Infection induced chronic inflammation and its association with prostate cancer initiation and progression
Dedicated to
My amazing grandmothers
Infection induced chronic inflammation and its association with prostate cancer initiation and progression
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Abstract


An association between cancer development and inflammation has long been suggested. Approximately 20% of all human cancers in adults are assumed to result from chronic inflammation. The aim of this thesis was to investigate if infection-induced chronic inflammation plays a role in prostate carcinogenesis.

Our results revealed a greater infiltration of the bacterium Propionibacterium acnes in the prostate tissue obtained from men with prostate cancer compared to men without any histological evidence of the disease. These findings indicate that prostate cancer could potentially be included in the list of cancers with an infectious etiology.

Further, we investigated whether chronic inflammation has a role in disease progression. Our results demonstrated that men with lethal prostate cancer had pronounced infiltration of immune cells with suppressive function of the anti-tumor immune response compared to men with a more indolent prostate cancer.

Confirmation of our results may open up avenues for targeted prostate cancer treatment by offering men with chronic inflammation alternative therapies such as anti-inflammatory drugs. If the involvement of P. acnes in prostate cancer development is replicated in other studies, vaccination therapies may be feasible. To further individualize prostate cancer therapy, bolstering the anti-tumor immune response in order to reduce tumor progression may be determined to be advantageous for some patients.

Keywords: Prostate cancer, chronic inflammation, CD4 helper T cells, CD8 cytotoxic T cells, regulatory T cells, Propionibacterium acnes.

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Opportunities to find deeper powers within ourselves come when life seems most challenging

- Joseph Campbell -
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Populärvetenskaplig sammanfattning

Idag vet man att ett stort antal cancersjukdomar har en infektionsrelaterad inflammation som bakomliggande orsak. Kronisk infektion av bakterien *Helicobacter pylori* ökar exempelvis risken för att utveckla magsäcks canc-

Inom ramen för denna avhandling ville vi studera om en kronisk inflammation, kopplad till infektion, kan vara en möjlig orsak även till utveckling av prostatacancer. Resultaten visade på en betydlig ökad förekomst av bakterien *Propionibacterium acnes* i prostatavävnaden hos män med prostatacancer jämfört med män utan sjukdomen. Detta ger en indikation om att prostatacancer kanske kan läggas till listan över cancertyper som kan orsakas av infektiösa agens.

Vidare ville vi studera om den kroniska inflammationen enbart har betydelse i den initiala fasen av prostatacancerutvecklingen eller även senare i sjukdomsutvecklingen. Våra resultat talar för att vårt vanligtvis skyddande immunförsvar förändras under prostatacancerutvecklingen och istället får en tumörbefrämjande effekt. De män som avled på grund av sin cancersjukdom hade en ökad mängd immunceller som istället för att hämma cancertillväxt agerade cancerstimulerande. Detta skulle kunna påvisa en möjlig mekanism cancerscellerna använder sig av för att skapa sig förutsättningar till fortsatt tillväxt.

Om resultaten i denna avhandling kan bekräftas i ytterligare studier kan detta öppna framtida möjligheter för att individanpassa förebyggande behandling genom att exempelvis erbjuda anti-inflammatoriska läkemedel till män med kronisk prostatainflammation. Skulle det kunna säkerställas att *Propionibacterium acnes* har en bidragande roll i prostatacancerutvecklingen skulle det också kunna öppnas möjligheter för framtida vaccinationsterapier. För den enskilde patienten kan möjligen även terapier där man stärker det skyddande immunförsvaret vara ett alternativ för att motverka prostatacancertillväxt och utgöra ett komplement till övrig behandling.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
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<td>CZ</td>
<td>Central zone</td>
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<td>DAB</td>
<td>Diaminobenzidin</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<td>FOXP3</td>
<td>Forkhead box P3</td>
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<tr>
<td>GSTP1</td>
<td>π class glutathione S-transferase</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IL-1</td>
<td>Interleukin 1</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
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<td>IL-8</td>
<td>Interleukin 8</td>
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<td>iT&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Induced regulatory T cell</td>
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<td>MLST</td>
<td>Multilocus sequence typing</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<td>nT&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Natural regulatory T cell</td>
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<tr>
<td>P. acnes</td>
<td>Propionibacterium acnes</td>
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<tr>
<td>PAH</td>
<td>Postatrophic hyperplasia</td>
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<tr>
<td>PCa</td>
<td>Prostate cancer</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PIA</td>
<td>Proliferatory inflammatory atrophy</td>
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<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
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<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
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<tr>
<td>PZ</td>
<td>Peripheral zone</td>
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<tr>
<td>rep-PCR</td>
<td>Repetitive sequence based PCR</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Radical prostatectomy</td>
</tr>
<tr>
<td>SA</td>
<td>Simple atrophy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature of DNA</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TURP</td>
<td>Transurethral resection of the prostate</td>
</tr>
<tr>
<td>TZ</td>
<td>Transition zone</td>
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This thesis is based on four papers, which will be referred to by their Roman numerals (I, II, III, and IV):

**Paper I**  

**Paper II**  

**Paper III**  

**Paper IV**  
INTRODUCTION

Cancer development is characterized by six fundamental hallmarks; self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replication ability, sustained vascularisation and tissue invasion and metastases (1). Cancer development can be considered as being comprised of three phases: initiation, promotion, and progression, where the initiation phase is characterized by point mutations, gene insertions and deletions or gene amplifications within the cells causing permanent genomic alterations. Tumor development is promoted by clonal expansion of the initiated cells, leading to a progression in the tumor development (2).

An association between cancer development and inflammation has long been suggested. Approximately 20% of all human cancers in adults are assumed to result from chronic inflammation triggered by environmental exposures or infectious agents (3). Regardless of etiology, chronic inflammation is suggested to support a malignant transformation by inducing repeated cell damage and subsequent stimulation of cell replication and tissue repair. These complex cascades of events, leading from chronic inflammation to cancer, are orchestrated mainly by cytokines, reactive oxygen, and nitrogen species.

Classic examples of inflammatory induced cancers are hepatocellular carcinoma arising in close connection with chronic hepatitis B or C or gastric cancer after a history of Helicobacter pylori infection (4-5).

Chronic inflammation has also been hypothesized to influence prostate carcinogenesis, since both acute and chronic inflammation are commonly observed in prostate tumor specimens (6-7). The multifocal pattern of inflammation seen in prostate tissue specimens cannot exclusively be explained as an anti-tumor immune response since it is scattered throughout the entire organ. Further the inflammation pattern is also frequently found in prostate glands with no evidence of malignancy. Interestingly, a similar histological inflammation pattern has been identified in tissues infected by Helicobacter pylori (8). In most cases the cause of the so common prostatic inflammation is unclear even though various potential sources exist including infectious agents. Many different pathogenic organisms have been reported able to infect and induce an inflammatory response in the prostate including sexually transmitted microorganisms, such as Chlamydia trachomatis and Trichomonas vaginalis (9-10). The most investigated pathogen connected to prostate cancer (PCa) is human papilloma virus due to its role in other genital cancers. Other viruses able to infect the prostate are
human herpes simplex virus type 2 and cytomegalovirus (11-13). The data from these studies are however contradictory where the pathogens in some investigations were associated with inflammation and PCa development while in others they were not. Recently the non-sexually transmitted bacteria Propionibacterium acnes, was added to the list of possible infectious agents of the prostate (14).

**PCa**
The prostate gland measures approximately 25 cm$^3$ and has a walnut-like shape. It is one of the male accessory sex glands and the main functions are to synthesize and secrete various proteins and fluids to support the semen. The prostate is located right under the bladder, in front of the rectum, and surrounds the upper part of urethra. The anatomical position makes it possible for infectious agents to reach the gland through the urine.

In humans the prostate consists of three histologically different zones: the peripheral, transition, and central zone. The peripheral zone, located near the prostatic wall, comprises 70% of the prostate volume and almost 70% of PCa originate in this zone. The transition zone surrounds the urethra and accounts for 5-10% of the prostate volume in young men. However, since benign prostate hyperplasia (BPH) originates in this part of the gland a dramatic increase in volume can be seen with increasing age. The transition zone is the initiation point for approximately 15-20% of the prostate tumors. The central zone constitutes about 25% of the prostate volume and is rarely the origin for prostate tumor development (Figure 1) (15-16).

![Figure 1. A schematic picture of the different zones in the prostate gland. PZ = Peripheral zone, TZ = Transition zone, and CZ = Central zone.](image)
The prostate consists of two histological structures, glands and fibromuscular stroma. The glands consist of basal epithelial cells, luminal epithelial cells and neuroendocrine cells surrounded by physically supporting stroma. The fibromuscular stroma is composed by smooth muscle cells, blood vessels, immune cells, fibroblasts, and nerves. Almost all cancer in the prostate arises in the glandular epithelium and is therefore considered as adenocarcinomas and can be confirmed by the absence of basal epithelial cells.

PCa is the most common cancer among European and American men; approximately 339 000 and 240 000 men, respectively, are diagnosed annually (17-18). In addition, PCa is the most prevalent form of cancer in Swedish men affecting nearly 10 000 men each year (19). In the US, PCa is the second greatest contributor to cancer mortality and in Sweden almost 2400 (24%) of the men diagnosed with PCa will die from this disease.

