnes* could be involved in prostate
carcinogenesis. After 2005, two independent studies (one from our group and one from Germany) have detected *P. acnes* in prostate tissues and found a positive association to prostate cancer development.

Two large epidemiological studies have found an association between a history of cutaneous acne and increased risk of prostate cancer. However, contradictory results regarding *P. acnes* involvement in prostate cancer development are also reported. Severi et al. found in a population based case-control study that plasma concentration of *P. acnes* antibodies were significantly inversely associated with prostate cancer (OR: 0.73), in particular for advanced disease (OR: 0.59).

Three different phenotypes of *P. acnes*, Type I (Type IA and Type IB), II and III, have been categorized according to sequence comparison of the housekeeping gene recA and a putative hemolysin gene tly. Moreover, a multilocus sequencing typing (MLST) approach, based on nine housekeeping genes, have further subdivided Type I into I-1a and I-1b and I-2. There are several studies indicating that certain *P. acnes* strains participate in specific pathogenic process. For example, members of the *P. acnes* type I-1a have been shown to be associated with severe acne whereas *P. acnes* type I-1b and II is more prevalent in orthopedic implants and in radical prostatectomies. Furthermore, the ability to trigger production of proinflammatory cytokines in keratinocytes is different for different phenotypes.

Interestingly, due to its immunostimmulatory capacity this bacterium has been tried as immunotherapy against a variety of neoplasms in both animals and humans. The mechanism of the anti-tumour activity of *P. acnes* is not fully understood but it is speculated that the inflammation caused by *P. acnes* could induce a cytotoxic response that kill tumour cells.
2. AIMS

2.1. General aim
The general aim of this thesis was to evaluate if microorganisms have a role in the aetiology of prostate cancer.

2.2. Specific aims

Aim 1
To study the presence of eight potentially tumour promoting DNA viruses (Epstein-Barr virus, Herpes simplex virus 1 and 2, Cytomegalovirus, Adenovirus, Human Papilloma virus, Polyoma viruses BK and JC), the fungus *Candida albicans* and bacteria in prostate samples from men with benign prostate hyperplasia and to evaluate if presence of microorganisms was different in cases that later developed prostate cancer compared to controls that did not.

Aim 2
To characterize the inflammatory response in prostate derived epithelial cells upon infection with *Propionibacterium acnes* in an *in vitro* model system.

Aim 3
Establish if *Propionibacterium acnes* can induce an inflammatory response in the rat prostate *in vivo*, and if so characterize this response.

Aim 4
Examine to what extent *Propionibacterium acnes* induced chronic inflammation can promote preneoplastic lesions in the rat prostate.
3. Materials and methods

3.1. Human samples (paper I and II)
Samples from men (<75 years) diagnosed with benign prostate hyperplasia and treated with transurethral resection of prostate (TURP), were collected at the University hospital in Umeå during the years 1982-1997. The TURP-samples formed the base for a nested case-control study. In this TURP population, 201 prostate cancer cases were identified by utilization of the Regional Cancer Registry. Selected cases received their cancer diagnosis at least 6 months after the TURP treatment and before October 31, 2002. If the patient had received several TURP treatments, the first TURP specimen was selected for this study. Controls were randomly selected to match the cases according to year of birth, year of TURP treatment and life survival. In total 201 matched pairs, collected during the years 1982-1996, were studied to evaluate if presence of genetic traces of microorganisms (virus, bacteria and fungi) were correlated to histological inflammation and subsequent prostate cancer diagnosis. The study was approved by the ethical review board in Umeå (permit no 05-160M).

3.2. In vitro cell line (paper III)
The non-neoplastic prostatic epithelial cell line RWPE-1 was used for the in vitro studies. RWPE-1 is derived from a white male donor and immortalized with human papilloma virus 18. The RWPE-1 cell line expresses luminal cytokeratines 8 and 18 but it also co-express basal cytokeratines. It is an androgen sensitive cell line since growth is stimulated upon androgen exposure. RWPE-1 does not grow in agar nor does it form tumours in nude mice.

RWPE-1 was grown in keratinocyte serum-free medium supplemented with 5ng/epidermal growth factor, 0.05mg/L bovine pituitary extract and 100U/ml penicillin/streptavidine. The cells were maintained in a humidified incubator at 37°C containing 5% CO₂ according to the manufacturer’s instructions.

3.3. Propionibacterium acnes strains (paper III, IV and V)
P. acnes, serotype 1a (CCUG 41530), was used in the in vitro and in vivo infection studies. This bacterial strain was isolated at the University hospital...
of Northern Sweden, from a patient that suffered from a cerebral shunt infection.

Furthermore, four different clinical prostate isolates, P1 (serotype 1a), P2 (serotype 1b), P3 (serotype 2) and P4 (serotype 2) were used in the \textit{in vivo} infection experiments. The prostate isolates were cultivated from prostatectomies at the university hospitals of Umeå and Örebro, Sweden.

Bacteria were grown in brain-heart infusion broth + 5\% horse serum in 37°C at micro-aerobic conditions. The bacteria were grown to a density of 10^9 per ml and resuspended in sterile PBS to a concentration of 10^8 bacteria per ml.

### 3.4. Animals and treatments (paper IV and V)

Immunocompetent adult male Sprague Dawley rats (3-4 months old) were used for \textit{in vivo} infection experiments.

To establish an \textit{in vivo} infection model with \textit{Propionibacterium acnes}, male Sprague Dawley rats were used. The bacteria were injected directly into the prostate lobes; the left ventral prostate (VP) and the left dorso-lateral prostate (DLP), in order to be sure that the bacteria reached the infection site.

During anaesthesia an incision was made in the lower abdomen to expose the prostate area. \textit{Propionibacterium acnes}, 5 x 10^7 diluted in 5\µl PBS (serotype 1a in animals infected 5 days and 3 weeks and prostate isolate \textit{P. acnes} mix in animals infected for 3 weeks, 3, 6 and 12 months) or vehicle was injected into the left VP and left DLP lobes using a 100\µl Hamilton syringe. At time of sacrifice, after 5 days, 3 weeks, 3 months or 6 months, the animals were sedated and the prostate lobes from the left and right sides were dissected. The removed tissue was utilized for bacterial counts or was fixed in formalin, 24h, for subsequent morphological analysis. One hour before sacrifice the animals were injected intra-peritoneally (i.p.) with bromodeoxyuridine (BrdU, 50mg/kg, Sigma Aldrich) in order to label cells with active DNA synthesis (proliferating cells). All animal work was approved by the local ethical committee for animal research (permit no A73-09).

