Prostate Cancer and Inflammatory Genes

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Umeå 2005
To my Family
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Abstract

Prostate cancer remains a significant health concern for men throughout the world. Accumulating epidemiologic and molecular evidence suggests that inflammation is an important component in the etiology of prostate cancer. Supporting this hypothesis, population studies have found an increased risk of prostate cancer in men with a prior history of certain sexually transmitted infections or prostatitis. More general evidence of a relationship between inflammation and prostate cancer has been provided by reports indicating that daily use of non-steroidal anti-inflammatory drugs (NSAIDs) may be associated with a lower incidence of prostate cancer. The exact mechanism whereby inflammation might act in tumour development and progression remains to be elucidated, but is likely to be complex. The genetic contribution to inflammatory responses involved in the development of prostate cancer has not yet been extensively or systematically studied. However, this thesis evaluates the role of various inflammation-related genes in the pathogenesis of prostate cancer.

The macrophage scavenger receptor 1 (MSR1) is a transmembrane protein that is mainly expressed by macrophages. This receptor mediates the binding, internalization and processing of a wide range of macromolecules, and is suggested to play a major role in the recognition and clearance of pathogenic and damaged cells. Recent reports have suggested MSR1 to be a candidate gene for hereditary prostate cancer. Therefore, we screened the MSR1 gene among men with hereditary prostate cancer and identified 18 sequence variants. One previously reported truncation mutation was found more frequently in men with prostate cancer than in unaffected men, in accordance with previously published results. However, the difference in frequencies we found between these groups was not statistically significant. In addition, we genotyped five common polymorphisms in MSR1 in 215 men with unselected prostate cancer and 425 controls. No association between any of the five common variants and prostate cancer were found.

We then performed a comprehensive genetic study using extensive population-based case-control material to evaluate possible associations between sequence variants in inflammation-related genes and prostate cancer. The first gene to be examined was interleukin-1 receptor antagonist (IL-1-RN), encoding a cytokine that plays an important role in regulation of the inflammatory response by binding to the IL-1 receptor and thus inhibiting the binding of the pro-inflammatory cytokines IL-1α and IL-1β. Collectively, these three cytokines exert a central role in the protection against diverse lesions, ranging from microbial colonisation to infection and malignant transformation. The genetic analysis of IL-1RN revealed that the most common haplotype was significantly associated with prostate cancer risk for patients with prostate cancer, and further this association appears to be stronger in cases with advanced disease.

The macrophage inhibitory cytokine-1 (MIC-1), a member of the transforming growth-
factor-\( \beta \) superfamily has been shown to exert diverse biological functions, including regulation of macrophage activity in the inflammatory response and both growth inhibition and induction of apoptosis in epithelial and other tumour cell lines. The genetic analysis of MIC-1 revealed that a sequence variant (H6D) appears to be associated with a decreased prostate cancer risk. We also performed measurements of MIC-1 serum levels among patients with prostate cancer and healthy controls. These data indicate that serum MIC-1 levels are associated with an increased risk for prostate cancer. Further, the clear relation between clinical stage and MIC-1 level also suggest that MIC-1 may be useful as a prognostic factor, where high serum concentration is associated with a poor prognosis.

In summary, our results provide further support for the assumption that polymorphisms in inflammatory genes play critical roles in prostate cancer susceptibility. Additional studies are needed to elucidate the mechanisms whereby the demonstrated variations contribute to prostate cancer development.
Populärvetenskaplig sammanfattning

Antalet män som insjuknar i prostatacancer har ökat markant under de två senaste decennierna, vilket lett till att den blivit den vanligast diagnostiserade cancerformen bland män i de flesta industrialiseradeländerna. År 2002 diagnostiserades 679,000 nya fall av prostatacancer världen över, medan 221,000 män avled av samma sjukdom. Orsaken till incidensökningen beror delvis på förbättrade diagnosmetoder, men detta förklarar inte hela stegringen utan andra okända faktorer är också involverade. De senaste decennierna har ett flertal studier visat att inflammationsprocesser i prostatakörteln är en betydande faktor i utvecklingen av prostatacancer. Den exakta mekanismen för hur inflammation medverkar till utveckling och progression av tumörer i prostatakörteln är fortfarande höljd i dunkel men är antagligen en komplex process.

Det mänskliga genomet består av ungefär 30000 olika gener. Alla dessa gener uppvisar olika grad av strukturell variation mellan olika individer. Denna variation kan i sin tur leda till en förändrad funktion hos det protein som en gen kodar för, eller att mängden protein som produceras varierar. Även gener involverade i inflammationsprocesser uppvisar en sådan genetisk variation. Om denna variation har någon betydelse för risken att utveckla prostatacancer har inte tidigare blivit utförligt studerat. I denna avhandling har vi studerat hur genetisk variation i olika inflammationsrelaterade gener påverkar risken att utveckla prostatacancer. Tre gener, vilka allt är involverade i reglering av inflammationsresponsen eller kroppens förvar mot invaderande patogener har blivit utförligt studerade.

I det första arbetet undersöktes om sekvensvariationer i en gen kallad *Macrophage Scavenger Receptor 1* (MSR1), kan påverka risken att insjukna i prostatacancer. MSR1 är ett protein lokalisert i cellens membran och är i huvudsak uttryckt av makrofager. Proteinets funktion som en receptor med möjlighet att binda till sig en mängd olika substanser, som lipopolysackarider (fragment från bakterier), och oxidera lipoproteiner. Efter inbindning tas dessa upp av den aktuella cellen. Funktionen hos MSR1 är inte helt klarlagd men den är bland annat involverad i igenkänning och eliminering av patogener och skadade celler. Tidigare studier i en amerikansk population har visat att mutationer i denna gen skulle kunna ha betydelse för utvecklingen av prostatacancer. Vi screenade MSR1 genen hos män med årfiltg prostatacancer och identifierade ett antal sekvensvariationer. Ett urval av dessa sekvensvariationer undersöktes bland 215 män med oselekterad prostatacancer och 425 kontroller, men ingen av dem medförde en förändrad risk att insjukna i prostatacancer. Våra resultat stödjer ej tidigare publicerade resultat där sekvensvariationer i MSR1-genen skulle ha betydelse för uppkomsten av prostatacancer.

I nästföljande studier använde vi oss av ett stort populationsbaserat fall/kontrollmaterial med 780 prostatacancerfall och 1383 friska kontroller för att studera om genetisk variation hos inflammationsrelaterade gener medför en ökad eller minskad risk att insjukna i prostatacancer.
Den första genen kallad *Interleukin-1 receptor antagonist* (IL1-RN), är ett lösligt protein, en cytokin, som reglerar kroppens inflammationsresponser genom att binda till en Il-1 receptor och därigenom förhindra inbindning av två andra cytokiner, Il-1α och Il-1β, som normalt driver på inflammationsresponser. Tillsammans spelar dessa tre cytokiner en central roll i kroppens skydd mot många olika skador, från invasion av mikrober till malign transformation. Balansen mellan pådrivande och inhiberande cytokiner bestämmer utvecklingen av en inflammationsresponser. Den genetiska analysen av IL-1RN visade att bärare av den vanligast förekommande sekvens-kombinationen (haplotypen) av IL1-RN-genen medför en ökad risk att insjukna i prostatacancer.