**PCa diagnosis**

Currently there is no recommended PCa screening strategy, but the prostate specific antigen (PSA)-test is commonly used to assess the risk. In Sweden, a PSA-value less than 3ng/ml is considered a normal value (20). A higher PSA-value indicates that the patient may have a prostatic disorder such as BPH, prostatitis or PCa. Men presenting with an elevated PSA-value will undergo further investigations of their prostate, most commonly through biopsies, where about 8-12 needle biopsies are taken from different locations of the prostate and subsequently histologically evaluated by a pathologist. Alternatively, men can be diagnosed with incidental PCa after a transurethral resection of the prostate (TURP), which is a treatment procedure for BPH. In both cases, if PCa is discovered, the tumor stage (TNM) and Gleason grade is determined.

The TNM staging system is based on the extent of the tumor. The T describes the size of the tumor, the N describes regional lymph node involvement, and the M if there is metastasis at distal locations other than the lymph nodes. The T-stages range from T1 to T4, where T1a, T1b, and T1c represent no palpable tumor, T2 are organ-confined tumors, T3 tumors have grown outside the prostatic capsule, and T4 refers to tumors that extended into adjacent organs. N0 represents no regional lymph node involvement and N1 represents the presence of metastases in the lymph node. The M parameters include M0 classifying no distant metastasis and M1 which is identification of metastasis at distant parts of the body.

The Gleason grading system used for PCa today was created by Donald F Gleason in 1966 (21). This system remains a fundamental tool to determine the biology and prognosis of the disease and it is the single most im-
portant prognostic factor in PCa. The system is based on a microscopic evaluation of the degree of loss of the normal glandular structure. The classic Gleason scoring consists of five tissue patterns. Gleason grade 1 is assigned to well differentiated cells which are still organized in glandular structures, while Gleason 5 is assigned to poorly differentiated cells where the glandular structure have disappeared completely. By adding the primary Gleason grade and the secondary Gleason grade, i.e. the two most common tumor patterns, the Gleason score (GS) is obtained, ranging from 2-10. The higher the GS is, the more aggressive tumor, thereby indicating a worse prognosis for the patient. Approximately 75% of the PCa cases are diagnosed with a GS of 6 or 7, and the outcome for these patients are variable and highly unpredictable (22).

Curative and non-curative treatments
Patients with clinically localized PCa are usually offered treatment with curative intention. Today there are two major alternatives, either surgery (radical prostatectomy, RP) or radiotherapy. Hugosson et al. reported that RP is probably still the most commonly used procedure for treatment of localized PCa even though a substantial amount of men will end up suffering from side-effects such as incontinence and erectile dysfunction (23).

Active surveillance, or watchful waiting, has emerged in recent years as options for men who decide not to undergo surgery or radiation therapy. During active surveillance, localized PCa is carefully monitored for signs of progression. If the tumor starts to progress the patients is offered treatment with intent to cure. Watchful waiting is eligible for older PCa patients where curative treatment no longer is feasible. The fundamental basis for active surveillance and watchful waiting is to avoid giving treatment for as long as possible to optimize the patient’s quality of life.

Challenges in PCa management
PCa is often a multifocal disease, i.e. each patient has evidence of more than one tumor simultaneously in the prostate gland. Some of these tumors are well differentiated and will most likely never become clinically significant (indolent) while others are poorly differentiated and may develop into aggressive tumor. However, the majority of tumors are somewhere in between and the nature of these are difficult to predict. The current diagnostic and prognostic tools - PSA, GS, and tumor extent can not provide sufficient information about whether a tumor will result in lethal or indolent disease. The fact that the tumor can not be adequately distinguished as “good” or “bad” at time of diagnosis results in a substantial overtreatment
and loss of quality of life for many men. Therefore, there is an urgent need for new and better prognostic markers.

**Chronic inflammation in PCa**

Even though PCa is a very common disease the etiology is largely unknown. The most established risk factors are family history, age, and African-American ethnicity (24-25).

Epidemiological, molecular pathological, and histopathological studies have provided evidence highlighting the role of chronic inflammation as a trigger for cancer development (3). Regardless of etiology, the proposed mechanism for inflammation-induced tumors includes repeated tissue damage and regeneration in the presence of reactive oxygen (ROS) and nitrogen species (RNS) (26). Various immune cells such as neutrophils, leucocytes, and macrophages, release these free oxygen radicals in order to kill, for example, different pathogens. It is known that ROS have the capacity to induce different types of DNA damage such as DNA base modifications and DNA-strand breaks (26-27). It has further been demonstrated that excessive production of ROS has an impact on altered DNA methylation such as hypermethylation of tumor suppressor genes.

Increased levels of cytokines and ROS in a persistently inflamed tissue are implicated in all three phases (initiation, promotion and progression) of carcinogenesis. The initiated cell undergoes proliferation to produce a clone of mutated cells, forming a pre-malignant mass. Some of the pre-neoplastic cells may during the promotion and progression phase encounter additional mutations and subsequently become malignant. Proliferating tumor cells, their surrounding host stromal cells, and tumor-infiltrating macrophages and T-lymphocytes (T cells) create a tumor environment that reflects a persistent inflammatory state. This microenvironment also contains several cytokines such as interleukin 6 (IL-6), IL-8, and IL-1, which also have the capability to participate in tumor initiation, promotion, and progression. IL-6 is a cytokine that modulates the expression of genes involved in cell cycle progression (28). It also inhibits apoptosis and has been associated with the pathogenesis of various cancers, including prostate cancer (28). IL-1α is a regulatory cytokine that induces activation of the transcription factor nuclear factor kappa B (NF-κB) and thereby promotes cell survival, proliferation, and angiogenesis (29). IL-8 is a potent angiogenic factor in several cancers and elevated IL-8 is correlated with angiogenesis, tumor progression, and poor survival in non-small cell lung cancer patients (30).

Under normal circumstances, epithelial cells are protected from DNA damage by the π class glutathione S-transferase (GSTP1) gene. GSTP1 has
been described as a “caretaker” gene as it protects the cell from oxidative genome damage mediated by electrophilic compounds (31).

**Prostatic atrophy**
In addition to inflammation, atrophy is extremely common in prostate specimens. Atrophy in the prostate is related to aging and is identified as a reduction in the volume of pre-existing glands and stroma. It can be divided into two major patterns: diffuse and focal (15). Diffuse atrophy is the result of a decrease in circulating androgens and aging and involves the entire gland in a uniform manner. Focal atrophy occurs frequently as heterogeneous foci in the peripheral zone and it has been observed in close proximity to the precancerous lesion called prostatic intraepithelial neoplasia (PIN) and also to PCa tumors (15, 32). To formalize the terminology of the various atrophic lesions, a new classification was proposed in 2006 (33). Here, the focal atrophies were categorized into four distinct subtypes; simple atrophy (SA), simple atrophy with cyst formation (SACF), postatrophic hyperplasia (PAH), and partial atrophy (Partial). The key histological criteria of each focal atrophy subtype are presented in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Key Histological Criteria of Focal Prostate Atrophy</th>
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<tr>
<td>Cytoplasm amount</td>
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<tr>
<td>Cytoplasm color</td>
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<tr>
<td>Papillae</td>
</tr>
<tr>
<td>Gland size</td>
</tr>
<tr>
<td>Gland shape</td>
</tr>
<tr>
<td>Gland packing</td>
</tr>
<tr>
<td>Inflammation</td>
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<tr>
<td>Morphology</td>
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**Proliferative inflammatory atrophy**
In 1999, De Marzo proposed the term proliferatory inflammatory atrophy (PIA) to designate foci of proliferative glandular prostate epithelium with-
morphological appearance of SA or PAH occurring associated with inflammation (34). He further suggested that PIA may give rise to prostate adenocarcinoma either directly or via progression into PIN, based on evidence from morphological and molecular investigations (Figure 2).

![Figure 2. De Marzo et al. proposed model of cellular changes in early PCa progression. (The figure is adapted with permission from De Marzo and Nature Publishing Group).](image)

Independent studies have identified SA and PAH located in close vicinity to both PIN and PCa in the peripheral zone of the prostate gland (32, 35-37). Furthermore, the proliferation was higher and the apoptosis was lower in SA and PAH compared to nonatrophic epithelial cells. Observations in prostatectomy specimens have revealed a gradual increase in the median number of proliferating nuclei from benign prostate tissue (1.20%), to SA (2.67%), to PAH (3.62%), to PIN (6.14%), to PCa (12%) (38).

Although the majority of cells in PIA are protected from DNA damage due to over expression of GSTP1 approximately 6% of the cells lack the expression of this gene (39). Since hypermethylation of GSTP1 is found in almost 70% of the cells in PIN and in practically all cells in PCa, the specific population in PIA lesions lacking GSTP1 may be more vulnerable for genetic alterations and cellular transformation (34). These are some of the findings supporting the hypothesis of PIA as a precancerous lesion. However, it should be noted that the results vary since in some investigations only PAH is associated with PCa while in others only an association between SA and PCa has been found (38, 40). Furthermore, not all studies found an association between atrophy and PCa or between atrophy and PIN (35, 41).
Propionibacterium acnes as a possible source of prostatic chronic inflammation

Prostatic inflammation can be caused by several different sources including urine reflux, physical trauma or infectious agents such as viruses and bacteria (42-43).

P. acnes, is a facultative anaerobe, Gram-positive rod bacterium whose end product of fermentation includes propionic acid, from which the name is derived. P. acnes is a common member of the skin microbiota and resides in pilosebaceous follicles predominantly in the face and the back of most part of the human population. The microorganism present on the human skin is suggested to have beneficial effects since it have the ability to lower the pH on the skin thereby protecting the follicles against the colonization by other more harmful pathogens. In addition to the prevalence on the skin, P. acnes has also been identified at other body sites such as the oral cavity, intestinal tract, and conjunctiva (44).