### 3.5. DNA extraction (paper I and II)

Formalin-fixed, paraffinised prostate tissues were sectioned to 20\µm thickness by use of a microtome. Tissues were deparaffinised as described in \textsuperscript{202} and DNA was purified by using QIAamp DNA Blood minikit (Qiagen,
Hilden, Germany). Special care was taken to avoid contamination between samples; the sectioning was done in an area physically separated from post-PCR samples and microtome and microtome knife blades were cleaned with chlorine and ethanol after each sample. The DNA extraction process was performed in a fume hood that was free from PCR products and care was taken to avoid cross contamination of the samples. Negative controls (using all reagents except for tissue) were run in parallel with the DNA extractions.

3.6. PCR assays (paper I and II)

PCR was used to detect viral, bacterial and fungal genomes in the TURP samples. Nested PCR systems were used (except for HPV and Candida albicans) in order to increase the specificity of the reaction.

For the eight DNA viruses and Candida albicans, specific PCR primers and programs were used (as described in paper I). For bacteria, a 16S ribosomal DNA PCR assay was used, amplifying highly conserved sequence regions shared by bacterial species (as described in paper II). Great care was undertaken to avoid DNA contamination; all PCR mixes were performed in a DNA free area with dedicated pipettes, small reagent and primer aliquots, and the use of aerosol resistant pipette tips. For the 16s ribosomal DNA PCR working areas, PCR components except for Taq polymerase, primers and template DNA were UV irradiated before use. Negative (sterile water as template) and positive controls (specific for each organism, produced at the Department of Clinical Microbiology, University Hospital of Northern Sweden) were performed with PCR assays to monitor potential reagent or laboratory contamination.

PCR products were electrophoresed through a 2% agarose gel containing ethidium bromide and visualized by UV transillumination.

To control for DNA degradation and PCR inhibition, all DNA preparations from archival prostate samples were tested for the presence of the human beta-globin gene by two different PCRs with different product length, 110bp and 268bp, respectively.

3.7. Cloning and sequence analysis (paper I and II)

Amplicon products from the 16S rDNA PCR were cloned using the pT7Blue Perfectly Blunt cloning kit (Novagen) to get high concentration of the PCR product and to be able to identify individual sequences in the mixture of several 16s rDNA gene products that made direct sequencing impossible. In brief, plasmids were transformed into Nova Blue Shingles competent cells
according to the manufacturer’s instruction. Colony PCR analysis was performed on 5-10 randomly selected white colonies and plasmids containing cloned 16s rDNA gene inserts were selected for sequencing.

All positive samples from virus, fungi and bacterial PCR were sequenced with the BigDye Terminator cycle sequencing kit 1.1 (Applied Biosystems) according the manufacturer’s instruction and analysed in ABI PRISM 3700 DNA ANALYSER (AME Bioscience). Homology searches were performed with the alignment search tool BLAST on the NCBI database (www.ncbi.nlm.nih.gov). Sharing >98% nucleotide identity to a species was used as the criteria for defining a species.

3.8. ELISA (paper III and IV)

Secretions of cytokines IL-6, IL-8 and GM-CSF in P. acnes infected prostate epithelial cells were measured by ELISA (R&D Systems) as described in paper III. In brief, cells seeded into 24-well plates were grown to 75% confluence. The cells were then infected with P. acnes at a multiplicity of infection (MOI) of 16. Supernatants were harvested after 24h and 48h, stored at -20°C and later assayed by ELISA. To examine if secretion of cytokines were mediated via the TLR 2, cells were blocked with an anti-TLR2 monoclonal antibody (100 ng/ml, InVivoGen) 1h prior to infection.

C-reactive protein (CRP) was measured in serum from P. acnes infected rats and control. Levels were determined utilizing an ELISA method (Rat Serum CRP M-1010) according to the manufacturer’s instructions (Alpha diagnostics intern. San Antonio, TX, USA).

3.9. RNA preparation and reverse transcription (paper III)

RNA was prepared from P. acnes infected RWPE-1 cells after 24h using the RNesy Mini kit (Qiagen) as described in paper III. In brief, cells were seeded at a density of 1 x 10^6, grown to 75% confluence and then infected with P. acnes at a MOI of 16. After 24 h, cells were trypsinized, lysed, homogenised and total RNA was extracted according manufacturer’s instruction. RNA concentration and purity were assessed in a Nanodrop ND-1000 spectrophotometer (Thermo scientific) at 260nm. Complementary DNA (cDNA) was generated from 1µg total RNA using RT² First Strand Kit (SABiosciences) according to the manufacturer’s instruction.
3.10. Real-time Quantitative PCR and PCR-array analysis (paper III)

Gene expression analysis was performed using the RT² Profiler PCR array, Human Toll-Like receptor Signalling Pathway (SABiosciences) according manufacturer’s instruction. Real time PCR detection was performed with an IQ™5 instrument (BIO-RAD). The relative gene expression was calculated with the \( \Delta\Delta C_T \) method of the web-based software package RT² profiler PCR array systems (SABiosciences).

3.11. Quantification and characterisation of inflammation in human and rat prostate samples (paper I, II and IV)

Histological quantification of inflammation was done in the PCR positive TURP samples and matched controls (paper I and II). Inflammation was characterized in a dichotomous scale as either severe or moderate/minimal. Severe inflammation fulfilled the following criteria: infiltrating inflammatory cells were seen in the majority of the slides; three or more single inflammatory foci or one focus that took up one third of the slide or more. Moderate/minimal inflammation was defined as no areas of confluent sheets of inflammatory cells or very small ones (less than one third of the slides).

In the rat prostate lobes (VP and DLP) the type and magnitude of the inflammation was characterized and quantified (paper IV) according to the following criteria: tissues were graded according to the intensity of the inflammation (i.e. according to the number of inflammatory cells present) as no or minimal inflammation (0), mild/moderate (1) or severe (2). The inflammation was classified as either diffuse or focal. Diffuse inflammation involves the entire tissue in a relatively uniform manner. Focal inflammation occurs as patches of inflamed tissue areas in a background of normal appearing tissue. In the case of focal inflammation the proportion of total prostate volume inflamed was calculated using a stereological method. In summary, using a light microscope mounted with a square-lattice in the eye-piece the number of grid intersections falling on inflamed vs. non-inflamed tissue was measured \(^{203}\).
3.12. Immunohistochemistry and morphology (paper V)

Tissue sections were stained using primary antibodies against: BrdU (Dako) for counting proliferating cells, Factor VIII (Dako) for blood vessels, γH2AX (Novus) for detecting DNA damage.

Tissue sections were also stained for: AR (UPstake Lake Placid), GSTP1 (Leica Microsystems), smooth muscle actin (Dako), Desmin (Dako), CK 18 (PROGEN Biotechnik), p63 (DAKO) and CK 14 (Biosite), as described in more detail in paper V.