*Macrophage inhibitory cytokine-1* (MIC-1) är en medlem i en stor familj av proteiner kallade transforming growth factor β superfamily, som har visat sig medverka i en mängd olika viktiga biologiska funktioner, inkluderat reglering av inflammations-responser, tillväxthämmning, men också induktion av apoptosmekanismer i olika tumörcelliner. Den genetiska analysen av MIC-1 visade att en genetisk variant i genen (H6D), medför en minskad risk att insjukna i prostatacancer. Vidare mättes nivåerna av MIC-1-proteinet i serum hos patienter med prostatacancer och hos friska män för att se om MIC-1 nivåerna var förändrade hos patienter med prostatacancer. Analyserna visade att serumnivåerna var tydligt förhöjda hos patienter med prostatacancer. Nivåerna var också positivt korrelerade med ökande ålder och tumörstadie. Resultaten indikerar att MIC-1 skulle kunna vara en användbar prognostisk faktor, där höga serumnivåer är associerade med en dålig prognos.

Således visar våra resultat att genetisk variation i inflammationsrelaterade gener har betydelse för uppkomst av prostatacancer. För att förstå med vilka mekanismer denna variation kan påverka utvecklingen av cancer är vidare studier nödvändiga. Vi fann också att mätning av MIC-1-nivåer i serum skulle kunna vara av kliniskt värde som prognostisk markör vid prostatacancer.
Original studies

This thesis is based on the following studies.


IV. Lindmark F, Wiklund F, Hunter M, Xu J, Bälter K, Adami HO, Breit S, Grönberg H. Serum levels of macrophage inhibitory cytokine-1 in prostate cancer cases and controls. Manuscript

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List of 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>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>Cancer prostate in Sweden</td>
</tr>
<tr>
<td>CHEK2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>CEPH</td>
<td>Centre d’Etude du Polymorphisme Humain</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>fPSA</td>
<td>free PSA</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Glutathione-S-transferase P1</td>
</tr>
<tr>
<td>htSNP</td>
<td>Haplotype tagging SNP</td>
</tr>
<tr>
<td>HPC</td>
<td>Hereditary prostate cancer</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy Weinberg Equilibrium</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin-1 alpha</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL1-RN</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysacharide</td>
</tr>
<tr>
<td>MIC-1</td>
<td>Macrophage inhibitory cytokine-1</td>
</tr>
<tr>
<td>MSR1</td>
<td>Macrophage scavenger receptor 1</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygotic</td>
</tr>
<tr>
<td>NSHDC</td>
<td>Northern Sweden health and disease cohort</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<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>Prostate specific antigen</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operator characteristic</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNASEL</td>
<td>Ribonuclease L gene</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPC</td>
<td>Sporadic prostate cancer</td>
</tr>
<tr>
<td>SR-A</td>
<td>Class A scavenger receptor</td>
</tr>
<tr>
<td>SRD5A2</td>
<td>5α-Reductase type 2</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmitted infection</td>
</tr>
<tr>
<td>TEAA</td>
<td>Trietylammonium acetate</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TMHA</td>
<td>Temperature modulated heteroduplex analysis</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour-node-metastasis</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>tPSA</td>
<td>total PSA</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
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Introduction

In most developed countries, prostate cancer is not only the most frequently diagnosed malignancy in men, but also the second leading cause of cancer-related deaths in males. In 2002, an estimated 679,000 prostate cancer diagnoses were made worldwide, accompanied by an estimated 221,000 prostate cancer deaths (Parkin et al., 2005). In Sweden, over 9000 new diagnoses of prostate cancer were recorded in the year 2003, accounting for 35.3 % of cancer diagnoses among men that year (fig.1). Further, 2350 men died from this disease 2003. Although the incidence of prostate cancer is increasing steadily in almost all countries, the aetiology and molecular mechanisms underlying the development and progression of the disease is still large unknown.

Prostate cancer is often regarded as a disease of older men, but nearly a third of men aged between 30 and 40 years have been found to harbour small foci of prostate cancer (Sakr et al., 1994). The majority of these lesions are diagnosed at a much later age or do not progress to clinically detectable tumours within the lifetime of the affected men. Cancer lesions can develop in two different regions of the prostate gland, most commonly (in ~80% of cases) in the periphery zone, while most of the remaining lesions are found in the transition zone, which is located in the perirectal region (McNeal, 1968, 1988). Prostate cancer is commonly multifocal, i.e. there is generally more than one tumour with different origins in the prostate at the time of diagnosis (McNeal, 1988). Each of these tumours can show remarkable differences in gene expression and behaviour, which are associated with varying prognoses. In its clinical form, prostate cancers are diagnosed upon histological evaluation of needle biopsy samples of prostate tissue, and classified as either local or advanced. In localized disease, the treatments range from radical prostatectomy, radiotherapy and application of anti-androgens to “watchful waiting” (Afrin et al., 2000; Drachenberg, 2000). If the cancer has spread outside the prostate, with lymph nodes and bone being the most common sites for metastasis, the most common treatment is surgical or chemical castration, to lower androgen levels, causing reductions in tumour growth and symptomatic relief, but not a cure. However, after one to two years most tumours will relapse to an androgen-independent, but still androgen receptor-dependent, state that normally kills the patient (Afrin et al., 2000; Drachenberg, 2000; Feldman et al., 2001).
Risk factors for prostate cancer

The etiological factors associated with prostate cancer are varied and particularly poorly understood compared to other common cancers.

The observation that prostate cancer risk increases for Japanese immigrants to Hawaii and Japanese immigrants to Los Angeles, suggests that diet and environmental differences play important roles (Shimizu et al., 1991; Minami et al., 1993). However, the increase is only to about 50% of the rate for Caucasian people and to 25% of that for African-American people in the USA, suggesting that differences between ethnic populations are substantial and that differences in incidence rate cannot be explained solely by differences in the environment and lifestyle (Grönberg, 2003).

Dietary factors

Dietary habits are probably an important factor that contributes to the geographic variations in prostate cancer rates. A large number of epidemiological studies have investigated the association between dietary factors and prostate cancer, but the findings have been mixed.

**Dietary fat.** There is considerable consistency across studies indicating that a high intake of fat, especially total fat and saturated fat, is a risk factor for prostate cancer (Kolonel, 2001). The mechanisms mediating the effects of fat on prostate carcinogenesis are not known, but it is hypothesized that the effect of dietary factors, such as fat, may be mediated through endogenous hormones (Bosland, 2000).

**Phytoestrogens.** Soy products contain isoflavonoids that have been shown to have a prophylactic effect on prostate cancer (Holzbeierlein et al., 2005). People in Asian countries eat more soy products than people in Western countries and higher levels of phytoestrogen metabolites have been
observed in Asian men compared to European men. This may provide a partial explanation for the low incidence of prostate cancer in Asia (Adlercreutz et al., 1993). Mice studies with human androgen-sensitive prostate cancer cells have shown that a diet based on rye bread and soy protein reduces tumour size and secreted PSA levels, while increasing apoptosis, and may thus inhibit tumour growth (Landström et al., 1998; Bylund et al., 2000). A subsequent pilot study of men with prostate cancer indicated that a high intake of rye bread increases apoptosis in prostate tumour cells (Bylund et al., 2003).

**Vegetables and fruits.** While chronic consumption of animal fat and red meats may promote prostate cancer, intake of vegetables and fruit may protect against prostate cancer development (Chan et al., 2001). A large prospective study of 130,544 men found no association between the total consumption of fruits and vegetables and risks for prostate cancer among 1104 prostate cancer cases. However, it still remains possible that there may be an association with specific types of fruits and vegetables (Key et al., 2004).