Two distinct phenotypes of P. acnes, Type I and Type II, were first identified based on serological agglutination tests and cell wall carbohydrate analysis (45). This classification was followed by a phylogenetic analysis of the housekeeping gene recA and a putative hemolysin gene tly, revealing that Type I and Type II represents two distinct groups (46). By comparing the recA gene further a subcluster of strains within P. acnes Type I was separated into IA and IB (46-47). Recently, a third linage has been identified with a filamentous cell morphology and with a slightly different recA gene sequence and this linage was designated to subtype Type III (48).

Currently (2011), three complete P. acnes genome sequences have been reported: KPA171202 (Type IB), 266 (Type IA), and SK137 (Type IA). All the genomes have similar GC content of approximately 60% and a genome size averaging 2509 kb. Comparative genomic analysis has been performed and the results show a low overall variation between the different types although a number of regions, encoding possible virulence properties, differed between the phylotypes (49).

P. acnes is generally regarded as commensal of the skin but has been associated with the skin disorder acne vulgaris. P. acnes has further been suggested as the etiological agent of a number of low-grade infections such as in postoperative and device related infections, prosthetic joint infections, prosthetic valve endocarditis, dental infections, and sternal wound infections after cardiothoracic surgery (44, 50-54). In recent reports P. acnes has also been frequently identified in prostate tissue from men with BPH and PCa. In 2005, Cohen et al. detected P. acnes in 35% of the cultures from prostate samples obtained from men undergoing RP (14). In addition
they found a significantly higher degree of inflammation in cases positive for *P. acnes* compared to those negative for the bacteria. These findings raised the question whether *P. acnes* could be involved in inflammation-induced prostate carcinogenesis. The prevalence of the bacteria in the various conditions may indicate that *P. acnes* exhibits pathogenic potential. Evidence for a number of virulence attributes in *P. acnes* has been observed, including haematolytic and cytotoxic properties as well as the capacity to avoid the immune response by intracellular location or by the production of biofilm (55-57).

Additional findings supporting the hypothesis of *P. acnes* as a trigger for PCa development and progression was recently presented by Fassi Fehri et al. (58). Their results showed an increased cell proliferation, a reduction of E-cadherin, and a strong production of IL-6 in normal prostate cells when infected with *P. acnes*. An interesting observations was also made by Alexeyev et al., when detecting *P. acnes* in prostate samples obtained from individual patients taken up to six years apart, indicating a sustained *P. acnes* infection of the prostate (55). This together with evidence revealing *P. acnes* as a trigger of ROS production in infected keratinocytes, highlights the speculation that *P. acnes*, by creating a chronic inflammation, may promote PCa initiation (59).

It has been proposed that *P. acnes* subtype I is predominantly associated with moderate to severe acne and that that *P. acnes* subtype II is the most prevalent phylotype cultured from endodontic infections and orthopedic implants (53, 60-62). More knowledge is needed about the existence of prostate-specific subtypes of *P. acnes*, which could be more prone to cause an infection related chronic inflammation and thereby participate in the pathogenic process. Two studies have been performed investigating the presence and subtype of *P. acnes* in prostate tissue obtained from men with PCa. Subtype II was found to be the most common subtype in both studies (14, 63). However, major limitations in these studies were the small number of *P. acnes* isolates included and also the lack of appropriate negative control tissue. Future studies should unravel if an invasive *P. acnes* subtype predominantly colonizes the prostate gland and if a certain subtype elicits a specific host response including initiation of transformation of the prostate cells. The outcome and severity of the prostate disease may depend on the *P. acnes* subtype, causing the infection-related inflammation. However, contradicting results regarding *P. acnes* involvement in PCa development has also been reported (64).
**Typing of P. acnes**

Pulsed field gel electrophoresis (PFGE) is regarded as the gold standard method for genotyping of various bacteria and has also been applied for P. acnes (54). In PFGE, the bacterial DNA is cut in pieces by a restriction enzyme and separated under an electrical field. This results in specific band patterns by which alterations in the genome can be detected by differences in the band pattern. The method has high discriminatory power but is time-consuming and the evaluation is subjective. Therefore results are difficult to compare between different laboratories. Because of these limitations the majority of contemporary subtyping studies are based on different polymerase chain reaction (PCR) or sequencing methods.

**Conventional PCR**

PCR is a technique based on enzymatic reactions used for amplifications of specific DNA fragment (65). The PCR imitates the natural synthesis of DNA sequences in the cell. To initiate a PCR, two oligonucleotide primers are needed to indicate the region of interest in the genome. The DNA synthesis is performed by a heat-stable DNA polymerase during repeated cycles of denaturation, annealing, and extension. In the denaturation step the double stranded DNA is separated at high temperature (>90°C), which make it possible for the primer, a short nucleotide sequence, to anneal to the complementary region on the DNA and function as a start for the polymerase. The optimal temperature for the polymerase is usually 72°C. During the extension step the temperature is increased again and a new DNA sequence of the region of interest is created. By repeating this cycle an exponential increase in the amount of the specific DNA sequence will be produced and 25-40 cycles generate approximately $10^7$-$10^{12}$ copies of DNA. To visualize and confirm right amplicon gel electrophoresis is commonly used. The negatively charged PCR products are migrating against the positive anode and separate bands can be visualised and compared with bands of known size. Large amplicons migrate more slowly through the agarose gel than small because they are retarded by the agarose particles.

**Real-time PCR**

One of the major advancement in the PCR technology was the introduction of real-time PCR (also called quantitative PCR, qPCR). With this technique, amplification of DNA and detection of the product can be performed in a closed system. This has several advantages such as speed, lower risk for contamination, and higher sensitivity. The real-time instrument used in this thesis is the Light Cycler System PCR in which the heat-
ing and cooling is controlled by air and not with heating block as in most terminal cyclers. By using air, which results in rapid temperature exchange, and glass capillaries, with a high ratio of surface area to volume, this is a fast method.

In this thesis, SYBR Green I has been used for detection even though probes are a more specific alternative. The fluorescent SYBR Green I dye binds to double stranded DNA and the fluorescence will increase when bound. During amplification the dye will bind to the newly synthesized DNA, leading to a fluorescent signal proportional to the DNA concentration. To characterize the end product a melting curve analysis is performed subsequently to the amplification. Here the melting temperature (Tm) of the PCR product, 50% each of single stranded DNA and double stranded DNA are calculated. Initially, the temperature is raised to the melting point of the DNA and the fluorescence is simultaneously measured. The melting point differs between different amplicons due to their GC content and length. Thus, the determination of melting temperature of a specific DNA fragment can be used to evaluate the PCR product. A schematic illustration of the procedure is shown in Figure 3.

**Figure 3. LightCycler PCR using SYBR Green.** The increase in PCR copies is detected directly during cycling in real-time PCR. 1. A SYBR Green colour binds to the minor groove of double stranded (ds) DNA. 2. The colour’s fluorescence is measured at the end of each cycle. 3. A melting curve analysis is performed.

**Nucleotide sequencing**

To learn more about the structure of DNA and thereby gain more knowledge about the abundant amount of proteins produced, Fred Sanger developed a method to determine the exact order of the nucleotides within a DNA sequence in 1977 (66). The Sanger sequencing method is based on an ordinary PCR. However, the DNA synthesis is performed using merely one primer in each reaction making the synthesis of one strand separately. In-
cluded in the PCR reaction mix is also fluorescence labelled dideoxynucleotides (ddNTPs) lacking the 3’OH group. These will be incorporated randomly in the DNA synthesis but when incorporated they will terminate the continuing synthesis. As in an ordinary PCR, the sequencing is performed in repeated cycles but will generate DNA sequences that differ in size and where each terminal dideoxy-dNTP is labelled with four different fluorescent colours. The products are then separated by an automated capillary electrophoresis-based genetic analyzer. By applying a high voltage, the negatively charged DNA fragments migrate through capillaries and subsequently a laser will emit the dye on each size separated, fluorescently labelled, DNA fragment which is detected by an optical device. A schematic illustration of the procedure is shown in Figure 4.

**Figure 4. DNA Sequencing.** Each of the four terminator bases is labelled with a different fluorescent dyes. Thus, when the template is copied, the growing chain is simultaneously terminated with a labelled base. This cycle is repeated 25 times to reach detectable amounts. The fragments are then separated by capillary electrophoresis and each fragment is registered by its laser excited dye and the order of the bases can be read.

**Multilocus sequence typing**

Multilocus sequence typing (MLST) is a subtyping method used for identification of clones within microorganisms such as bacteria. MLST is a highly discriminatory technique and is based on naturally occurring mutations in housekeeping genes (67). Housekeeping genes are present in all bacteria since they are essential and they can be used to provide information about relationships between isolates since they diversify very slowly. This method can further be used to differentiate the isolates into different clones. Isolates of bacterial species that are indistinguishable in genotype
are assigned as a clone. The technique starts with amplification and sequencing of internal fragments of usually seven housekeeping genes. For each gene the different sequences present are assigned with a specific allele number. The different allele numbers from the seven genes are then put together representing the sequence type (ST), which describe various clones within the bacteria. The majority of the bacterial population belongs to a small number of related genotypes referred to as clonal complexes (CC). The grouping will result in different CCs based on similarity in STs in six of seven internal fragments. Since the first MLST scheme was developed for Neisseria meningitides, approximately 20 additional schemes have been developed but when this thesis was started no P. acnes scheme was available. Although MLST has high discriminatory capacity, the method is both time-consuming and costly and therefore less demanding techniques are appreciated such as repetitive-sequence-based PCR methods.