3.13. Western blot based serology (paper IV)

Whole blood samples were collected from rats by cardiac puncture prior to sacrifice. Blood samples were allowed to coagulate and serum was collected after centrifugation at 1400 rpm for 10 minutes at room temperature. Presence of anti-*P. acnes* IgG was assessed by a Western blot procedure where the rat serum functioned as the primary antibody. A total bacterial lysate (1 x 10^{10} bacterial cells dissolved in 400μl sample buffer) was submitted to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidine fluoride (PVDF) membrane. The filter was blocked by normal rat serum, cut into strips and incubated for 1h with individual rat serum diluted 1:2000. After washing, the strips were collected into a single container and incubated with goat-anti- rat –horseradish peroxidase antibody (1:5000) for 1h, washed and developed with ECC solution (Amersham).

3.14. Culture and *Propionibacterium acnes* genotype identification (paper IV)

Whole lobes were aseptically homogenized in PBS and 1/10 of the slurry obtained was subjected to 10-fold serial dilutions and plated on anaerobic blood plates. Plates were incubated for 1 week in 37°C under anaerobic conditions and then *P. acnes* colonies were counted on 1-2 plates.

Bacterial strains were genotyped utilizing the recA gene. Strains from biotype 1 and 2 differ in the sequence of the recA gene at base 71 (type 1= G, type 2= A), base 183 (type 1= A, type 2= G), base 214 (type 1= C, type 2= T), base 424 (type 1= A, type 2= G).
3.15. Immunofluorescence staining of *Propionibacterium acnes* in rat prostate tissue (paper IV)

Antigen retrieval of deparaffinised tissue sections was performed by boiling in citrate buffer (10mM, pH 6.0) at 2atm for 1h. Following blocking with 1% BSA in PBS, slides were incubated with *P. acnes* polyclonal rabbit antiserum (Agrisera, Umeå, Sweden) diluted 1:1000 in blocking solution for 1h. Slides were washed in PBS and incubated for 1h with goat anti-rabbit monoclonal antibodies labelled with Alexa 488 (Invitrogen) diluted 1:1000 in blocking buffer. Following washing and dehydration, slides were mounted and examined with epifluorescence (Axioscope) or confocal fluorescence microscope (Nikon).

3.16. Statistical analyses (paper I-V)

Fisher exact test was used in paper I to determine if an association between viral genomic finding and subsequent prostate cancer in TURP samples was present.

Logistic regression was used in paper II to estimate the association between presence of *P. acnes* and prostate cancer risk, presence of *P. acnes* and histological association, odds ratio (OR) and corresponding 95% confidence interval (CI). Chi-square test was used to test for differences in Gleason score and *P. acnes* positive cases.

In paper III, a permutation test was used to compare means of gene expression regulation and secretion of cytokines in infected cells vs. non-infected cells.

In paper V, a Mann-Whitney U-test was used for comparison between *P. acnes* infected rats and controls.
4. Results and discussion

4.1. Paper I and II

The prevalence of microorganisms in clinical prostate tissue samples and evaluation of their association with prostate cancer development (Aim 1)

To test whether microorganisms could be identified in the prostate prior to cancer we used a novel experimental design. We identified men with benign prostate hyperplasia where some were later diagnosed with prostate cancer and age-matched men who did not develop prostate cancer during the study period. In this way we hoped to detect agents that could potentially promote the development and progression of cancer. In previous studies aiming to establish connections between cancer and infectious agents in the prostate, agents were searched for in cases where cancer was already established. In such studies we cannot know whether the microorganism was there before the cancer or whether it was there as a result of the disease. It is also possible that agents causing a cancer-inducing inflammation are cleared and not present at the stage of analysis.

We screened the material for traces of virus, bacteria and fungi. Eight different DNA viruses were examined and chosen by the criteria that they have the ability to infect the urogenital tract region and that they are known to be involved in cancer development. Specific PCRs for the virus were: adenovirus, herpes simplex virus 1 and 2, Epstein-Barr virus, cytomegalovirus, human papilloma virus and the polyoma viruses BK and JC. The presence of bacteria was studied by using a universal 16S rDNA PCR. Furthermore we studied the presence of the fungus Candida albicans with a specific C. albicans PCR.

Great care was taken during DNA extraction and PCR amplification to avoid DNA contamination. Negative controls were performed with the DNA extraction and in the PCR reactions. Before screening, DNA from all prostate tissue samples was checked for integrity by amplifying the human beta-globin gene. This was done because fixation, paraffin embedding and long-term storage (we used archival samples, 8-22 years old) may affect the DNA quality of the samples. Out of 402 DNA samples tested, 352 (87.6%) were positive for the human beta-globin and were therefore considered to have sufficient quality for subsequent PCR analysis of the different microorganisms. For the C. albicans PCR 240 samples were available.
Of the eight viruses investigated, EBV and JCV were found in prostate tissue. Out of 352 samples tested, 31 (8.8%) were positive for EBV and 10 (2.8%) were positive for JCV. Two samples were positive in the C. albicans PCR. We then assessed whether the presence of virus and fungi had any association with prostate cancer risk. In total, 159 matched case-control pairs were available for virus analysis. There was no association between positive EBV and JCV finding and prostate cancer risk. Of 29 positive EBV samples, 15 (9.4%) were in the case group and 14 (8.8%) were in the control group. Of 10 positive JCV samples, three (1.9%) were in the case group and seven (4.4%) were in the control group. The two samples positive for C. albicans were in the case group (Table 3). There were no differences in the occurrence of severe inflammation in EBV or JCV positive samples compared to virus negative samples.

There are several tissue-based studies that have investigated the presence of virus in association to prostate cancer 139, 140, 142, 147, 148, 166, 204. Today there is no known virus with a strong association to prostate cancer development.

HPV is the most investigated pathogen in relation to prostate cancer because of its well-established role in cervical cancer development. The results from these studies are contradictory and in a recently published meta-analysis no association between HPV infection and prostate cancer could be found 138.

We did not find any HPV DNA in our prostate tissue material. Several other studies have also reported null-results for HPV DNA in the prostate 142 205. Whether that is because of methodological matters or true negative results have been discussed. However, Hrbacek et al. reviewed different studies in regard to material and methodological issues in detecting HPV by PCR in prostate tissue and found that these variables were not likely to influence the results 206. Furthermore, we tested our HPV PCR on 14 archival paraffin-embedded tissue samples from cervical cancers and found that all 14 samples were positive, thus proving that our PCR can detect HPV DNA in archival samples (data not shown).

Three other studies have detected EBV DNA in prostate tissues. Grinstein et al. reported EBV to be present in 37% of prostate carcinomas and in 0% of control samples using immunohistochemistry 140. Sfanos et al reported EBV as the most frequent microorganism detected (16 of 200, 8%), in a study analysing different microorganisms by PCR in cancer patients 166. Recently, Whitaker et al. reported high frequencies of EBV gene sequences to be present in both benign (2 of 10, 20%) and malignant prostate tissue samples (4 of 10, 40%) 141. Furthermore, high-risk HPV gene sequences were seen in
the same tissue specimen, suggesting that EBV and HPV can collaborate to transform prostate cells.