**Vitamins and trace elements.** A variety of vitamins, trace elements have been elucidated for their role in prostate cancer pathogenesis, but the results of the epidemiology studies are inconsistent (Chan et al., 2001). A promising substance is selenium; an essential, non-metallic trace element that has been demonstrated to promote apoptosis in prostate cancer cells and, possibly, impairs their proliferation through antiangiogenic activity. Selenium intake has been significantly associated with a decreased risk for prostate cancer, for a review see Combs GF, 2004 (Combs, 2004).

**Hormonal and other physiological factors**

The growth and development of the prostate is dependent on androgens. Ablation of androgens by surgical or medical castration is an effective strategy in the treatment of advanced prostate cancer. Moreover, men with congenital abnormalities in androgen metabolism and those who undergo castration before puberty do not develop prostate cancer (Haas et al., 1997).

Several epidemiologic studies have evaluated the possible association between plasma testosterone concentrations and prostate cancer risk, but the results have been inconsistent (Hsing, 2001). A recent well designed study published in 2004 reported that high levels of circulating testosterone are not associated with increased prostate cancer risk (Stattin et al., 2004). However, if testosterone is important for the onset of prostate cancer, then it may need to be measured in early adulthood, years before the prostate cancer is actually detected (Crawford, 2003).

Other factors such as smoking, weight, alcohol consumption, physical activity, socioeconomic status, and vasectomy have also been evaluated for their role in prostate pathogenesis, but the results have generally indicated that they are not significant (Grönberg, 2003).

**Familial and genetic factors**

Over the past two decades multiple pieces of evidence have been accumulated supporting the view that genetics plays a role in prostate cancer susceptibility and pathogenesis. However, the evidence also indicates that
the genetic basis of prostate cancer is much more complex than was initially anticipated. Large families have been observed in which prostate cancer cluster together. Early observations were made in large families collected and studied in Utah, but the first description of familial aggregation of prostate cancer was reported as far back as 1956, (MORGANTIG et al., 1956). This initiated a series of case-control studies, cohort studies, and twin studies that generated evidence of a familial component.

**Case-control and cohort studies**

Several published reports have demonstrated an increased risk for prostate cancer among men with a positive family history for prostate cancer. In 2003, Bruner et al. published a meta-analysis of 16 case-control studies and eight cohort studies supporting this working theory, in which family history was defined as cases where a father, brother, any first- or second-degree relative or other relative had been affected by prostate cancer (Bruner et al., 2003). Pooled Risk ratios (RRs) for men with any affected family member, one affected first-degree relative and one affected second-degree relative were 1.9 (95% CI, 1.6-2.2), 2.2 (95% CI, 2.1-2.4) and 1.9 (95% CI, 1.6-2.3), respectively. Further increases in RR have consistently been observed (i) when more than one relative is affected, (ii) as the closeness of the affected relative to the unaffected individual increases, and (iii) as the age of onset of the affected cases decreases. Taken together, this is all strong evidence for the involvement of a genetic component in familial disease.

**Twin studies**

The most straightforward way to estimate the impact of genetic factors on the development of prostate cancer is to analyze its incidence in mono- and di-zygotic twins. This is because monozygotic twins (MZ) derive from the fission of a single fertilized egg, thus they inherit identical genetic material and the only genetic differences between them originate from somatic changes. By contrast, dizygotic (DZ) twins derive from two distinct fertilized eggs and thus have the same genetic relationship as full siblings, although they may be more “biologically” related because they share the same prenatal intra-uterine experience. Since the environmental factors affecting pairs of MZ and DZ twins are generally very similar, since they are usually raised in the same environment, it is assumed that any differences in concordance rates between them solely reflect genetic factors. Two large twin studies from America and Scandinavia have been published and the results they present show surprisingly consistency (Page et al., 1997; Lichtenstein et al., 2000). Both studies found that an MZ twin whose brother has been reported as having had prostate cancer is nearly four times more likely to have prostate cancer than a DZ twin whose brother has reportedly had prostate cancer. Furthermore, Page et al. and Lichtenstein et al. estimated that the proportion of the risk of prostate cancer that can be explained by heritable factors was estimated to 57% and 42%, respectively.

**Family-based segregation analyses**

Further support for a genetic component in prostate cancer comes from family-based segregation analyses, which have been used to derive models for the prevalence (rare,
common), mode of inheritance (dominant, recessive, autosomal, or sex chromosome linked), and penetrance (the likelihood of carriers developing the disease by a certain age) of genes that may be involved in familial prostate cancer. In 2004, Daniel Schaid reviewed the eight segregation analyses that had published thus far (Schaid, 2004). In summary, the segregation analysis of prostate cancer give strongest support for autosomal dominant inheritance of susceptibility genes, but there are also evidence for autosomal recessive or X-linked mode of inheritance, a model with 2 or 3 genes contributing to the disease and a multifactorial mode of inheritance. Nevertheless, it has become obvious that prostate cancer is most likely caused by a number of genes – each with different modes of inheritance, population frequencies, and penetrance.

Prostate cancer susceptibility genes

Although more than 40% of prostate cancer cases are estimated to be an effect of genetic variation (Lichtenstein et al., 2000), rare, highly penetrant genes probably account a minority of prostate cancer cases. Mutations in high-penetrance susceptibility genes increase the risk of cancer several fold, and tumours with such mutations are often called hereditary cancers. So far three high-risk candidate genes have been identified; HPC/ELAC2 on 17p, RNASEL on 1q25, and MSR1 on 8p22-23 (see descriptions below). Attempts to confirm these findings in different populations have been problematic, and the importance of these genes in prostate cancer pathogenesis is still obscure (Schaid, 2004). Nevertheless, these three genes clearly do not account for the majority of hereditary prostate cancer cases. Carriers of mutations of another gene, BRC-A2, are known to be at high risk of breast and ovarian cancer, but BCR-A2 has also been implicated in prostate cancer predisposition. A large collaborative study from the Breast Cancer Linkage Consortium estimated, based on a survey of 173 families harbouring BRC-A2 mutations, a relative risk of 4.65 (95% CI, 3.48-6.22) for prostate cancer in male BRC-A2 gene carriers (The Breast Cancer Linkage Consortium, 1999). However, since BRC-A2 mutations are rare in families with hereditary prostate cancer, they only account for a very small fraction of hereditary prostate cancers.

The discovery of highly penetrant prostate cancer genes has coupled problems for at least two major reasons. First, due to the late onset of prostate cancer, it is difficult to identify representatives of more than two generations of a family that could be included in molecular studies. Increases in the number of generations that can be examined increases the possibility of finding relevant and true susceptibility loci that affect prostate cancer risks. Another significant problem is the lack of clear distinguishing features between hereditary and sporadic forms of the disease, making it difficult to distinguish, within high-risk pedigrees, men who have developed cancers that are sporadic rather than due to an inherited germline mutation (Rubin et al., 2004).

Polymorphisms associated with prostate cancer

The remainder of the genetic influence is most likely mediated by more common genetic variants or polymorphisms. Low-penetrance polymorphisms increase the risk of cancer only modestly, but the prevalence of such polymorphisms may be higher than
the prevalence of mutations in high-penetrance susceptibility genes, and thus their overall impact or attributable risk can be substantial. The list of these variants is long, but the major pathways currently under investigation include those involved in androgen action, DNA repair, carcinogen metabolism, and inflammation pathways. Numerous polymorphisms have already been suggested to be associated with the risk of prostate cancer.