**Repetitive-sequence-based PCR**

Repetitive-sequence-based PCR (rep-PCR) use primers that target noncoding repetitive sequences interspersed throughout the bacterial genome and thus can be used for subtyping investigations of bacteria (68). The DiversiLab System, which was recently made commercial available, is based on rep-PCR technology and is able to discriminate between different bacteria. After DNA extraction, the strain specific rep-PCR primers binds to many repetitive sequences interspersed throughout the genome and multiple fragments of various lengths are subsequently amplified. By using microfluidics chips the amplicons are then separated by size. Inside the lab-chip, the DNA fragments bind to an intercalating dye and unique rep-PCR fingerprint patterns are created containing multiple bands of varying sizes and intensities. The results are then displayed as electropherograms, virtual gel images, and demographic data. Figure 5 shows a schematic illustration of the procedure.
Immunology of the prostate

To protect the body from infection-related diseases humans are equipped with the innate and the adaptive immune system. Both these immune systems have the ability to distinguish between self and non-self molecules. One specific class of non-self molecules is termed antigens. Different parts of the bacterium such as cell wall structures are examples of antigens and they all have the capacity to evoke an immune response.

The immune response in the normal prostate is mainly based on the adaptive immune system including T and B cells (69). The distribution of immune cells is constant according to anatomic zonal location within the prostate. The lymphocytes are most numerous in the stroma but are also located in the intraepithelial compartments. The lymphocytes in the stroma are predominantly CD4+ helper T cells whereas intraepithelial T cells are mainly CD8+ cytotoxic T cells. This indicates that CD8+ cytotoxic T cells are the first line of defence in the prostate gland.

CD8+ cytotoxic T cells eradicate infected cells by releasing the cytotoxin perforin which form pores in the target cells plasma membrane, allowing water and toxin to enter. Another important toxin is granzyme B that stimulate the target cell to undergo apoptosis (70). The main function of CD4+ helper T cells is to aid in maintaining the expansion of CD8+ cyto-
toxic T cells. However, it has been reported that they also have the capacity to eradicate infected cells.

Another important role of the adaptive immune system is to identify and eliminate tumor cells. The transformed cells of tumors express antigens appearing as non-self, thereby initiating an immune cell attack. Both T cell populations (helper and cytotoxic) represent powerful components in this anti-tumor immune response, although CD8⁺ cytotoxic T cells have been reported as most essential (71-72).

**T cell infiltration and PCa**

Despite the potential of T cells to protect against cancer development conflicting results have been reported for the relationship between T cells infiltration in the prostate and survival of PCa patients. Vesalainen et al. reported that tumors with dense lymphocytic infiltration were associated with increased survival rates compared with men with fewer lymphocytes (73). On the other hand, poor disease outcome has been observed in men with greater numbers of CD4⁺ helper T cells in their prostate tissue (74-76). This suggests that, despite an ongoing anti-tumor immune response, tumors arise and become eventually clinically detectable. Thus tumors, by using different mechanisms, are able to escape from elimination by the T cells. The recruitment of a subset of T cells termed regulatory T cells with a suppressive function on the anti-tumor immune response, is one crucial mechanism used by the tumor to escape T cell elimination (77-78).

**Regulatory T cells and their association with cancer development**

Regulatory T cells (Tregs) can be defined as a T cell population that functionally suppresses an immune response by influencing the activity of other cell types. Tregs were initially described by Gershon in 1970 and where then called suppressive T cells (79). Five years later Sehon proposed, based on his mice experiments, that these cells negatively regulate tumor immunity and thereby contribute to tumor growth. Much effort was then directed at finding a marker for this cell population but not until 1995 could Sakaguchi et al. identify CD25 as a phenotypic marker for CD4⁺ Tregs (80). However, recently it has been reported the existence of a population of CD8⁺ T cells with suppressive function and the most specific marker to identify both CD4⁺ and CD8⁺ Tregs is the transcription factor forkhead box P3 (FOXP3) (81).

Even though several phenotypically distinct T reg populations have been suggested, Tregs can be divided in two major groups, natural Tregs (nTregs) and induced Tregs (iTregs). nTregs arise in the thymus and iTregs could be induced in
the periphery when FOXP3-negative T cells become FOXP3-positive by high local concentration of the cytokine transforming growth factor β (TGF-β) (82). Their suppression on CD8+ cytotoxic T cells and CD4+ helper T cells is performed in a cell-cell contact manner, using either the previously described perforin and granzyme B mechanism or by releasing high amounts of TGF-β (77-78).

There is accumulating evidence of an increased number of Tregs in patients with various types of cancer, such as gastric, esophageal, and colorectal cancers (83-84). Concerning PCa, Miller et al. was the first to assess human prostate tumors for the presence of Tregs (85). The results showed an increase in Tregs in PCa tissue compared to normal prostate tissue from the same patient. In a subsequent study Sfanos et al. found an enrichment of Tregs in the prostate of the majority (126 of 146) of men undergoing RP (86). Further, an enhanced frequency of Tregs was observed in lymphocytes obtained from peripheral blood and from prostate tissue of PCa patients (87-88). The identification of Tregs in peripheral blood from men with PCa was also made by Sotosek et al., showing significantly higher levels of Tregs compared to men with BPH and healthy volunteers (89). An increase in the proportion of Tregs is furthermore suggested to be strongly correlated with tumor progression. This hypothesis was supported by the evidence that patients with ovarian cancer showed a positive correlation between infiltrating Tregs and decreased survival (90). Similar suggestions have been made concerning PCa progression, but so far no conclusions have been made due to limitations in patient’s follow-up time (91).

There are several possible mechanisms behind the increased infiltration of Tregs in the prostate tumor environment. Curiel et al. have shown that tumor associated macrophages and the tumor itself produce the chemokine CCL22 which mediates Treg trafficking to the tumor due to its affinity for the receptor CCR4, which is expressed on Tregs (92). Another explanation might be the secretion of TGF-β by the prostate tumor cells which will lead to an up regulation of FOXP3 in otherwise FOXP3-negative T cells (93).

**Immunohistochemistry**

Immunohistochemistry (IHC) is a method to detect antigens in tissue sections by the use of monoclonal or polyclonal antibodies (94). When using formalin-fixed-paraffin-embedded (FFPE) tissue sections, many antigens can be successfully demonstrated by using IHC, if correctly and carefully optimized. Most antigens require pre-treatment, termed antigen retrieval, to break protein cross-links formed by formalin fixation and thereby uncovering hidden antigenic sites. Antigen retrieval is usually performed by
using either heat, which is termed Heat Induced Epitope Retrieval (HIER) or enzyme digestion, termed Proteolytic Induced Epitope Retrieval (PIER).

Various enzymes can be used for detection and two of the most common are peroxidise or alkaline phosphatase. In this thesis peroxidise was chosen and because one of the main causes of non-specific background staining is the endogenous peroxidise activity the tissue section had to be pre treated with hydrogen peroxidise prior to incubation of primary antibody.

There are several methods suitable to detect antigens such as the direct method and indirect method. The direct method is a one step staining method including a labelled antibody reacting directly with the antigen in the tissue. In this technique only one antibody is utilized and the procedure is fast. However, due to small signal amplification the method is insensitive and rarely used since the introduction of indirect method. The indirect method, used in this thesis, involves an unlabelled primary antibody which will bind to the epitope of interest and a labelled secondary antibody which will bind to the primary antibody. A schematic illustration of the procedure is shown in Figure 6.

![Image](image-url)

**Figure 6. Immunohistochemistry.** A. Direct method of IHC using one labelled antibody that binds directly to the antigen. B. Indirect method of IHC using one antibody against the antigen and a second labelled, antibody against the first.

To increase the sensitivity, a specific type of HRP-polymere system can be used. This polymere system contains a backbone to which multiple enzy-me molecules and secondary antibodies are attached.

The detection system in IHC is based on the principle of enzyme-substrate reactions and is performed to convert colourless chromogens into coloured products to allow visualization with the microscope. Several
chromogens are available and the choice is generally based on the enzyme selection. In this thesis the selected chromogens include Diaminobenzidin (DAB), Warp Red, and Vina Green. DAB and Vina Green are substrates for horseradish peroxidase (HRP) and Warp Red is a substrate for alkaline phosphatase. The chromogens will generate brown, green, and red staining, respectively.
AIMS

The aims of this thesis were to investigate if infection-related inflammation and focal atrophy have a role in prostate cancer initiation and/or progression and more specifically to:

1. Evaluate chronic inflammation, type of focal atrophy, and PIN in adjacent tumor tissue as predictors of lethal PCa.

2. Investigate the ability of two techniques to discriminate *P. acnes* isolates of diverse origin: an MLST protocol and a repetitive-sequence-based PCR.

3. Investigate the frequency of *P. acnes* isolated from prostate tissue of men with PCa and from negative controls without histological evidence of PCa.

4. Evaluate possible prostate specific *P. acnes* subtypes.

5. Evaluate the role of CD4<sup>+</sup> helper T cells, CD8<sup>+</sup> cytotoxic T cells, CD4<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells, and CD8<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells in tumor tissue with respect to acute or chronic inflammation, type of atrophy, PIN, and lethal PCa.
MATERIAL & METHODS

Patient material

For paper I and IV, patients were recruited from a cohort of men with localized PCa diagnosed in the Örebro and South East Health Care Region of Sweden between 1977 and 1999, termed the Swedish Watchful Waiting cohort (n=1,367). These men were diagnosed with incidental PCa through TURP or adenoma enucleation, i.e. category T1a-b tumors. In accordance with standard treatment protocols patients with early stage/localized PCa were followed expectantly (“watchful waiting”). The study cohort was followed for cancer-specific and all-cause mortality until March 1, 2006, through record linkages to the Swedish Death Register and Migration Register. Information on cause of death for each individual was obtained through a complete review of medical records by a study end points committee. Deaths were classified as cancer specific when PCa was the primary cause of death. The study was approved by the ethical committee in the Uppsala-Örebro region (M58-05). We utilized a novel nested study design that included men who either died from PCa during follow up (lethal PCa “cases”) or men who survived at least 10 years following their diagnosis (indolent PCa “controls”). By using this design we excluded men with non-informative outcomes, namely those who died from other causes within 10 years after cancer diagnosis. In paper I, 228 cases were included, together with 387 controls and in paper IV, 261 cases and 474 controls were included. Cases without complete data for both IHC and inflammation/atrophy variables in paper IV were also excluded.