The oncogenic potential of the human polyoma viruses BK and JC in humans is still debatable but they have been shown to transform mammalian cells in vitro and to induce tumours in animal models 207. Because of their long-term latency in the urinary tract, several studies have investigated their possible role in prostate cancer development 146, 147, 204, 208. In a study with a small sample size Zambrano et al. detected JCV DNA in prostate cancer tissue samples (38%) 204 but other studies have reported null-results 208, 209. Together with our result it demonstrates that these viruses can be found in prostate tissue but our results indicate that they are unlikely to contribute to prostate cancer development.

96 (27%) of the 352 TURP samples were positive in the universal bacterial 16S PCR. Sequence analysis revealed *Propionibacterium acnes* as the most predominant bacterium, found in 22 (6.3%) of the prostate samples. The second most frequent isolate was *Escherichia coli*, found in 12 (3.6%). The other isolates included *Pseudomonas sp.*, found in 3 samples, *Actinomyces sp.*, 2 samples, *Corynebacterium sp.*, 2 samples, *Veillonella sp.*, 2 samples. *Streptococcus mutants*, 1 sample *Nocardioides sp.*, 1 sample, *Rhodococcus sp.*, 1 sample. The remaining 35 clones were considered as background noise.

There was no association between positive 16S findings and subsequent cancer diagnosis. However, when we assessed whether the presence of *P. acnes* was associated with prostate cancer risk, we found that *P. acnes* was twice as common in the case group (14) as compared to the control group (8) (Table 2), but the indicated association was not statistically significant (OR: 2.17, 95% CI 0.77-6.95). Furthermore, severe inflammation appeared somewhat more common in *P. acnes* positive samples compared to *P. acnes* negative samples (62% in *P. acnes* positive samples compared to 50% in bacterial negative samples, p=0.602).

Of the 12 *E. coli* positive samples, 8 were in the case group and 4 in the control group (Table 3).

The finding that *P. acnes* was the most prevalent bacterium is interesting since two recent studies have reported *P. acnes* as a prevalent finding in prostate cancer tissue specimens 158, 169. Cohen et al showed that *P. acnes* could be isolated in 35% from prostate cancer tissue samples and that *P. acnes* was positively associated with prostatic inflammation 188. Fassi Fehri et al. found that *P. acnes* was present in high percentage in both prostate
cancer tissues and in precancerous PIN tissue samples, but was absent in healthy prostate tissues samples 169.

Table 3. Microorganisms found in prostate tissue, prevalence and distribution in case and control groups.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Total (n= 352)</th>
<th>Case (n= 159)</th>
<th>Control (n= 159)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>31 (8.8%)</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>JCV</td>
<td>10 (2.8%)</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>C. albicans</td>
<td>2 (0.8%) *</td>
<td>2 *</td>
<td>- *</td>
</tr>
<tr>
<td>16S positive</td>
<td>97 (27%)</td>
<td>47</td>
<td>49</td>
</tr>
<tr>
<td>P. acnes</td>
<td>22 (6.3%)</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>E. coli</td>
<td>12 (3.4%)</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

* 240 samples were available for C. albicans PCR and 115 matched case-control pairs.

Both these studies suggested that P. acnes induced prostatitis could have a role in prostate cancer development. Interestingly, one study found a correlation between the development of severe acne in adolescence (measured indirectly as tetracycline use for 4 or more years) and increased risk of prostate cancer 191. The authors speculated that men having severe acne are more immune sensitive to P. acnes infections and therefore more likely to develop stronger inflammatory immune responses to intraprostatic P. acnes infections.

Whether P. acnes can induce an inflammatory response in the human prostate is not established. We have shown that P. acnes can induce proinflammatory cytokines, such as IL-6 and IL-8, when co-cultivated with prostate epithelial cells in vitro (paper III) and in our recently developed animal infection model, we showed that P. acnes induced a chronic inflammation in the dorso-lateral prostate in rats (paper IV). Moreover, the P. acnes induced prostatitis lead to glandular and stromal changes commonly seen in precancerous prostate tissues (paper V).

Although P. acnes well-established involvement in the skin inflammatory disease acne vulgaris, it is generally believed to have low pathogenic potential and is often considered to be a contaminant when cultured from clinical biopsies. However, the bacterium has been reported to cause severe infections at various body sites, particularly after surgery 176, 210. Since we
used PCR technique to detect *P. acnes* DNA in prostate tissue there is always a threat that the positive findings are false-positive results because of contamination during tissue sampling or during laboratory processing. We performed negative controls for both DNA extraction and PCR amplification and thereby controlling for possible contamination during laboratory processing. Furthermore, 6.3 % of 352 TURP samples were *P. acnes* positive and if there had been a systematic contamination from laboratory reagents it would have been present in a much higher amount. To prove that *P. acnes* PCR positive samples really represent tissue-invasive infections Alexeyev et al. showed that 7 out of 10 PCR positive TURP samples were confirmed by visualizing *P. acnes* in the prostate gland by using a fluorescent in situ hybridization assay (FISH) \(^{189}\). *P. acnes* was mainly detected in the stroma with intracellular localization (most likely within macrophages) or as bacterial aggregates. The intracellular form further strengthens a genuine *P. acnes* infection in the prostate gland. Moreover, *P. acnes* could be detected in sequential prostate samples from individual patients, taken up to 6 years apart \(^{189}\).

*P. acnes* might enter the prostate gland by several routes. Since *P. acnes* could be isolated from urine \(^{211}\), it might ascend into the prostate gland through the urethra. *P. acnes* has also been reported in the vagina \(^{212, 213}\) and it is therefore possible that *P. acnes* could be sexually transmitted. *P. acnes* can also be introduced to the prostate during preceding bladder catherization or surgical manipulation (biopsy or TURP). Another way of route is haematogenous spread, either via intracellular infected macrophages or as free living bacteria in the blood. One hypothesis on why *P. acnes* could establish an infection in the prostate could be the hypoxic environment seen in the prostate \(^{214}\) and that this impairs the local immune defence and favours growth of a facultative anaerobic bacterium.