**Androgen receptor (AR).** The most widely studied polymorphic gene may be the androgen receptor (AR) gene. The AR gene, located on chromosome Xp11-12, encodes a ligand-activated transcription factor that mediates the androgenic response, and has been shown to play an important role in prostate development and function. Exon 1 of this gene contains two polymorphic microsatellites: a highly polymorphic CAG repeat and a less polymorphic GGC repeat. Since the length of the polymorphic CAG trinucleotide repeat has been shown to be inversely correlated with the transcriptional activity of the AR gene, it has been hypothesized that variation in the length of the repeat are also associated with variations in prostate cancer risks. Several genetic epidemiological studies have shown a correlation between an increased risk of prostate cancer and the presence of short androgen-receptor CAG repeats, but other studies have reported inconsistent results (Simard et al., 2003). At present, no study has detected any correlation between GGC repeats and functional activity of the AR gene. Furthermore analyses of the possible association between GGC repeats and prostate cancer have been inconclusive (Gsur et al., 2004).

**SRD5A2.** The 5α-reductase type II gene (SRD5A2) is a membrane protein of the endoplasmatic reticulum that catalyzes the conversion of testosterone into the more active form androgen dihydrotestosterone and maps to 2p22-23. SRD5A2 is primarily expressed in androgen-sensitive cells of genital tissues and the prostate gland. Certain SRD5A2 polymorphisms may contribute to different enzyme activities of 5α-reductase variants. A dinucleotide (TA) repeat polymorphism in the untranslated region of SRD5A2 has been identified. Although no clinical significance for these polymorphisms has yet been determined, some TA repeat alleles may promote an elevation of enzyme activity, which may in turn increase the level of DHT. Thus, these alleles may increase the risk of prostate cancer. (Coughlin et al., 2002)

In addition, two nonsynonymous polymorphisms, A49T and V89L, have been identified. These two polymorphisms may also alter the DHT levels and consequently influence prostate cancer risks. However, the evidence for prostate cancer risk associated with these three SRD5A2 variants has been conflicting (Ntais et al., 2003).

Other genes with sequence variants that have suggested association with the risk of prostate cancer include CHEK2 and the vitamin D receptor (VDR) and 17α-hydroxylase (CYP17) (Hughes et al., 2005). However, a typical feature of many of these genes is that highly conflicting findings regarding their putative involvement in prostate cancer have been published (personal communication, Sara Lindström). Thus, at the moment, none of the sequence variants can be considered to be definitely associated with prostate cancer.

In conclusion, a large number of studies have evaluated the risk of dietary, environmental and genetic risk factors, with mixed results. Since all these risk factors are likely to cluster within families, discriminating genetic from non-genetic
risks is difficult, especially since the magnitude of the risks is similar for both genetic and non-genetic risk factors (Schaid, 2004).

Inflammation and cancer

Various carcinomas (including cancers of the liver, bladder, colon, stomach, and oesophagus) have been shown to arise from areas of infection and inflammation. Over 15% of all malignancies worldwide are attributable to infectious agents, and inflammation is a major component of these chronic infections (Kuper et al., 2000). Colon cancers arising in individuals with inflammatory bowel disease (e.g. chronic ulcerative colitis or Crohn’s disease) and stomach cancers caused by chronic Helicobacter pylori infection are among the most intensively studied and well established types of cancer associated with inflammation of different origins (Coussens et al., 2002).

Inflammation involves the induction of complex, coordinated chemical signals and associated physiological processes following injury that promote “healing” of damaged tissues. Early responses include increases in vascular permeability and activation, together with the directed migration of leukocytes (neutrophils, monocytes and eosinophils) towards the site of injury, where the ground-work is being laid for the formation of a new extracellular matrix. The directional migration is mediated by secreted chemokines that form a concentration gradient towards the site of inflammation. The extracellular matrix provides the structure upon which cells (fibroblasts and endothelial cells) can migrate and proliferate, regenerating new tissue and a vascular network. In later stage of the inflammatory response, the macrophages are the dominant cell type, orchestrating and directing the healing process. Normally, inflammation is a self-limiting process due to the production of anti-inflammatory cytokines that buffer the effect of pro-inflammatory cytokines. The cytokine/chemokine pattern persisting at the inflammatory site is important in the development of chronic disease. Dysregulation of any of the cooperating factors can lead to prolonged inflammation with chronic exposure to cytotoxic mediators. (Coussens et al., 2002). Chronic inflammation can be caused by a variety of factors, including bacterial, viral, and parasitic infections, chemical irritants, and non-digestible particles, but often the underlying cause is unknown. The longer the inflammation persists, the higher the risk of associated carcinogenesis (Shacter et al., 2002).

At the site of inflammation, caused by either wounding or infection, phagocytic cells (e.g. neutrophils and macrophages) generate reactive oxygen and nitrogen substances, but these cells also synthesize and secrete large quantities of growth factors and a number of potent angiogenic factors, cytokines, and proteases, all of which are important mediators in the tissue regeneration, but can also potentiate neoplastic tumorigenesis. The focus of interest has been the individual function of the different mediators, but in the inflammation response there is significant interaction and synergy among them – for example, prostaglandins induce the
expression of certain inflammatory cytokines which can in turn, induce the production of reactive oxygen and nitrogen species. The following description summarises the role of three types of inflammatory mediators in inflammation and tumour development.

**Reactive oxygen and nitrogen species.** At the site of inflammation activated phagocytes produce large amounts of reactive oxygen (ROS) and nitrogen species (RNS). These substances are normally produced in response to infection, killing invading agents. Nevertheless, they can also cause tissue damage and thereby act as initiators of tumorigenesis. ROS and RNS have been shown to alter protein structure and function, cause lipid peroxidation, and damage DNA, thereby contributing to the carcinogenic process. ROS/RNS-induced damage to DNA may result in mutagenesis or altered expression of transcriptional factors involved in carcinogenesis. Damage may also occur to vital DNA repair enzymes, altering their activity, which may increase mutagenesis (Wiseman et al., 1996). These processes can cumulatively lead to an imbalance between the rate of mutagenesis and the ability to repair mutations, resulting in the accumulation of multiple mutations and, thus, increases the risk for carcinogenesis.

**Prostaglandins.** In addition to ROS and RNS, prostaglandins are synthesized in large amounts by inflammatory cells in response to both acute and chronic inflammatory stimuli. Prostaglandins are lipid mediators of the inflammatory immune response that have a wide range of physiological functions, such as vasodilatation/constriction and inhibition/enhancement of inflammatory cell functions. Studies on humans and animals have generated evidence that suggesting that prostaglandins contribute to the development of cancer (Shacter et al., 2002). Two different cyclooxygenase (COX) enzymes catalyse the rate-limiting first step in prostaglandin synthesis. COX-1 is constitutively expressed in most cell types, while COX-2 is primarily expressed by monocytes and macrophages during inflammation. Long-term intake of non-steroidal anti-inflammatory drugs (NSAIDs; drugs that inhibit the activity of both COX-1 and COX-2) have been shown to reduce the incidence and progression of colon cancer (Baron et al., 2000), and similar effects has been indicated for prostate cancer (Basler et al., 2004). However, believing that the tumour-repressing effect is mediated solely through the inhibition of cyclooxygenase activity is an over simplification (Zha et al., 2004). Finally, other metabolites derived from arachidonic acid, such as leukotrienes and tromboxanes, may also be important mediators in the development of cancer, but the knowledgde of these aspects is still very limited.