In paper III a case-control study was performed including 100 cases and 50 controls. Cases were men diagnosed with PCa undergoing RP and controls were men diagnosed with bladder cancer undergoing cystoprostatectomy. In the latter operation the prostate will be removed even though no pathological conditions are detected. The samples from the cases were collected from consecutive RP operations between January 2009 and September 2012 and from the controls from consecutive cystoprostatectomy operations between January 2009 and October 2012. A pathologist examined these prostates with the same routine procedure as after RP and assessed the tissue for PCa without any histological findings. From each prostate six tissue biopsy samples were collected. The study was approved by the ethical committee in the Uppsala-Örebro region (2008/293).
Bacterial isolates
In paper II, a panel of *P. acnes* isolates with diverse origin were used. Twenty-five clinical isolates and four reference strains of *P. acnes* were examined. The clinical isolates, collected in Sweden, were selected to represent infections affecting different locations of the human body including prosthetic infections of the knee joint (PJI, n=3), hip joint (PJI, n=1), femur implant (PJI, n=1); deep sternal infections following cardiothoracic surgery (Thx, n=10); as well as isolates from perioperative tissue samples from the prostate (Pro, n=4). Skin isolates (Sk, n=6) and four *P. acnes* reference strains isolated from different locations were also used; hip prosthesis (CCUG 35749), facial acnes (CCUG 1794), human pustule (CCUG 36609) and one with unknown location (CCUG 38584).

Culture conditions and characterization
All the bacteria isolates and reference strains in paper II and III were cultured in an anaerobic atmosphere on FAA agar plates at 37°C in 5% CO₂. The isolates were characterized by colony morphology, Gram-staining, catalase and idole tests. All suspected *P. acnes* isolates were confirmed to the species level by using API 20 A.

DNA preparation
Two different DNA preparation methods were used in this thesis. For the real-time PCR analysis in paper II and III, DNA was extracted from *P. acnes* isolates by incubating two colonies, suspended in sterile distilled water including 100 units of Mutanolysin, for 30 min at 37°C. Subsequently, the QIAamp DNA Mini Kit (Qiagen) was used for extraction of DNA. For the DiversiLab analysis in paper II and III, the DNA from *P. acnes* isolates was extracted by using the UltraClean Microbial DNA Isolation kit (bioMérieux).

Real-time PCR
To obtain DNA amplicons for subsequent DNA sequencing (paper II and III) and to compare sequence variations within the genes, real-time PCR was used. At the MLST internet site www.mlst.net the internal fragments of the housekeeping genes; *lac* (encoding L-lactate dehydrogenase), *oxc* (Cytochrome c oxidase subunit II), *fba* (Fructose bisphosphate aldolase), *coa* (O-succinylbenzoate-CoA synthase), *zno* (Zn-dependant alcohol dehydrogenase), *gms* (Glutamyl-tRNA synthetase), *pak* (Pantothenate kinase), *cob* (Cobalamin), and *cel* (Transcription regulator CelR) were recommended for genetic analysis of *P. acnes* although no optimized protocol
was available. In addition to the recommended genes, the \textit{recA}, \textit{tly}, and \textit{Tc12S} were amplified. The primers used for PCR amplification were obtained from Scandinavian Gene Synthesis AB with exceptions for \textit{recA}, \textit{tly}, and \textit{Tc12S} (Sigma-Aldrich) and are summarized in Table 2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{lac} up</td>
<td>GCCGCAGCCCTTTGGGACTCT</td>
<td></td>
</tr>
<tr>
<td>\textit{lac} dn</td>
<td>GAAATGCTGTCGCCGCGG</td>
<td>469</td>
</tr>
<tr>
<td>\textit{oxc} up</td>
<td>GTGCTGCCGGAAAGTGCG</td>
<td></td>
</tr>
<tr>
<td>\textit{oxc} dn</td>
<td>CACCGGCGTCAGGATTGT</td>
<td>426</td>
</tr>
<tr>
<td>\textit{fba} up</td>
<td>AGGACCCGCCTATTTCACACTTC</td>
<td></td>
</tr>
<tr>
<td>\textit{fba} dn</td>
<td>AGCCGGTGCTAGTACCTTTT</td>
<td>532</td>
</tr>
<tr>
<td>\textit{coa} up</td>
<td>GCGGGAATCGAGGGTGCTA</td>
<td></td>
</tr>
<tr>
<td>\textit{coa} dn</td>
<td>AGGCCGGCCGCTAGATAAGTA</td>
<td>541</td>
</tr>
<tr>
<td>\textit{zno} up</td>
<td>CGCCGGCATCACACACTTTT</td>
<td></td>
</tr>
<tr>
<td>\textit{zno} dn</td>
<td>TCTCACATCGGCCGCAACC</td>
<td>507</td>
</tr>
<tr>
<td>\textit{zno} new dn*</td>
<td>CTCGGCGAGAATCGAGCA</td>
<td></td>
</tr>
<tr>
<td>\textit{gms} up</td>
<td>CCGGCTACACGTCCAGCA</td>
<td></td>
</tr>
<tr>
<td>\textit{gms} dn</td>
<td>CACATCGAGAACCGCATCACTC</td>
<td>538</td>
</tr>
<tr>
<td>\textit{pak} up</td>
<td>CGAGCCCTCAAATAAC</td>
<td></td>
</tr>
<tr>
<td>\textit{pak} dn</td>
<td>GTCGGCTCTCGACGAGGA</td>
<td>454</td>
</tr>
<tr>
<td>\textit{cel} up</td>
<td>GCGGCGACACACACGTTTCTACAGTGAC</td>
<td></td>
</tr>
<tr>
<td>\textit{cel} dn</td>
<td>GGCAGTGAGGGTGCCATTCA</td>
<td>432</td>
</tr>
<tr>
<td>\textit{cob} up</td>
<td>CATCTCTGGCTCGGGAAGG</td>
<td></td>
</tr>
<tr>
<td>\textit{cob} dn</td>
<td>TAGAAGAAGACATGGGCGAC</td>
<td>637</td>
</tr>
<tr>
<td>\textit{tly}-PAT-1</td>
<td>CAGGAGTTGAGTGCAATGCA</td>
<td></td>
</tr>
<tr>
<td>\textit{tly}-PAT-2</td>
<td>TCGTACCAAGACAGTACGA</td>
<td>830</td>
</tr>
<tr>
<td>\textit{recA}-PAR-1</td>
<td>AGCTCGGTTGGGTCTC</td>
<td></td>
</tr>
<tr>
<td>\textit{recA}-PAR-2</td>
<td>GTCTTGCTCATTACACCTGG</td>
<td>1030</td>
</tr>
<tr>
<td>\textit{Tc12S}-MF</td>
<td>CGGTAAACGCAATGGTCT</td>
<td></td>
</tr>
<tr>
<td>\textit{Tc12S}-MMR</td>
<td>GGAATATTGAAACGAGTCGCAGA</td>
<td>562</td>
</tr>
</tbody>
</table>

* used only in sequencing

The PCRs for all genes, except \textit{cob}, were performed in a real-time LightCycler system (Roche Diagnostics) using SYBR Green I fluorescence melting curve analysis for detection of specific amplicon. Each reaction mixture contained 2 µl of SYBR Green I and 2 µl DNA template in a total volume of 20 µl. PCR cycling conditions were optimized for each gene, including MgCl$_2$ and primer concentrations, annealing temperature and extension time. The PCR programs started with a pre-incubation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at different temperatures (55°C for \textit{lac}, \textit{fba}, \textit{pak} and \textit{cel}; 58°C for \textit{oxc},...
coa, gms, zno, tly and Tc12S; 66°C for recA) for 10 s, and extension at 72°C for 37 s.

Because of problems when optimizing the PCR for the cob gene in the Light Cycle system, this PCR was performed in the real-time Rotorgene system (Qiagen). Each reaction mixture contained 2 µl of SYBR Green PCR Kit and 2 µl DNA template in a total volume of 20 µl. The thermal cycling conditions included an initial pre-incubation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 10 s and annealing and extension at 60°C for 30 s.

**Purification and Sequencing**

Prior to sequencing the PCR products were purified using MultiScreen PCR plates (Millipore), according to the manufacturer’s instructions. The cycle sequencing was performed using Big Dye Terminator v3.1 according to the manufacturer’s instructions. One µl of the purified PCR products were cycle sequenced using 2 µl Big Dye Terminator v3.1. The cycle sequencing PCRs consisted of 25 cycles of denaturation at 96°C for 10 s, annealing at different temperatures (55°C for lac, fba, pak and cel; 58°C for oxc, coa, gms, zno, tly and Tc12S; 60°C for cob and recA) for 5 s and extension at 60°C for 4 min. Subsequently, the reactions were purified with ethanol-sodium acetate precipitation and resuspended in 10 µl formamide. The nucleotide sequences were determined by capillary electrophoreses using an ABI PRISM 3130XL genetic analyzer (Applied Biosystems). Alignments were made in ChromasPro software.