Previous studies investigating a bacterial aetiology for prostate cancer have mainly found (either by culturing or 16S PCR) urogenital pathogens such as *Escherichia* spp., *Pseudomonas* spp., *Ureaplasma* spp., *Streptococcus* spp. or commensal such as *Staphylococcus* spp. and *Actinomyces* spp \(^{163-167}\). The reason why previous studies have failed to detect *P. acnes* could be that the culture conditions have not been optimal for *P. acnes* growth. *P. acnes* is extremely slow-growing and require extended anaerobic culture \(^{158}\). In addition, the *P. acnes* cell wall is resistant to digestion by lysozyme which is used to lyse Gram-positive bacteria prior to DNA extraction \(^{215}\).
The second most prevalent bacteria found in this study, *Escherichia coli*, is a well established prostate pathogen. It is the most isolated pathogen in bacterial prostatitis and certain uropathogenic *E. coli* strains is known to harbour specific virulence factors that increase infectivity or disease severity \(^{216, 217}\). Elkahwaji et al. showed that mice intraurethrally infected with *E. coli* developed a chronic prostatitis that was associated with preneoplastic lesions similar to PIA and PIN \(^{159}\).

**Potential weaknesses in the study:** Since the nested 16S PCR system used in this study had a sensitivity of 300 cfu/reaction, bacteria present in lower amounts were most likely missed and the true portion of bacteria positive samples could have been underestimated. On the other hand, using a too sensitive PCR increases the risk of amplifying exogenous contamination.

Another difficulty in detecting infectious agent in clinical tissues by using PCR techniques is that only a small fraction of the tissue is examined. Knowing that inflammation, and probably also bacteria and viruses, are unevenly distributed in the prostate, the risk of missing focally infected areas is high.

An important limitation of this study is the lack of appropriative negative controls. The material in this study came from patients with BPH and since both prostate cancer and BPH are suggested to have an inflammatory aetiology this might bias the result. Inflammation is a common finding in patients undergoing treatment for BPH and a proposed source for that inflammation is infectious agents \(^{62}\). Ideal control material, without inflammation and hyperplasia, is difficult to obtain and since we do not have such material, the prevalence of microorganism in healthy prostates could not be determined.

**4.1.1. Conclusion**

In this study we examined whether some infectious agents are present in the prostate prior to the detection of cancer. We found that this is the case but we were unable to demonstrate that the presence of these microorganisms is firmly related to development of cancer. Interestingly, although not statistically significant, both *P. acnes* and *E. coli* appeared to be more common in men that later were diagnosed with cancer. This together with experimental findings in animal models, where these bacteria can cause chronic prostatitis and induce epithelial lesions similar to precancerous
lesions in humans, suggests that the pathogenic role of these two bacteria in the prostate should be examined in more detail.

Such studies could preferably use case-control cohorts of the type used in this study but the assays used to detect infectious agents should probably be more sensitive and specific than the ones used in this thesis. When interpreting results one should also be open to the possibility that infectious agents could have opposing roles at different phases of disease, cancer promoting at early stages but perhaps cancer inhibiting in manifest tumours. Parallel studies in human samples and in experimental models are probably necessary to examine this.

4.2. Paper III

The inflammatory response of prostate epithelial cells to *Propionibacterium acnes* infection (Aim 2)

Having found that *P. acnes* was the most prevalent bacterium in BPH samples and that it was potentially associated with histological inflammation and prostate cancer development (paper II) we wanted to extend these observations to investigate the effect of *P. acnes* infection on prostate epithelial cells *in vitro*. A bacterium that causes prostatitis reaches the prostate gland, via the prostatic ducts, from urine or through ascending STIs. Therefore glandular epithelial cells will be the first to be exposed to the infection.

Prostate epithelial cells of non-malignant origin (RWPE-1) were infected with *Propionibacterium acnes* 24h or 48h at a multiplicity of infection (MOI) of 16 and the early inflammatory response of infection was studied. Secretion of three important inflammatory cytokines, IL-6, IL-8 and GM-CSF, was measured and gene expressions of different pro-inflammatory genes were evaluated. Since *P. acnes* have previously been shown to stimulate pro-inflammatory cytokines in monocytes via activation of TLR2, we investigated if that could be the case also in prostate epithelial cells. This experiment was carried out by a TLR2 receptor blockage with an anti-TLR2 monoclonal antibody prior to infection.

We found that the secretion of IL-6, IL-8 and also to a lesser extent GM-CSF was markedly elevated upon 24h and 48h of *P. acnes* infection. Furthermore, gene expression analysis of these cytokines showed that they were highly up-regulated after 24h of infection.
*P. acnes* is known to be a potent stimulus to the immune system. Studies examining the pro-inflammatory response in *acne vulgaris* have shown that *P. acnes* stimulates monocytes to secrete IL-6 and IL-8 \(^{184}\) and keratinocytes to secrete IL-8 \(^{187}\).

The inflammatory response to *P. acnes* in the prostate has prior to this study never been investigated. Following our article, two *in vitro* studies have explored similar issues \(^{169, 218}\).

In one of these investigations, Fassi Fehri et al. confirmed our observation of a strong induction of IL-6 and IL-8 secretion and a moderate induction of GM-CSF secretion in *P. acnes* infected RWPE-1 cells. Furthermore they showed that the transcription factor NF-κB and many down-stream inflammatory related genes were up-regulated upon *P. acnes* infection \(^{169}\). Dysregulation of the transcription factor NF-κB have been proposed to be one molecular mechanism leading to chronic inflammation and cancer \(^{219}\) and constitutive or increased activation of NF-κB has been observed in prostate cancer cells \(^{220}\). Fassi Fehri et al. also compared the inflammatory response of RWPE-1 cells upon infection of prostate derived *P. acnes* strains and a *P. acnes* skin isolate and found that the overall induced transcriptional changes were the same after 24h of infection.

A recently published investigation compared the transcriptional inflammatory response in keratinocytes and prostate epithelial cells upon *P. acnes* infection and found that the response was acute and transient in keratinocytes whereas it was delayed and sustained in prostate epithelial cells. Moreover, *P. acnes* was shown to invade prostate cells (intracellular growth) but not keratinocytes \(^{218}\). This study shows that the host cell tropism is important for the pathogenicity of *P. acnes*.

Prostate epithelial cells have previously been shown to secrete inflammatory cytokines in response to different inflammatory stimuli \(^{221, 222}\). Although the absolute quantities of the secreted cytokines have varied between these studies, they show that prostate epithelial cell can contribute to the inflammatory response during infection.

IL-6, IL-8 and GM-CSF are important for recruitment and differentiation of macrophages and neutrophils to sites of infection \(^{223-225}\).

Interestingly, both IL-6 and IL-8 have been shown to be involved in prostate cancer development and progression. Clinical studies have revealed that increased serum IL-6 concentrations in prostate cancer patients are associated with advanced tumour stages and short survival \(^{226, 227}\). IL-6 act as an autocrine growth factor on prostate epithelial cells *in vitro* \(^{228, 229}\) and expression of IL-6 and its receptor are increased in prostate cancer tissues.
Importantly, increased expression of IL-6 receptor was correlated with increased proliferation. Increased expression of IL-8 has also been characterized in prostate cancer cells and correlates with angiogenesis, tumorigenesis and metastasis.