**Cytokines.** Cytokines are generally small molecules that alter the behaviour of the cells that secrete them and/or other cells, generally within the heamatopoietic system. They act in a in a complex coordinated network in which they initiate or suppress their own synthesis as well as that of other cytokines and cytokine receptors. The complexity arises from the fact that there is often substantial overlap and redundancy between the functions of individual cytokines (Howell et al., 2002). In response to both antigen-specific and non-specific stimuli, inflammatory cells secrete a wide array of cytokines, which play critical roles in the generation of both pro- and anti-inflammatory immune responses. For example, cytokines such as TNFα and IL-1 are usually classed as key pro-inflammatory cytokines, while cytokines such as IL-10 are anti-inflammatory. The balance between the
effects of pro-inflammatory and anti-inflammatory cytokines determines the outcome of the inflammatory process. Cytokines can contribute to cancer development in several ways. TNFα has been shown to enhance the formation of ROS by inflammatory cells, and thereby increase the risk for DNA damage and inhibition of DNA repair in tumour cells. Other mechanisms include direct stimulation of cell growth, induction of angiogenesis, and recruitment of inflammatory neutrophils (Shacter et al., 2002). In addition, an imbalance of pro- and anti-inflammatory cytokines, caused by point mutations or polymorphisms, may prevent the normal self-limiting nature of the immune response, leading to prolonged inflammation with chronic exposure to cytotoxic mediators.

In conclusion, multiple pieces of evidence have demonstrated that inflammatory cells have powerful effects on tumour development, but recruitment of inflammatory cells may also be inhibitory to tumour development, and may represent an attempt by the host to suppress tumour growth. This process requires further evaluation. One approach is to elucidate whether functional polymorphisms in genes that regulate inflammatory processes, such as cytokines, confer altered risks for developing cancer or are potential prognostic factors. See (Shacter et al., 2002) and (Coussens et al., 2002) for a comprehensive review.

The role of inflammation in the pathogenesis of prostate cancer

Although it has been established that chronic inflammation plays a causative role in the development of many human cancers, the contribution of inflammatory processes to the development of prostate cancer has not been extensively studied. However, data from epidemiological, genetic epidemiological and molecular pathology studies have been accumulated recently suggesting that inflammatory processes are also involved in the development of prostate cancer. The chronic inflammatory microenvironment, characterized by the accumulation of macrophages and lymphocytes, is extremely common in the tissue stroma and epithelium of the prostate and may support both the initiation and progression of prostate cancer (Lucia et al., 2004). The following description highlights connections between inflammatory processes and the development of prostate cancer.

Molecular pathology

Focal areas of epithelial atrophy in the prostate were first observed by pathologists more than fifty years ago (FRANKSLM, 1954; Rich, 1979). Atrophy of the prostate is identified as a reduction in the volume of the glands and can be divided into two major patterns: diffuse and focal. Diffuse atrophy is a reduction that involves the entire prostate and is caused by a decreased level of circulating androgens. Focal atrophy, on the other hand, occurs primarily, but not exclusively, in the outer part of the gland, where prostate cancer normally arises.
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(McNeal, 1988; Feneley et al., 1996; Ruska et al., 1998; De Marzo et al., 1999). Focal atrophy is not related to reduced levels of circulating androgens, compared with normal prostatic epithelium, and the condition is highly proliferative without any increase in apoptosis. Focal atrophy is often associated with acute or chronic inflammation, reflected by infiltration of inflammatory cells (lymphocytes, macrophages and polymorphonuclear cells) (Ruska et al., 1998; De Marzo et al., 1999). Due to the combined findings of high proliferation indices and inflammatory infiltrations seen in these lesions, the term proliferative inflammatory atrophy, or PIA, was introduced (De Marzo et al., 1999). The underlying causes of PIA lesions are still not clearly understood, but they may arise either as a consequence of epithelial damage (from infection, ischemia, or toxin exposure) or as a direct consequence of inflammatory oxidant damage to the epithelium (Platz et al., 2004).

Accumulating evidence from morphological, immunohistochemical and genetic studies have provided support for the concept that PIA lesions may be precursors to prostate cancer, partly because the vast majority of such lesions are found adjacent to or near early adenocarcinomas or high grade prostatic intraepithelial neoplasms, or both (Feneley et al., 1996; De Marzo et al., 1999; Putzi et al., 2000). Further support for the hypothesis comes from the fact that PIA shares several molecular alterations found in both Prostate Intraepithelial Neoplasia (PIN) and prostate cancer. Nakayama et al. reported that approximately 6% of PIA lesions show evidence of somatic hypermethylation of the GSTP1 gene promoter (Nakayama et al., 2003). Corresponding levels of GSTP1 hypermethylation in PIN lesions and prostate cancer tissue are approximately 70% and 90%, respectively (Jerónimo et al., 2001; Lin et al., 2001).

In addition, chromosome 8 abnormalities, which are common in prostate cancer, have been found in areas of PIA lesions. Acquired somatic chromosome 8 abnormalities have been noted in about 1% of normal prostate tissue, 4% of PIA lesions, and about 6% of adenocarcinomas (Shah et al., 2001). Other studies have reported p53 mutations in 5% of PIA lesions, a rate similar to that seen in high-grade PIN, compared to 20% of prostate cancer cases (Tsujimoto et al., 2002). Thus, these are genetic changes that are associated with PIN and prostate cancer.

De Marzo et al. examined expression patterns of three molecular markers implicated in prostatic carcinogenesis: p27, Bcl-1 and GSTP1 (De Marzo et al., 1999). p27 is a cyclin-dependent kinase inhibitor whose expression suppresses the cell cycle and is usually reduced in prostatic adenocarcinomas and high-grade PIN. The cited authors reported a down-regulation of p27 in PIA. They also observed an increase in Bcl-2 expression, as previously reported by Ruska et al. (Ruska et al., 1998), which is consistent with the very low levels of apoptosis. Moreover, they found increased levels of GSTP1 in many lesions suggesting, together with the elevated levels of GSTA1 and COX-2 reported in other studies (Parsons et al., 2001; Zha et al., 2004), that a stress-induced response has occurred in these cells. Accumulating evidence also indicates that many of the atrophic luminal cells in PIA represent a form of intermediate epithelial cell with high proliferative activity. These cells share features of both basal and luminal secretory cells, and hence are postulated to be targets of neoplastic
transformation in the prostate (van Leenders et al., 2003).

Even though PIA lesions may be an early histological precursor to prostate cancer, it is important to note that some studies have not found an association between PIA and prostate cancer (De Marzo et al., 2003) and, moreover, not all cancers occur within or in the vicinity of PIA. In fact, PIA may simply indicate an intraprostatic environment that favours prostate cancer development. Nevertheless, further studies are needed to elucidate the relationship between focal atrophy and cancer in the prostate.

Chronic inflammation, with a predomination of lymphocytes and macrophages, is extremely common in the prostate. However, examination of prostatectomy specimens seldom shows inflammatory infiltrates in the malignant tissue, whereas directly adjacent benign tissue can be heavily infiltrated (Lucia et al., 2004). To date, the relationship between lymphocytic infiltration in the tumour and progression of tumour development in the prostate is uncertain, but the results from two studies evaluating the connection between inflammatory infiltration and survival indicate that lymphoid aggregates within the tumour are associated with a poor outcome in patients with prostate cancer (Irani et al., 1999; McArdle et al., 2004). Furthermore, increased macrophage activity in the tumour has also been found to be related to poor prognosis in prostate cancer (Lissbrant et al., 2000). Why the presence of increased lymphocytic aggregates would be associated with poor cancer specific survival rates remains unexplained.