**MLST and phylogenetic analysis**

MLST was performed on 29 P. acnes isolates in paper II. Multiple-sequence alignments of the nucleotide sequences were performed using BioEdit software. Each distinct gene sequence was assigned an allele number or allele type (AT) and each unique combination of the nine genes ATs was assigned a new sequence type (ST). The most common ST was assigned the lowest ST number. The phylogenetic analysis was performed with the software TREECON (version 1.3b) using the Jun and Nei substitution model, the Kimura evolutionary model, value of 0.5 and the neighbor-joining method (95-96). To identify clonal complexes, the eBURSTv3 program (http://eburst.mlst.net) was used.

To evaluate the discriminatory capacity a discrimination index (D-index) was calculated. The D-index represents the likelihood that two randomly picked strains from the sample are recognized as two different strains. The maximum possible D-index is one (100%).
Repetitive-sequence-based PCR

All the samples in paper II and III were amplified using DiversiLab Propionibacterium Fingerprinting kit (bioMérieux). Each reaction mixture contained 18 µl kit-supplied rep-PCR mix and 2 µl DNA template in a total volume of 25 µl. The thermal cycling conditions included an initial preincubation at 94°C for 2 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 70°C for 90 s, and a final extension at 70°C for 3 min. To detect the genomic fingerprints of each *P. acnes* isolate, the genotyping system DiversiLab was used. The amplified fragments were separated by electrophoresis performed in microfluidics DNA LabChip and detected with an Agilent 2100 Bioanalyzer. The similarity between the isolates was analyzed with the Kullback-Leiber method and the Unweighted Pair Group Method with Arithmetic mean (UPGMA) was used as clustering method (97).

IHC

For the IHC analysis in paper IV, six TMAs were initially constructed. Three tissue cores with a diameter of 0.6 mm were collected from each patient TURP specimens. The IHC was performed using Target Retrieval Solution (DAKO) for deparaffination, rehydration, and antigen retrieval. Primary antibodies used for the single staining were mouse ready to use antibodies against CD4 (clone 4B12, DAKO) and CD8 (clone C8/144, DAKO) and the staining procedure was performed using the DAKO autostainer LINK system. The slides were incubated with primary antibodies at room temperature for 20 min and detected with EnVisionTM FLEX+, high pH (DAKO). For the triple staining a mouse monoclonal and rabbit monoclonal ready to use multiplex cocktail against CD4 and CD8 and a mouse monoclonal antibody against FOXP3 were used as primary antibodies. After primary antibody incubation for 30 min at room temperature the slides were incubated with secondary antibodies and chromogens. To identify CD4 and CD8, Mach 2 doublestain 2 (Biocare Medical), DAB (Biocare Medical) and Warp Red Chromogen kit (Biocare Medical) were used, respectively. For the detection of FOXP3, Mach 2 mouse-HRP-polymer (Biocare Medical) served as secondary antibody followed by Vina Green Chromogen kit (Biocare Medical) for visualisation. Finally the slides were counterstained with hematoxylin.

Evaluation of IHC

The staining evaluation was performed by two independent observers blinded to all the clinical data. Expression of CD4⁺ helper T cells, CD8⁺
cytotoxic T cells, \( \text{CD}4^+ \text{T}_{\text{reg}}, \) and \( \text{CD}8^+ \text{T}_{\text{reg}} \) were quantified using a light microscope at 40 X magnification. All \( \text{T}_{\text{reg}} \) were counted; \( \text{CD}4^+ \) helper T cells and \( \text{CD}8^+ \) cytotoxic T cells were counted up to 50 cells; greater numbers were recorded within a single category.

**Statistical methods**

In paper I, chi-square tests were used to evaluate associations between type of focal atrophy, presence of PIN, chronic inflammation, and tumor characteristics. In paper III, the same test was used to evaluate the association between the presence of \( \text{P. acnes} \) and PCa and in paper IV to evaluate the association between clinical covariates and lethal case PCa versus indolent control PCa status. In paper I, T tests were used to compare age of diagnosis according to inflammation, atrophy, and PIN status. To estimate odds ratios (OR) and 95% confidence intervals (CI) for lethal PCa according to type of focal atrophy, degree of chronic inflammation, and PIN, an unconditional logistic regression was used. Unconditional logistic regression was also employed in paper IV to estimate ORs for the association between the mean number of the different T cells populations across an individual patient’s three tissue cores with respect to the following outcomes: presence of histopathological characteristics and lethal PCa. Multivariate models adjusted for age at diagnosis, calendar year of diagnosis, GS, and presence of atrophy and inflammation, tumor stage, tumor percent were performed in paper I and IV.
RESULTS & DISCUSSION

Evaluation of chronic inflammation, focal atrophy, and PIN in adjacent tumor tissue and their prognostic significance for PCa

The aim of paper I was to investigate chronic inflammation, focal atrophy, and PIN in adjacent tumor tissue as predictors of lethal PCa. Prostate specimens from 615 men who were diagnosed with incidental PCa through TURP were evaluated. In line with results from several previous studies, both inflammation and focal atrophy were frequently observed. In approximately 75% of the specimens there was evidence of chronic inflammation, appearing as moderate or severe in 26% of the cases. The focal atrophy classification revealed that SA was the most common type, identified in 59%, followed by PAH in 20%, and PIN in 13% of the specimens. The evaluation further showed that men with moderate or severe chronic inflammation were more likely to have PAH lesions and that PIN was more frequently observed in tumor specimens with evidence of PAH. These observations are consistent with the hypothesis proposed by De Marzo et al., suggesting that the onset of chronic inflammation, initiated by infections or some other source, might have an influence on normal prostate epithelial transformation into PAH, which may later on give rise to PCa (34). These findings support the suggestion that a subgroup of cells within the PAH lesion are more vulnerable for genomic alterations and cellular transformations, possibly by down regulation of the caretaker gene GSTP1. However, the results contradict the theory suggesting that chronic inflammation alone is able to transform normal cells into PIN since neither acute nor chronic inflammation was related to PIN in this material.

In order to investigate the prognostic significance of chronic inflammation, SA, PAH, or PIN, their association with the odds of dying of PCa were examined (Table 3).
Table 3. ORs and 95% CIs of lethal PCa

<table>
<thead>
<tr>
<th>Chronic inflammation</th>
<th>OR¹ (95% CI)</th>
<th>OR² (95% CI)</th>
<th>OR³ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Mild</td>
<td>0.70 (0.47-1.03)</td>
<td>0.96 (0.58-1.60)</td>
<td>0.94 (0.55-1.62)</td>
</tr>
<tr>
<td>Moderate/Severe</td>
<td>1.10 (0.70-1.70)</td>
<td>1.61 (0.89-2.93)</td>
<td>1.73 (0.92-3.27)</td>
</tr>
<tr>
<td>SA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Yes</td>
<td>0.96 (0.69-1.34)</td>
<td>1.07 (0.71-1.63)</td>
<td>1.20 (0.76-1.87)</td>
</tr>
<tr>
<td>PAH</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Yes</td>
<td>0.89 (0.59-1.34)</td>
<td>0.82 (0.48-1.39)</td>
<td>0.92 (0.53-1.61)</td>
</tr>
<tr>
<td>PIN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Yes</td>
<td>2.16 (1.35-3.47)</td>
<td>1.89 (1.04-3.42)</td>
<td>1.75 (0.94-3.28)</td>
</tr>
</tbody>
</table>

OR¹ unadjusted
OR² adjusted for age at diagnosis, calendar period of diagnosis, GS, inflammation, SA, PAH, and PIN
OR³ additionally adjusted for tumor stage and tumor percent

No association was found between chronic inflammation, SA, or PAH and odds of death from PCa. However, men with PIN adjacent to their tumor were nearly two times as likely to die of their disease. This association remained significant even after adjusting for clinical variables. In the multivariate model there was also a trend for a positive association of moderate and severe chronic inflammation with respect to lethal PCa. In addition, this association was modified by the presence of PAH. Among men with PAH, who simultaneously expressed moderate/severe chronic inflammation, a nearly five-fold increase in odds of lethal PCa was found. These findings indicate that SA and PAH differ from each other not only morphologically, but also potentially in how they relate to PCa development and progression.

Previous studies have identified a number of hallmarks of PIN and PCa present in PAH such as down regulation of p27Kip1 and gain of chromosome 8 (38, 98-99). However, a recent study, made on prostate biopsy cores, showed that PAH was more frequently found in tissues with evidence of mild or no inflammation and that SA was the type of atrophy present found in moderate/severe inflammatory cores (40). They suggested that PAH may represent a post-inflammatory atrophic lesion.

Potential limitations in the study: All 615 PCa patients included were diagnosed by TURP and thus the evaluation was performed on prostate tissue obtained from the transition zone. The majority of PCas arise in the pe-
ripheral zone but approximately 25% of prostate carcinomas are thought to develop within the transition zone.

**Conclusions:** In this population-based case-control study of men diagnosed with localized PCa and followed up to 30 years after diagnosis, the use of chronic inflammation, focal atrophy, and PIN as predictors of lethal PCa was evaluated. The results suggest that PIN, and perhaps chronic inflammation in the presence of PAH may have prognostic significance and also that lesions in tumor adjacent tissue may aid in identification of a clinically relevant disease.