The innate immunity uses specific receptors, the pathogen recognition receptors (PRR), to recognize and induce an inflammatory response to invading microorganisms. Prostate epithelial cells lines have previously been shown to express different TLRs and that the cells induce inflammatory cytokine secretion through TLR mediated signalling pathways. In acne, the *P. acnes* induced inflammation has been shown to be triggered through TLR2, a PRR for gram-positive bacteria, but also TLR4 and TLR9 have been implicated.

To investigate if the pro-inflammatory cytokine secretion was induced via the TLR2 signalling pathway we made an experiment where the receptor was blocked before infection. In this experiment we saw that the secretion of IL-8 was reduced by blocking the TLR2, indicating that induction of this cytokine is mediated via TLR2 signalling pathway in prostate epithelial cells. In line with this, our gene expression analysis revealed up-regulation of several transcription regulators, JUN, REL, RIPK2, NFKB2, NFIA, IRF1, and the co-factor TICAM1 that are involved in the TLR2 signalling pathway.

However, we did not observe any significant reduction in secretion of IL-6 and GM-CSF, suggesting that other pathogen recognition receptors are responsible for the induction of these cytokines.

**Potential weaknesses in study:** In this study we used an immortalized benign prostate epithelial cell line. These cells allow us to study *in vitro* behaviour; however, cells may behave different *in vivo*. Furthermore several cytokines may be activated as a result of the immortalization.

We only measured the secretion of three cytokines, probably, as indicated by the PCR array, several other cytokines is secreted upon *P. acnes* infection that may influence the outcome of the infection.

The *P. acnes* strain used in this study was isolated from a patient that suffered from a cerebral shunt infection. Since it is reported that *P. acnes* strains isolated from the prostate differ genetically and phenotypically from cutaneous isolates it is possible that the inflammatory response would have been different if the prostate cells were infected with a prostate *P. acnes* isolate.

Prostate epithelial cells are probably the first to be exposed to the infection but since Alexeyev et al. showed that a majority *P. acnes* bacteria in
prostate cancer samples were located in the stroma it would have been interesting to evaluate how these cells respond to a *P. acnes* infection.

### 4.2.1. Conclusion

In this study we demonstrate for the first time that *P. acnes* induce a strong inflammatory response in prostate epithelial cells, partly, as when this bacterium infects other tissues, mediated by activation of TLR2. Many of the cytokines induced are known to play essential roles in promoting prostate cancer growth.

This study suggests that the role of *P. acnes* as a potential pathogen in the prostate *in vivo* needs to be examined further.

### 4.3. Paper IV and V

**A rat prostate infection model to study the pathogenic potential of *Propionibacterium acnes* (Aim 3 and 4)**

The finding that *P. acnes* can be found in benign (paper II) and malignant prostate tissues \(^{158, 169, 189}\), the association to prostatic inflammation \(^{158, 189}\) and that the bacterium is able to trigger a strong inflammatory response in prostate epithelial cells *in vitro* (paper III) prompted us to investigate if *P. acnes* can induce an infection/inflammation in the prostate *in vivo*.

Live *P. acnes* were injected in the ventral and dorso-lateral lobes of the rat prostate and the effects of the infection were examined 5 days, 3 weeks, 3 months and 6 months after injection. Two types of *P. acnes* agents were used; a monoculture of *P. acnes* strain 1a (CCUG 41530), isolated from a cerebral shunt infection and a mixture of four different prostate derived *P. acnes* strains. Prostate tissues were analyzed for bacterial content, histological inflammation and serum levels of CRP and anti-*P. acnes* IgG (paper IV). Furthermore, prostate tissues were evaluated for possible pre-neoplastic changes caused by the infection/inflammation using immunohistochemistry (paper V).

**Is *Propionibacterium acnes* a pathogen in the prostate?**

We found that injection of *P. acnes* in the prostate of Sprague Dawley rats induced a strong inflammatory response and that the immune reaction was different in the ventral prostate compared to the dorso-lateral prostate. In
the ventral prostate, *P. acnes* induced a transient and diffuse inflammation that was almost cleared after 3 weeks. In contrast, the dorso-lateral prostate showed a focal chronic inflammation that could be detected up to 3 months post-infection. Live *P. acnes* could be recovered from the dorso-lateral lobe up to 3 months post-infection whereas the ventral lobe was cleared from bacteria at that time. *P. acnes* specific immunofluorescence also revealed that bacteria could be detected in prostate tissue up to 3 months post-infection. Bacteria were located both in the stroma and in the glands and coincided with foci of histological inflammation.

The finding of different infection responses between the lobes indicates that DLP is more susceptible to bacterial infections than VP. The anatomical distinctions between the lobes are well established but the physiological character of the lobes are less defined. Most functional studies are performed in the VP, despite accumulating data that DLP is the lobe that is most similar to the cancer prone peripheral zone in humans. A similar study to ours, experimentally infecting mice with *E. coli*, also reported DLP to be the most affected lobe. However, others have reported no differences in susceptibility or VP to be the most inflamed lobe. Whether these differences are due to methodological aspects or physiological differences in rodent strains and/or bacterial strains used is unknown. Interestingly, we found that VP was more prone to spontaneous prostatic inflammation compared to DLP. The VP from PBS injected rats showed spontaneous inflammation already at the age of 4 months, and one third of the animals were inflamed after 9-10 months. In contrast, the DLP from PBS injected animals stayed free of inflammation throughout the experiment. This result is in concordance with earlier studies reporting spontaneous prostatitis.

Although it is probably a more natural way of infection to inoculate the bacteria intra-urethral (using a catheter), we injected the bacteria directly into the prostate lobes. Pilot studies done in our lab found that intraurethral inoculation compared to direct injection of bacteria to the prostate gave the same results (in regard to infection/inflammation) but the intraurethral method was not as reproducible as the direct injection (data not shown).

We found no differences in the amount of bacteria recovered from the animals infected with the non-prostate derived *P. acnes* compared to those infected with a mixture of prostate isolates nor did we see any differences in histological inflammation comparing the two groups. Even though studies have reported that *P. acnes* isolates derived from prostate is genetically and phenotypical different from skin isolates we did not observe any
differences in pathogenicity of the prostate between the different strains in the prostate in our study.

Although the inflammatory response was localized to the prostate, serological analysis of CRP and \textit{P. acnes} specific IgG demonstrate elevated levels with maximum antibody concentration after 3 weeks and 3 months, respectively. Similar serum immune reactions have been reported in rats infected with \textit{E. coli} \textsuperscript{241}. The normally high CRP levels in uninfected rats and also the age-related increase are in line with CRP levels reported for other rat strains \textsuperscript{242}.