Epidemiology

Prostatitis and prostate cancer. Prostatitis is a common urological disorder worldwide; 2-10% of men experience it during their lifetime (Habermacher et al., 2005). The clinical presentation is characterised by uncomfortable symptoms, such as dysuria, and rectal and suprapubic pain (Roberts et al., 1998). Prostatitis is classified into four categories: I, acute bacterial; II, chronic bacterial; III, chronic nonbacterial/chronic pelvic pain syndrome; and IV, asymptomatic prostatitis. Both acute bacterial prostatitis (which is usually caused by Escherichia coli) and chronic bacterial prostatitis (caused by E. coli and several other infectious agents) are normally treated with antibiotics. Chronic non-bacterial prostatitis/chronic pelvic pain syndrome, is the most common form, affecting 90-95% of patients with prostatitis. Men with this type of prostatitis may experience symptoms for weeks to years, and current therapy has limited success in alleviating the pain that the patients experience. Asymptomatic prostatitis is a histological diagnosis of prostate inflammation that is found subsequent to pathological examination of the prostate tissue. This category is found in a significant number of patients; epidemiologic studies have estimated the prevalence to be as high as 32.2% in a population of men with elevated PSA (van Leenders et al., 2003).

Several case-control studies have investigated the association between prostate cancer and prostatitis, with variable results. In 2002, Dennis et al. performed a meta-analysis of 11 case-control studies that evaluate the possible relationship between prostatitis and prostate cancer. The meta-analysis found an increased risk of prostate cancer among men with a history of clinical, or symptomatic prostatitis (OR=1.6; 95% CI, 1.0-2.4), particularly in population-based case-control studies (OR=1.8; 95% CI, 1.1-3.0) (Dennis et al., 2002b). However, the
complexities involved in diagnosing prostatitis and the risks for potential recall or detection bias when performing association studies between prostatitis and prostate cancer make the analyses of the results problematic. The results could reflect an effect of detection bias, since patients with prostatitis may be more likely to be monitored by a urologist and thus more likely to be screened for prostate cancer, or the results could reflect a recall bias, since patients with prostate cancer may try to remember more about potential exposure than controls do (Dennis et al., 2002b).

Epidemiological studies investigating the possible association between prostatitis and prostate cancer have relied on patient reports for information on prostatitis history. Therefore, there are serious problems in using studies of this type to identify valid relationships between category IV prostatitis and prostate cancer. The reason why inflammation leads to symptoms in some men but not others are not known. It is possible that a distinguishing feature between symptomatic and asymptomatic prostatitis may be the anatomical localisation of the prostatic inflammation. Inflammation in the peri-urethral region, or transition zone, may be more likely to manifest in urinary symptoms and pain than inflammation in the outer areas of the gland, or the peripheral zone, where prostate cancer more commonly develops (Palapattu et al., 2004).

In addition, according to two epidemiological studies, the prevalence of prostatitis correlates with the prevalence of prostate cancer worldwide, with rates of prostatitis and prostate cancer being much higher in North America than Asia (Roberts et al., 1998; Tan et al., 2002).

More general evidence of a relationship between inflammation and prostate cancer was provided by reports of an association between the use of anti-inflammatory drugs (NSAIDs) and prostate cancer risk. However, the results have been inconsistent (Basler et al., 2004). The most interesting data showing an inverse association with NSAID use come from a large (n=1362) population-based longitudinal study in Olmsted County, MN, USA, the results of which suggest that daily use of NSAIDs may be associated with a lower incidence of prostate cancer among ≥60-year-old men (RR 0.45; 95% CI, 0.28-0.73) (Roberts et al., 2002). Furthermore, the inverse association with NSAID use increased with increasing age.

Sexually transmitted infections and prostate cancer. Sexually transmitted infections (STIs) have long been hypothesized to increase the risk for prostate cancer. STIs are theorized to increase the risk of prostate cancer by causing inflammation of the prostate, which may in turn lead to the initiation of carcinogenesis (Nelson et al., 2003). Several studies have investigated the potential association between prostate cancer and STIs, using various approaches, including self-reports, serology, and detection of infectious agents in the prostate.

Self-report: In a meta-analysis (Dennis et al., 2002a) reviewed 36 independent analytical studies, published between 1971 and 2000, that measured some aspect of sexual activity in relation to prostate cancer. The data suggest there was an elevated relative risk of prostate cancer among men who reported a history of any STI (RR=1.44; 95% CI, 1.24-1.66). The risk associated with syphilis alone was higher (RR=2.30; 95% CI, 1.34-3.94), while the risk associated with gonorrhoea was not as strong as that for the presence of all STIs (RR=1.36; 95% CI, 1.15-1.61). The association between prostate cancer and
STIs was further supported by RR increasing with increasing frequency of sexual activity (RR=1.20; 95% CI, 1.11-1.30), for men reporting more than 30 sexual partners (RR=1.27; 95% CI, 1.08-1.49), and for men who visited prostitutes (RR=1.19; 95% CI, 1.01-1.41).

Recently, Taylor et al. performed a new meta-analysis, including 29 case-control studies, which corroborated the previous findings (Taylor et al., 2005). The study demonstrated a significantly elevated OR for prostate cancer for all STIs (OR=1.48, 95% CI 1.26-1.73), and for gonorrhea (OR=1.35; 95% CI, 1.05-1.83). However, the results should be interpreted with caution, since most of these studies were retrospective case-control studies, and thus may have been affected by bias in recall between cases and controls. Also, a history of STIs may not be causally associated with prostate cancer, but may be simply a surrogate for other lifestyle risk factors for prostate cancer (Platz et al., 2004). Furthermore, since these two meta-analyses, a number of studies have been published reporting inconsistent association between STIs and prostate cancer risk (Giles et al., 2003; Fernández et al., 2005; Patel et al., 2005).

Serological markers: Several epidemiological studies have also reported associations between prostate cancer and circulating serum IgG antibodies against agents infecting the genito-urinary tract. One study reported an increased risk for prostate cancer among men who showed serological evidence of syphilis (OR=1.8; 95% CI, 1.0-3.5) (Hayes et al., 2000), while studies investigating possible serological links between Chlamydia trachomatis and prostate cancer reported null results (Dillner et al., 1998). Five studies investigating possible associations between human papilloma virus (HPV) and prostate cancer have found variable results, ranging from no association to statistically significantly higher risks for HPV-seropositive men (reviewed in (Palapattu et al., 2004)).

The meta-analysis by Taylor et al. also examined the associations between HPV infection and prostate cancer risk detected in ten studies using either serological or PCR-based evidence of HPV infections. The results showed an OR of 1.39 (95% CI, 1.13-1.71). Notably, while HPV is known to be an oncogenic virus, its influence on prostate carcinogenesis may be independent of inflammation.