**DiversiLab and MLST techniques for molecular epidemiological characterization of P. acnes isolates**

Given the consistency of data on the common occurrence of prostatic inflammation together with the findings in paper I, we wanted to investigate a potential source of such inflammation and its relationship with PCa development. In recent reports *P. acnes* has been frequently identified in prostate tissue from patients with PCa, indicating that *P. acnes* infection could contribute to chronic inflammation and more speculatively to PCa initiation (14, 55, 57, 100). It has also been proposed that specific subtypes of the bacteria may be more prone to cause infection than others. Due to the limitations using PFGE as a subtyping method, the aim of paper II was to investigate the ability of two other typing techniques, MLST and DiversiLab, to discriminate between *P. acnes* isolates. For this investigation, a collection of *P. acnes* isolates was used including 25 clinical isolates of heterogeneous origin and four reference strains.

The MLST analysis, based on the concatenated sequences of nine genes, revealed 23 different STs among the 29 isolates as shown in Table 4. The most common ST, ST1, was shared by six isolates, all of subtypes IA but with different origin.

To investigate the genetic diversity among the *P. acnes* isolates phylogenetic trees based on individual MLST genes were constructed. The comparison of the trees showed concordant phylogeny, with clustering according to the major subtypes I, II, and III. Subsequently, a phylogenetic tree based on concatenated sequences of the nine MLST genes was constructed to illustrate the observed diversity when using more than one gene in the analysis. An improved diversification was observed among subtype I isolates, resulting in further separation into IA and IB indicating the limitation of just using a single gene.
MLST has been proven to be a reliable and applicable method to characterize other bacterial species than *P. acnes* due to its high discriminatory power. It is in general based on seven genetic loci. In the present study the highest D-index of 0.96 was displayed when combining the seven MLST genes; *fba, gms, zno, cel, lac, cob*, and *coa*. Including the two remaining genes did not increase the D-index further. Given the D-index close to one, the MLST-protocol proposed here, including seven genes, can be used as a subtyping tool to study populations of *P. acnes* isolates. Since MLST is a costly and time-consuming method, we made a comparison with the capacity of DiversiLab to diversify the *P. acnes* collection. In general the dendrogram showed clustering according to the four major subtypes IA, IB, II, and III and the method differentiated the collection into 11 rep-PCR fingerprint patterns (Table 4). The D-index of the DiversiLab method was 0.87, i.e. slightly lower than for the MLST method.

Some studies have been performed to evaluate the discriminatory ability of MLST compared to DiversiLab. The major conclusions were in line with our results, showing that DiversiLab can be used as a first-line tool for rapid typing of *P. acnes* isolates since the method is less technically demanding and time consuming compared to MLST. However, a higher D-index was found using MLST which implies that MLST is the preferable method for more thorough genetic investigations of *P. acnes* isolates.

### Table 4. Dendrogram and typing results based on DiversiLab and MLST of *P. acnes* isolates.

| Sample ID | Sample Type | Fingerprint pattern | DiversiLab | MLST | D-index | mlst
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0 12</td>
<td>TH1</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
</tr>
<tr>
<td>0 30</td>
<td>TH1</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
</tr>
<tr>
<td>0 30</td>
<td>TH1</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
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<tr>
<td>0 30</td>
<td>TH1</td>
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<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
</tr>
<tr>
<td>0 30</td>
<td>TH1</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
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<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
<td>1 2 3 4 5 6 7 8 9 10 11</td>
</tr>
</tbody>
</table>

*See Svan, The Thoracic, Pneumothorax, Pros Postoperative, CTU Acute Hemorrhage, University of Gothenburg, Sweden, ST Sequence type, P0 Present*
Potential limitations in the study: In the present study, 25 clinical isolates of *P. acnes* collected in Sweden represent infections affecting various locations of the human body. The small number of *P. acnes* isolates included can be viewed as a limitation and could potentially cause a bias of the D-index.

Conclusions: The present study shows that both DiversiLab and MLST are valuable techniques for molecular epidemiological investigations. The results propose that DiversiLab is the subtyping method to use for rapid screening of larger collections of *P. acnes* and that the herein presented MLST protocol is preferable for more thorough molecular genetic investigations of the clonal heterogeneity.

The prevalence and subtype of *P. acnes* in the prostate gland and its potential role in PCa initiation

Considerable lines of evidence suggest that PCa initiation may be related to an infectious agent. Although a specific pathogen responsible for this has not been determined involvement of *P. acnes* has been proposed. In paper III the first aim was to investigate the prevalence of *P. acnes* in prostatic tissue among 100 men with PCa and compare these findings with those obtained from 35 men without diagnosed disease. By including control tissue, i.e. specimens from patients showing no sign of PCa, the present study addressed a major limitation of previous studies.

The culturing and species verification revealed a, in line with previous studies, frequent colonization of *P. acnes* in prostate tissue obtained from men with PCa (14, 55, 57, 100). Interestingly, the cultivation showed a significantly greater percentage of men with PCa to have the bacteria present compared to men with healthy prostates (p=0.001). *P. acnes* was identified in 60% of men with the disease compared to only 29% in men with no diagnosed or histological signs of prostatic malignancy. This finding adds evidence for the suggestion that *P. acnes* may have a role in PCa initiation. Additional support to this theory comes from recent reports, showing that *P. acnes* has the capability to trigger both a strong immune response and production of ROS (101-102). Moreover, an interesting observation was recently reported by Fassi Fehri et al. They found that long-term exposure of prostate epithelial cells to *P. acnes* resulted in a cellular transformation (58). These results all together support the hypothesis that increased ROS production caused by sustained *P. acnes* infection may increase the risk for genomic alterations and subsequently PCa development.

One might speculate that that a specific subset of cells within PAH lesions are more vulnerable for transformation caused by infection, based on
our findings in paper I showing that men with chronic inflammation in the presence of PAH had an increased risk to die from their PCa.

Furthermore, the distribution of *P. acnes* regarding different locations in the prostate gland was investigated. Though not statistically significant *P. acnes* appeared nearly equally distributed throughout the gland in men with PCa but was rarer in the samples taken from the peripheral zone in men without evidence of the disease. Given that PAH is commonly observed in the peripheral zone and that almost 70% of all PCa originates from this area this may be of interest when investigating the role of this pathogen in PCa etiology.

In order to further evaluate the subtypes of the isolates classified as *P. acnes*, a repetitive-sequence-based PCR method, DiversiLab, evaluated in paper II, was used together with sequencing analysis of the *tly* gene. According to the dendrogram from the DiversiLab analysis and the typing based on *tly*, the prostatic isolates were separated into the three major subtypes, (I, II, and III) where I and II were the two most common ones. The distribution in men with PCa was 36 isolates subtyped as IA, 19 isolates as IB, 70 isolates as II, and three isolates as III. In men with no histological evidence of PCa only 15 isolates were characterized as *P. acnes* which included the following subtypes; five subtype IA, one subtype IB, and nine subtype II. Several results in the present study support the hypothesis that prostate specific *P. acnes* subtypes exist that may be more prone to cause a persistent infection and thus chronic inflammation (60-61). First, in line with previous reports, a major cluster composed by 65 subtype II isolates was observed in the phylogenetic analysis (14, 63). Second, this subtype was also the most common in men who where diagnosed with PCa after the histological examination (controls with PCa). This may indicate that *P. acnes* subtype II has a joint origin in the urethra and infects the prostate via that route.

The results further shows, that the presence of *P. acnes* in the prostate cannot simply be explained by performed biopsy sampling since no prostate biopsies are performed within the control group.

**Potential limitations in the study:** In the present study we were not able to evaluate the temporal relation between *P. acnes* infection and PCa initiation because we could not be ascertain that the included men were infected with *P. acnes* prior to the development of PCa.

**Conclusions:** In this unique cohort of men with and without PCa we evaluated the frequency and subtypes of *P. acnes* isolated from multiple peri-operative prostate tissue samples. The data provide evidence that men suf-
ferring from PCa are more likely to have prostatic *P. acnes* infections compared to men without PCa. Specifically, the findings suggest that *P. acnes* subtype II may be an important factor in the initiation of PCa.

**Evaluation of subpopulations of T cells in the tumor environment and their prognostic significance for PCa**

Several studies have demonstrated that T cells, specifically CD8⁺ cytotoxic T cells and CD4⁺ helper T cells, are capable of recognizing and eliminating various tumor cells (71-72, 103). However, conflicting results have been reported for the relationship between lymphocytic infiltration and survival in PCa patients (73-74, 104). One of the mechanisms suggested is the recruitment of Tregs (77-78). However, so far only one larger study has analysed the association of Tregs and clinical outcome, although the follow-up time was relatively short, (median 30.5 months) (91). Therefore, the aim in **paper IV** was to evaluate the role of the three different subpopulations of T cells in tumor tissue with respect to focal atrophy, PIN, and lethal PCa in a cohort of men with PCa who are followed up to 30 years.

The IHC showed that the lymphocytes were predominantly located in the stroma surrounding the tumor, either as single cells or as aggregates. The majority of the FOXP3 positive cells were simultaneously positive for CD4. CD8⁺ Tregs were only identified in three patients. Representative pictures are shown in Figure 7. The mean numbers of CD4⁺ helper T cells, CD8⁺ cytotoxic T cells, and CD4⁺ Tregs was positively associated with the presence of moderate or severe chronic inflammation. This observation demonstrates that IHC markers of inflammation are positively related to histopathological classification of inflammation.
Figure 7. CD4 (brown), CD8 (red), and FOXP3 (green) expression in prostate tissue. A. Immune cells located in the stroma. B. Immune cells located in the epithelia and in the stroma. C. Arrows indicate CD4+FOXP3+ T cells. D. Solid arrow indicates a CD4+FOXP3+ T cell and open arrow indicate a CD8+FOXP3+ T cell.