Corpora amylaceae (prostate concrements composed of calcified proteinaceous material) are commonly seen in human prostate samples and they may promote chronic inflammation by causing tissue damage \textsuperscript{2}. Corpora amylaceae are probably formed from cellular debris created during chronic inflammation \textsuperscript{243}. The finding of suspect corpora amylacea (CA) formation in one of the rats infected for 6 months support the hypothesis that infection and inflammation are contributing factors to CA formation \textsuperscript{243, 244}. It will be interesting to examine whether traces of \textit{P. acnes} (in addition to \textit{E. coli} \textsuperscript{243}), can be found in human CA.

Experimental animal models for bacterial prostatitis have mainly been studied with uropathogenic \textit{E. coli} \textsuperscript{159, 160, 236, 237, 241, 245-249} and a few studies with \textit{Chlamydia} organisms \textsuperscript{250, 251}, \textit{Pseudomonas aeruginosa} \textsuperscript{252} and \textit{Proteus mirabilis} \textsuperscript{253}. All these organisms were able to induce a chronic inflammatory state in the rodent prostate and showed many similarities to the natural history of human chronic bacterial prostatitis. Differences in susceptibility to infection between different mice and different rat strains have been reported \textsuperscript{11, 249} and indicate that host genetics is important for developing bacterial prostatitis. Also specific virulence factors carried by the bacteria play an important role for maintaining a chronic infection \textsuperscript{248, 252, 253}.

Whether the Sprague Dawley rat is an ideal model to study bacterial prostatitis and in particularly \textit{P. acnes} prostatitis, is poorly evaluated. Wistar rats have been reported to be more susceptible to chronic bacterial infection compared to Sprague Dawley rats when intraurethrally infected with \textit{E. coli} \textsuperscript{11}. However, the two studies did not use the same \textit{E. coli} strain and the DLP were not analysed \textsuperscript{241, 254}. Knowledge of \textit{P. acnes} virulence factors are scarce but genomics, transcriptomics and proteomics data have shown that \textit{P. acnes} harbours several proteins involved in host tissue degradation and inflammation and that the expression and secretion is different between different \textit{P. acnes} strains \textsuperscript{183, 255}. 


Is *Propionibacterium acnes* able to induce potentially neoplastic changes in the prostate?

In paper IV we showed that *P. acnes* can induce a chronic infection in the rat dorso-lateral prostate. Since *P. acnes* has also been shown to be potentially associated with prostate cancer in men, our *P. acnes* prostatitis model can be a useful tool to study the molecular connections between bacterial infection, inflammation and prostate carcinogenesis.

In paper V we examined if *P. acnes* induced prostatitis could promote pre-neoplastic lesions in the rat prostate. We examined the tissues for changes commonly seen in human PIA lesions such as altered cytokeratin, AR and GSTP1 expression, increased proliferation and changes in the stroma. Because DLP was found to be the most severely affected lobe we analysed it in more detail. *P. acnes* infected prostate tissues were examined for histopathological changes and stained for markers associated with cell differentiation, DNA damage and proliferation.

In DLP, we found that *P. acnes* infection was accompanied by major changes in glandular and stromal morphology. The well-defined order of basal and secretory luminal cells disappeared and the distinction between stroma and glandular epithelium was difficult to identify. In the glandular lumina, numerous necrotic and apoptotic cells were seen together with macrophages. We saw a decreased number of luminal cells (CK18 and AR positive) and an increased number of basal cells (CK14 positive) in the inflamed areas suggesting that the tissue damage induced by *P. acnes* infection resulted in proliferation of new epithelial cells. This was confirmed by enhanced BrdU (labelling proliferating cells) staining in epithelial cells. Furthermore, double-staining with basal (CK14) and luminal (AR, CK18) markers showed enrichment of epithelial cells expressing both CK14 and AR/CK18 in the inflamed areas. Such intermediate cells have been proposed as precursor cells for prostate cancer and are enriched in human PIA lesions 75, 256. Moreover, we saw that expression of the androgen receptor in the epithelium was decreased in inflamed areas. Studies with mice infected with *E. coli* in the prostates have revealed similar epithelial responses to inflammation 159, 160. Khalili et al. saw an increase of p63 (marker for basal cells) expressing cells, luminal cells expressing CK5 (marker for basal cells) indicating intermediate cells and down regulation of AR in inflamed ducts. Furthermore, these observations were accompanied by increased proliferation in the inflamed areas 160.
We also found that the *P. acnes* induced prostatitis was associated with stromal changes. The glandular walls were much thicker and consisted of an increased amount of smooth muscle cells. These cells showed an increase in smooth muscle actin (SMA, marker for myofibroblasts and smooth muscle cells) and a decrease in desmin (marker for fully differentiated smooth muscle cells) indicating a dedifferentiated phenotype. Furthermore, vasculature staining by factor VIII showed an increased density of blood vessels in the inflamed areas, suggesting angiogenesis.

Smooth muscle cells play a central role in the regulation of prostatic growth and function. By secreting growth factors they regulate epithelial proliferation and death. Therefore, the maintenance of the smooth muscle phenotype is critical for prostate gland homeostasis 257. The smooth muscle cell response to prostatic infection have also been examined in other studies 246, 247, 258, 259 observing similar results to ours. Smooth muscle cells responded to infection stimuli by changing their contractile phenotype toward a secretory profile both *in vitro* 258 and *in vivo* 246. Moreover, infection stimulated secretion of pro-inflammatory cytokines have growth promoting activities 258, 259. Dedifferentiated smooth muscle cells, increased production of growth factors and angiogenesis are common features in the reactive stroma accompanying the tumour cells in human prostate cancer. In this sense, the stromal response to infection is in many ways similar to that seen in prostate carcinogenesis.

Inflammation is known to induce oxidative stress as a consequence of the production of reactive oxygen released by the inflammatory cells 260. The association of oxidative stress with prostate cancer has been recognized in several studies 76, 159, 261. Furthermore the enzyme glutathione S-transferase pi (GSTP1), which inactivates electrophilic carcinogens, have been shown to be epigenetically silenced in PIA, PIN and prostate cancer by DNA methylation 81, 131. In our study, we showed that GSTP1 was initially up-regulated in the luminal cells after 5 days of infection. However, after 3 weeks of *P. acnes* infection, GSTP1 expression was reduced in the majority of both basal and luminal cells in the inflamed areas. De Marzo and colleagues have suggested that loss of GSTP1 gene expression (the most common and early genetic alteration noted in patients during prostate cancer development 132) will make the epithelial cells more vulnerable to DNA damage during a chronic infection 2. To investigate this hypothesis we stained our *P. acnes* infected tissue with the DNA damage marker γH2AX. Indeed we found a remarkably increase of γH2AX positive cells in the
inflamed areas. Increased γH2AX staining in combination with the increased proliferation could make these cells more exposed to mutation and as a late consequence of this, cell transformation. Actually, we could detect epithelial cells with mild atypia in the inflamed areas.