Detection of infectious agents in prostate tissue: Several studies have investigated the presence of infectious agents in the prostate tissue. Various viruses and bacteria have been found, but the results have been inconsistent, possibly due to methodological differences (such as differences in the tissue handling and detection methods), or possible contamination from agents in areas close to the prostate, e.g., the urethra. It is also worth noting that STIs detected in the prostate cancer tissues could have been acquired after the initiation of carcinogenesis. Furthermore, the failure to detect infectious agents in the prostate tissue does not necessarily mean that such agents play no role in prostate carcinogenesis, as some may be cleared after causing damage (Platz et al., 2004). Human papilloma viruses, human herpes virus-8, Herpes simplex virus-2, cytomegalovirus and Epstein-Barr virus are some of the agents that have been found, see the review by Palapattu et al. for further information (Palapattu et al., 2004).

In 2005, Cohen and co-workers performed an exploratory study to detect the presence of bacterial agents in prostate tissue from patients with prostate cancer (Cohen et al., 2005). They reported a predominant presence of the gram-positive
bacterium *Propionibacterium acnes*, which moreover showed a positive association with prostatic inflammation. *P. acnes* is a slow-growing microbe that is known to be a potent stimulant of the immune system, and it is highly resistant to killing and degradation by human neutrophils and monocytes, a characteristic which allows it to establish long-term low-grade infections that may persist for years to decades. A independent study using PCR-based technique, found *P. acnes* twice as common in prostate tissue from patients with benign prostatic hyperplasia that later developed cancer compared to those that did not (personal communication, Oleg Alexyev).

**Genetics**

As described above, multiple pieces of evidence have been accumulated supporting the view that genetic factors play a role in prostate cancer susceptibility and pathogenesis. Unlike some carcinomas such as those of the colon (Klump *et al.*, 2004) and pancreas cancer (Jaffee *et al.*, 2002), where relatively common somatic genetic alterations are observed (e.g. mutations in *p53* and *K-ras*), gene mutations in prostate cancer display a great deal of heterogeneity, not only from case to case, but also from lesion to lesion in a single cases. The heterogeneity of genomic defects in prostate cancer suggests that prostate cancers may arise as a consequence of either chronic or prolonged exposure to genome-damaging stresses, defective maintenance of genome integrity, or a combination of both, and thus no single dominant molecular pathway is responsible for prostate carcinogenesis (De Marzo *et al.*, 1999).

To date, a substantial number of germline prostate cancer susceptibility genes as well as somatic genome alterations have been identified, some of which indicate that inflammation may be an important mediator in the development of prostate cancer. The following sections summarise some of the germline and somatic genomic changes suggesting that infection or inflammation of the prostate contributes to the development of prostate cancer.

**GSTP1**

GSTPs represent a superfamily of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. GSTP1 appears to act as a “caretaker” gene, defending prostate cells against genome damage mediated by carcinogens. In the normal prostate epithelium, GSTP1 is expressed predominantly in basal cells, but not in the columnar secretory cells, although the enzyme may be induced in columnar epithelial cells that are subjected to genome-damaging stresses. An elevated level of GSTP1 is a histological characteristic of proliferative inflammatory atrophy, strongly indicating that a stress-induced response has been induced in these cells (De Marzo *et al.*, 1999). GSTP1 can undergo somatic alterations, especially hypermethylation of CpG islands of regulatory sequences at the GSTP1 locus, which prevents the transcription of GSTP1. Abnormal hypermethylation of CpG islands of regulatory sequences at the GSTP1 locus is associated with decreased transcriptional activity. Such hypermethylation occurs in many types of human cancers (Jones *et al.*, 2002), it has been reported to be the most common somatic genome alteration in prostate cancer and it represents an early event in neoplastic transformation. The hypermethylation occurs in approximately 70% of PIN lesions and in over 90% of
prostate carcinomas, but not in normal prostate tissue or benign prostatic hyperplasia (Jerónimo et al., 2001; Lin et al., 2001).

Recent published data have suggested that heterocyclic amine carcinogens present in well-done meat as well as oxidants may be detoxified by GSTP1 (Nelson et al., 2001). Experimental studies in which LNCaP cells (that do not normally express GSTP1) have been modified to express GSTP1, show that cells expressing GSTP1 more readily withstand DNA damage when exposed to promutagenic substances and oxidative stress, compared to unmodified LNCaP cells (Nelson et al., 2001, 2002). However, in response to oxidant stress, unmodified LNCaP cells survive better than LNCaP cancer cells that have been modified to express high levels of GSTP1. Thus, for LNCaP prostate cancer cells, loss of the GSTP1 function appears to increase genome damage and decrease cell mortality rates following exposure to oxidants. This finding of tolerance to oxidative genomic damage in the absence of functional GSTP1 may be a clue regarding the function of somatic GSTP1 inactivation in prostate cancer progression (Palapattu et al., 2004).

**MSR1**

In 2001, Xu et al. published a study with suggestive linkage to chromosome 8p22-23 among 159 families (Xu et al., 2001). Further mutation screening studies of hereditary prostate cancer families identified a number of mutations in the macrophage scavenger receptor 1 gene (MSR1) that co-segregate with prostate cancer (Xu et al., 2002).

MSR1 is a large 11-exon gene located on chromosome 8p22, an area that is frequently lost in prostate cancer (Matsumoto et al., 1990). It encodes a Class A scavenger receptor (MSR1, also known as SR-A, an important component of the innate immune system), which occurs in three different isoforms (I, II, and III) generated by alternative splicing of a single 11-exon mRNA. Isoforms I and II are functional while isoform III is a non-functional protein acting as a dominant negative isoform by blocking modified low-density lipoprotein uptake (Gough et al., 1998). The functional MSR1 protein is a trimeric transmembrane molecule composed of three identical protein chains. It has six distinct structural domains: the amino-terminal cytoplasmic domain, a transmembrane domain, a spacer domain, an α-helical coiled coil domain, a collagenous domain, and the scavenger receptor, cysteine-rich, carboxy-terminal domain (Kodama et al., 1990). MSR1 shows unusually broad ligand-binding properties. It binds a diverse array of macromolecules including bacterial lipopolysaccharide and lipoteichoic acid, and both oxidized high-density lipoprotein and low-density lipoprotein in the serum (Platt et al., 2001). The function of the receptor in vivo is still not entirely clear, but studies have suggested that these receptors may play a role in macrophage–host cell interactions, macrophage adhesion to substrata, endocytosis of ligands, and the recognition and clearance of foreign pathogenic substances and damaged or apoptotic cells (Platt et al., 2001).

The MSR1 receptor is mainly expressed in macrophages and related cells, and its expression is regulated by a number of factors including cytokines such as M-CSF, IFNγ, and TNFα (Shirai et al., 1999). Mice that are deficient in MSR1 are highly susceptible to infection by Listeria monocytogenes, Staphylococcus aureus, and herpes simplex type 1 (Thomas et al., 2000; Ishiguro et al., 2001), which may be relevant in
Prostate cancer susceptibility, since an infectious aetiology of prostate cancer has been proposed. Moreover, areas within the prostate that show evidence of inflammation are often populated by macrophages that express MSR1 (Nelson et al., 2003).

Several germ-line mutations in MSR1 have been linked to prostate cancer in some families with hereditary prostate cancer. In the mutation screening study by Xu et al., six rare missense and one nonsense mutation (R293X) were detected in MSR1 (Xu et al., 2002). A family-based linkage and association test indicated that these mutations cosegregate with prostate cancer (P=0.0007). Of these mutations, the nonsense mutation R293X was detected in approximately 2.5% of men with non-HPC compared to a 0.4% of unaffected men (P=0.05). In a follow-up study of 301 non-HPC cases and 250 controls, by the same group, five common variants within MSR1 were found, with statistically significant differences in allele and haplotype frequencies (Xu et al., 2003).