By using the mean cell count value we investigated whether CD4+ helper T cells, CD8+ cytotoxic T cells and CD4+T<sub>reg</sub> were associated with lethal PCa. Every additional CD4+T<sub>reg</sub> was associated with a 12% increase in odds of dying of PCa. When the mean number of cell counts was categorized into quartiles, a nearly two-fold increase in the odds of lethal PCa was found when comparing the highest to the lowest quartile of CD4+T<sub>reg</sub>. This provides additional evidence in support of the hypothesis that T<sub>reg</sub> cells are recruited to the prostate tumor where they may suppress anti-tumor immunity and thereby aid tumor progression. These data do not address the function of the infiltrating T<sub>reg</sub> although a recent study showed an enhanced frequency of T<sub>reg</sub> with suppressive function in lymphocytes obtained both from prostate tissue and peripheral blood of patients with PCa (88). In line with our results, Sfanos et al. found an enrichment of T<sub>reg</sub> in almost all cases when investigating 20 men undergoing RP for early PCa and Fox et al. identified FOXP3 positive T cells in 126 of 146 PCa tissue specimens (77, 86).
Interactions between chronic inflammation, SA, PAH and mean number of CD4+ helper T cells, CD8+ cytotoxic T cells, and CD4+Tregs on lethal PCa were further explored. The association between CD4+ helper T cells and lethal PCa was modified by the presence of PAH. No association was observed between CD4+ helper T cells and lethal PCa among men without PAH, but among men with PAH every one-unit increase in CD4+ helper T cell was associated with a 5% increase in the odds of dying of PCa.

Several possible mechanisms have been suggested for the increase of Tregs in the tumor area. Tumor-associated macrophages are able to secrete the chemokine CCL22, which has affinity for the Treg associated receptor CCR4. An alternative explanation could be the production of TGF-β performed by the prostate tumor cells. TGF-β has been described as a multi-functional cytokine which is able to up-regulate FOXP3 expression, on otherwise FOXP3 negative T cells, and thereby expand the Treg population (105).

Our data suggest that there are at least two distinct pathways leading to lethal PCa. The first involves moderate/severe chronic inflammation combined with evidence of PAH concurrently with a shift in the CD4/CD8 T cell ratio. An alternative pathway could be represented by an inflammation-independent, prostate tumor assisted increase of CD4+Tregs. This may explain the inconsistency in previous studies investigating T cell infiltration and PCa outcome. We believe that it is of great importance to characterize specific subtypes of T cells in studies investigating the role of chronic inflammation and its role in PCa initiation, promotion, and progression. To further strengthen the IHC evaluations in this field, it may be of value to exclude single staining for FOXP3 expression since a recent study identified FOXP3 expression also in prostate tumor cells (81).

Potential limitations in the study: All PCa patients in the present study were diagnosed by TURP before the PSA era. Given that PSA testing and needle biopsy are utilized more commonly in the clinical practice today, further studies in PSA screened populations and biopsy specimens will be required. Moreover, the use of TMAs for evaluation of T cells infiltration could be viewed as a limitation because of potential heterogeneity in the prostate tissue and that a limited amount of the tissue is evaluated.

Conclusions: Our data provide evidence that men with greater numbers of CD4+Tregs in their prostate tumor environment have an increased risk of dying of PCa. In addition, in men with evidence of PAH the outcome of the disease varies according to the number of CD4+ helper T cells. This sug-
gests that identification of CD4+ T_{regs} in prostate tumor tissue may predict clinically relevant disease at time of diagnosis.
GENERAL DISCUSSION & FUTURE PERSPECTIVES

The overall aim of this thesis was to investigate if infection-induced chronic inflammation has a role in the pathogenesis of PCa. Even though inflammation is commonly observed adjacent to both prostate tumors and precursor lesions, less is known about its consequences and on which specific phases of the cancer development process it exerts an influence.

Further, could it be that depending on the tumor microenvironment the infiltrating immune cells behave differently and thereby manoeuvre the tumor promotion and progression in opposing directions?

According to the injury and regeneration model, repeated injury to the prostate epithelium caused by inflammatory components will subsequently give rise to focal atrophy lesions termed PIA (SA and PAH in the presence of inflammation) (34). Furthermore, some cells within these lesions, with compromised caretaker functions, may progress to PCa either directly or via progression into PIN. Evidence for this hypothesis is based partly on the findings of up/down regulation of a number of proto-oncogenes and tumor-suppressors, such as NKX3.1, Ki67, Bcl-2, p53, GSTP1, and COX-2 in PIA in a similar extent as in PIN and PCa (39, 106-109). However, the issue whether PIA is a risk lesion for PCa is still controversial.

The results of this thesis suggest two distinct pathways leading to prostate tumor progression. (1), PAH combined with evidence of moderate or severe chronic inflammation, and (2) a pathway independent of chronic inflammation. This indicates that chronic inflammation alone is not capable of causing tumor progression but is likely to initiate tumor development. This conclusion can be made since the inflammation was frequently identified not only in men with lethal PCa, but also in patients with an indolent disease.

The findings furthermore highlight the importance of evaluating SA and PAH separately in future studies and thereby identifying precursor lesions for PCa. Additional support comes from a recent study where SA was identified as the atrophy lesion associated with moderate or severe chronic inflammation that is in contrast to our observation of PAH as the most common lesion (40).

To better clarify whether chronic inflammation and/or focal atrophy predispose development of PCa, future studies have to evaluate the presence of chronic inflammation and atrophy in prostate tissue prior to PCa. It is now also possible to study the potential link between inflammation and PCa in an in vivo system since Shinohara et al. developed a mouse
model of long-term bacterial induced chronic inflammation of the prostate (110).

The second pathway leading to lethal PCa proposed in this thesis, is an increased T\textsubscript{reg} population in the prostate tumor environment. The results provide evidence for the hypothesis that the type, not the quantity, of prostate tumor-infiltrating T cells may be a more critical determinant for the prognosis of the disease. Previous investigations, regarding other types of cancer, have associated a high infiltration of T\textsubscript{reg} with worse outcome, possible due to their capacity to suppress the anti-tumor immune response, performed by CD4\textsuperscript{+} helper T cells and CD8\textsuperscript{+} cytotoxic T cells (83). The results from this study suggest that PCa can be added to the list of cancer diseases were infiltration of T\textsubscript{reg} contributes to tumor progression, which is schematically presented in Figure 8.

![Figure 8. CD4\textsuperscript{+}T\textsubscript{reg} suppress antitumor immune response, performed by CD8\textsuperscript{+} cytotoxic T cells and CD4\textsuperscript{+} helper T cells, thereby aid in tumor progression.](image)

Furthermore, we believe that, if verified in a biopsy cohort, the identification of CD4\textsuperscript{+}T\textsubscript{reg} may serve as an additional marker for identification of clinically relevant disease at the time of diagnosis.

In the best scenario this thesis contributes valuable information that could be used to reduce the substantial overtreatment seen today and thereby improving quality of life for men with diagnosed PCa. One might further speculate that a combined therapy that attacks both malignant cells and tumor-infiltrating T\textsubscript{reg} may improve the cancer treatment by eliciting a long-lasting anti-tumor immunity to the transformed cells. To expand the knowledge about where in the cancer process the anti-tumor immune re-
The other major topic this thesis aimed to address was the question “Is it conceivable that an infectious agent is able to cause inflammation and thereby act as an etiological factor for PCa development?“. The current thesis identified, in line with others, frequent colonisation of *P. acnes* in men with PCa (14, 55, 57, 100). To expand the previous reports this study also analysed prostate tissue obtained from men without any histological signs of PCa. Interestingly, *P. acnes* was found almost two times as frequently in men with the disease compared to men without diagnosed disease. This may indicate that *P. acnes* has a role in PCa development. However, this study cannot answer the question when during the cancer process the prostate becomes infected with *P. acnes*. It is possible that the colonisation of *P. acnes* takes place before tumor initiation but on the other hand it is also possible that the microorganisms are able to infect pre-cancerous lesions or tumor cells and consequently participate in tumor promotion and/or progression. The presence of *P. acnes* in the prostate cannot however simply be explained by contamination after performed biopsy sampling since no prostate biopsies are performed within the control group. Interestingly, *P. acnes* was also found within 53% of the controls with histological findings of PCa.

Another factor to be explored is if some specific subtypes are more prone to cause infection related inflammation than others. It has been reported that only a subfraction of the individuals infected with the cancer-inducing microorganisms, *Helicobacter pylori* and human papilloma virus in fact develop cancer (111-112). The results of this thesis, contributes to the hypothesis that there exist prostate-specific subtypes of *P. acnes*. According to the identified subtypes, the data here reveals subtype II as a potential colonizer of the human prostate. If verified in future studies, it may open up avenues for vaccination therapies specific against *P. acnes* and specifically subtype II. However, it may be difficult to identify *P. acnes* only on biopsy specimens due to the small amount of tissue collected.

Besides further investigations to study the role of *P. acnes* in infection-induced chronic inflammation and its association to PCa development, focus should also be put on host variability. Mak *et al.* recently concluded that the host protein Vimentin was relevant for *P. acnes* invasion and thereby determine its inflammatory capability (113). The finding of inter-individual responses to invading *P. acnes* further highlights the importance of investigations regarding host differences associated with cancer initiation (114).
In conclusion, the results from this thesis may be of importance in future work trying to individualize the PCa treatment with the aim to improve the quality of life in PCa patients. In clinical practice, patients with chronic inflammation, in particular if associated with PAH may be considered at higher risk for lethal PCa development. Further, this may indicate the need of preventive strategies such as anti-inflammatory drugs. It may also be of value for the individual patient to reduce the levels of Tregs and thereby prevent prostate tumor progression and complement additional treatments.
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