Our *P. acnes* induced prostatitis model show similarities with human PIA lesions e.g. accumulation of epithelial cells with an intermediate phenotype, loss of GSTP1 expression and increased proliferation 75, 76, 85. Furthermore, the altered stroma cell morphology and increased vascular density seen in our infected rats are similar to that seen in human PIA 76. However, the *P. acnes* infection eventually cleared and when analyzing healed tissues for permanent changes such as increased proliferation, atypia and/or atrophy we did not find any differences in the *P. acnes* injected animals compared to the controls. This indicates that the infection/inflammation induced by *P. acnes* creates an environment favourable for many steps in the process to a neoplastic state but apparently some important features are lacking.

Apart from our investigation, two studies have evaluated the possible influence of chronic infection and the development of prostate cancer. These investigations both utilized a mouse model where the animal prostates were intraurethrally inoculated with uropathogenic *E. coli* 159, 160. In none of the models cancer was induced but potentially precancerous lesions were detected and the inflammation appeared to be more long lasting and to induce somewhat more epithelial atypia than in our *P. acnes* model. In one of these studies chronic inflammation was shown to be associated with reactive epithelial hyperplasia and oxidative stress 159. Furthermore many tumour suppressors (GSTP1, p27, PTEN, Nkx3.1) were down-regulated in response to the inflammation 159, 160.

To be able to investigate further if *P. acnes* can induce permanent precancerous lesions in the prostate the duration of the infection probably has to be more long-standing. Modifying the model by using rat strains more susceptible to prostatitis, repeated injections of bacteria or/and using other species and mice with specific alterations in their immune system (similar to those described in men with increased risk of acquiring prostate cancer) might be an opportunity. The effects of combined infection with the two bacterial species known to induce potential precancerous lesions in the prostate, uropathogenic *E. coli* and *P. acnes* could also be examined.

**Potential weaknesses in the study:** The species we used in this infection model (Sprague Dawley rats) is probably not ideal for induction of prostatitis
and prostate cancer development. Rats are in general resistant to infections and the Sprague Dawley rats in particular. Rats very seldom develop prostate cancer. A more ideal species could perhaps be dogs (prostate cancer is actually a very uncommon disease in animals, except in men and dogs) or mice genetically modified to increased susceptibility to chronic prostatitis and/or prostate cancer.

To investigate if *P. acnes* prostatic infection can promote prostate cancer direct studies of epigenetic changes and mutation should also be done. Furthermore, a study whether *P. acnes* can be found adjacent to PIA, PIN and prostate cancer lesions in humans is needed.

### 4.3.1. Conclusion

Our studies clearly showed that *P. acnes* is a potent pathogen in the rat prostate, able to induce chronic lobe-specific prostatitis and to induce some of the steps necessary in the formation of precancerous lesions. The role of *P. acnes* in the pathogenesis of prostate cancer therefore needs to be examined in more detail.
5. General discussion and future directions

The overall aim of this thesis was to investigate if microorganisms are present in prostate tissues and if so if they are related to the aetiology and pathogenesis of prostate cancer. The main and novel findings in this study are that microorganisms, particularly the bacterium *P. acnes* can be detected in prostate tissue prior to cancer diagnosis, that *P. acnes* trigger a strong inflammatory reaction in prostate epithelial cells *in vitro*, that *P. acnes* can induce chronic prostatitis in rats and that this inflammation causes some of the early cellular changes that is commonly seen during prostate tumour formation in humans. The changes observed are however generally reversible and when the inflammation eventually is cleared the potentially precancerous lesions present apparently disappear.

So what do the results of this thesis and a review of the literature add to the key questions regarding the role of inflammation and microorganisms in prostate cancer formation and progression?

**Is it likely that prostate cancer can be caused by inflammation and infectious agents in particular?**

As already reviewed in the introduction substantial indirect evidence suggest that inflammation plays an essential role in the development and progression of prostate cancer. Inflammation is seen adjacent to precursor lesions, around and inside tumours, and experimental inhibition of inflammation may retard tumour growth \(^{203,262}\). What is less known is if this inflammation is a cause or a result of certain (early and/or late) steps in the cancerogenic process. Is inflammation an aetiological agent and/or is the inflammatory system activated at a later stage to promote, or under other circumstances inhibit growth of tumours caused by other mechanisms?

It is well established that some types of bacteria can cause chronic prostatitis \(^{41}\) and in experimental models induce potentially precancerous lesions \(^{159,160}\), and the current thesis suggests that *P. acnes* can be added to this list of agents. Notably, however, bacteria and other microorganisms are not the only cause of inflammation in the prostate. Chronic prostatitis can among many things be induced by steroid hormones like estrogen, by corpora amylacea and by factors in the diet. So even if we accept that prostatitis is part of the aetiology of cancer this does not mean that infections
are the cause of this inflammation as non-infectious causes are also possible. In reviewing the literature it appears that inflammation induced in several different ways such as estrogen, diet and bacteria may cause similar precancerous changes, suggesting that it is inflammation as such and not the individual agents that promote cancer development. All these external factors apparently need to act for a substantial time to induce precancerous lesions suggesting that the duration is more important than the individual agent. So if several factors may all induce chronic prostatitis it becomes important to know whether they can act in synergy, something yet to be tested. Another factor to be explored is how various agents that may cause prostatitis interact with genetically determined factors regulating susceptibility to develop chronic prostatitis and effectiveness of host defence systems against cancer. For well-established cancer-inducing microorganisms such as *Helicobacter pylori* and HPV, it is known that only a sub fraction of the infected individuals develop cancer 1. Another critically important, but unanswered question, is whether a proposed aetiological agent causes the non-aggressive or aggressive forms of the disease. An agent that is only able to induce harmless forms is less important than an agent that causes the life threatening forms of the disease.

**Is it likely that infectious agents and inflammation affect prostate cancer progression and behaviour?**

Studies on the relationships between infections agents, inflammation and prostate cancer generally explores whether the microorganism could be involved in the aetiology of the disease but even if this was not the case other effects are also possible. Microorganisms could infect precancerous lesions or cancers and modify their behaviour. Whether this occurs or not is unexplored, but not unlikely as the immune system is inhibited in tumours 113.

In other tissues *P. acnes* is known to induce a prominent immune response of the Th1 type 199 and vaccination with *P. acnes* has already been proposed to be an anti-cancer treatment 198. Introducing the bacteria in tumours may transform a tumour promoting Th2/M2 type of inflammation to a tumour inhibiting Th1/M1 type. Clinical data have also demonstrated that prostate cancer patients with high antibody titres against *P. acnes* have a better prognosis that those with low 192. Recent or ongoing *P. acnes* infection may actually protect. Against this background the overall conclusion of this thesis is that there are strong reasons to explore the role of *P. acnes* infection in the prostate in more detail.
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