A number of other independent studies have evaluated the association between prostate cancer and the MSR1 gene, and the generated data have created doubt concerning the role of MSR1 sequence variants in prostate cancer susceptibility (Miller et al., 2003; Seppälä et al., 2003; Wang et al., 2003).

RNASEL

RNASEL is a constitutively expressed latent endoribonuclease that mediates the antiviral and proapoptotic activities of the interferon-inducible 2-5A system. Once activated by interferon, cells containing a functional RNASEL gene produce an enzyme that degrades single-stranded RNA, leading to apoptosis (Silverman, 2003). Defects in the RNASEL gene have been shown to result in reduced immunity to viral infections and cancer (Hassel et al., 1993).

A genome-wide scan for linkage in prostate cancer families found evidence for a prostate cancer susceptibility locus on chromosome 1q24-25 (Smith et al., 1996). In 2002, Carpten et al. identified RNASEL as a candidate gene for the HPC1 locus through a positional cloning and candidate approach. A truncating mutation (E265X) and an initiation codon mutation (M1I) were reported to co-segregate within two specific prostate cancer families (Carpten et al., 2002). In addition to the rare mutations, a number of relatively common RNASEL variants have been associated with prostate cancer risk. There is some confirmatory evidence for the association between variants of RNASEL and prostate cancer risk (Rennert et al., 2002), but other studies have showed no association (Wang et al., 2002; Kotar et al., 2003; Wiklund et al., 2004).

In summary, the role of RNASEL in the pathogenesis is still very unclear, due to inconsistencies in the association studies. However, as Palapattu et al. mention in a review from 2004, it is tempting to speculate that genetic variants of the RNASEL gene increase the risk for prostate cancer only in the presence of some environmental factor (e.g. viral infection) that may vary between different study populations (Palapattu et al., 2004).

Cytokines in prostatic inflammation and prostate cancer

As described above, cytokines are a diverse group of intracellular signalling peptides and glycoproteins that regulate local and systemic inflammatory and immune
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responses. Several pro- and anti-inflammatory cytokines have been identified in patients with prostatic inflammation and prostate cancer, and their roles have been studied. In 1998, Alexander et al. measured the levels of the pro-inflammatory cytokines TNFα and IL-1β in the semen of men with chronic prostatitis and compared them with levels in normal men (Alexander et al., 1998). They found that men with prostatitis had higher mean levels of TNFα and IL-1β than normal men, as would be expected in an ongoing inflammatory response. These results were confirmed by Nadler and co-workers, who showed that IL-1β was measurable in ~90% of patients with type III and type IV prostatitis, but rarely detectable in controls (Nadler et al., 2000). The level of TNFα could be measured in both cases and controls, but levels were significantly elevated in patients with type III and type IV prostatitis.

Genetic polymorphisms that alter cytokine gene expression or protein function could have an important impact on prostatic inflammation and the further development of prostate cancer. A polymorphism in the promoter of the anti-inflammatory cytokine IL-10 has been identified; and the IL-10-1082 AA genotype is associated with low IL-10 production. In a study by Shoskes et al., patients with chronic prostatitis were found to have higher proportions of the allele associated with low IL-10 production compared to controls, indicating a pro-inflammatory state in those patients (reviewed in Pontari M and Ruggieri M (Pontari et al., 2004)).

Only a few studies have examined the association between prostate cancer risk and sequence variants in cytokine genes such as TNFα, IL-8 and IL-10 (McCarron et al., 2002). In the cited study, cytokine sequence variants (IL-1β-511, IL-8-251, IL-10-1082, TNFα-308, and VEGF-1154) were analyzed among 247 prostate cancer cases and 263 controls. The results showed that IL-8-251 TT and VEGF-1154 AA genotypes were lower among patients compared with controls (OR=0.66; 95%, CI 0.44-0.99 and OR=0.45; 95% CI, 0.24-0.86, respectively), whereas the IL-10-1082 AA genotype was significantly increased in patients compared with controls (OR=1.78; 95% CI, 1.14-2.77). Although the influence of cytokine polymorphism on prostatic inflammation and prostate cancer is likely to be complex, the few relevant studies published to date indicate that SNPs in cytokine genes may have a significant impact on disease development. These results also highlight the critical need to further elucidate the impact of cytokines and their sequence variants on prostate cancer susceptibility.

MIC-1

The type β transforming growth factor (TGF-β) superfamily has more than 40 members, which are involved in regulation of many cellular functions and biological processes, including proliferation, apoptosis, extracellular matrix secretion and adhesion, terminal differentiation, and development (Derynck et al., 1997). To identify novel molecules that participate in the local inflammatory response, Bootcov et al. subtracted cDNA library enriched for genes associated with macrophage activation. The following screening resulted in the identification of a novel transforming growth factor β superfamily cytokine, designated macrophage inhibitory cytokine-1 (MIC-1). The MIC-1 gene, located on 19p13, is about 6 kb long and is composed of two exons. The protein is synthesized as a 308 amino
acid long precursor molecule. The propeptide is separated from the mature protein by a furin-like protease acting on a conserved cleavage site at amino acids 193-196. Following processing, the mature protein of 112-aa is secreted as a disulfide-linked homodimer. The mature protein subunits contain a conserved pattern of seven cysteine residues, which is a hallmark of the TGF-β superfamily (Bootcov et al., 1997). Since MIC-1 is a secretory protein it can function in cells secreting it as well as in their neighbouring cells (i.e. in both autocrine and paracrine fashions). It has been reported that MIC-1 activity (like that of TGF-β), requires an intact signalling pathway mediated by type I and type II TGF-β receptors, as well as receptor activated Smad4 (Tan et al., 2000).

The MIC-1 gene is also known by various names, including growth/differentiation factor-15 (GDF-15) (Böttner et al., 1999), placental bone morphogenetic protein (PLAB) (Hromas et al., 1997; Thomas et al., 2001), prostate-derived factor (PDF) (Paralkar et al., 1998), and the non-steroidal anti-inflammatory drug-activated gene (NAG-1). Like many TGF-β superfamily cytokines, MIC-1 is expressed very widely, but under resting conditions the placenta is the only tissue expressing large amounts of MIC-1 (Fairlie et al., 1999). Small amounts of MIC-1 mRNA have been detected in epithelial cells in the kidney, pancreas, colon, and prostate as well as macrophages. However, in cases of inflammation, injury or malignancy, MIC-1 expression is dramatically increased.

Shortly after cloning of the MIC-1 gene, a polymorphism was identified, resulting from alteration of the basic amino acid histidine (H), to the acidic amino acid aspartic acid (D), located at position 6 of the mature MIC-1 protein, next to the cysteine at position 7 that has been indicated to be important for the stability of this protein (Fairlie et al., 2001). Due to the differences in the properties of these two amino acids, this change may alter the stability of the protein and thus the function of MIC-1. Some studies have examined the possible relationship between MIC-1 genotypes and different diseases, including cancer, with variable results. A study by Brown et al. indicated that the presence of the HH genotype of MIC-1 was associated with a decreased risk of metastasis of colorectal cancer at presentation of the disease, but it should be noted that the numbers of patients in the investigated groups were relatively small (Brown et al., 2003).

It has been shown that MIC-1 has diverse biological functions in distinct cellular contexts. A number of direct and indirect lines of evidence suggest there is a link between MIC-1 and cancer. The promoter region of this gene contains two p53 target sites; and a number of studies have demonstrated that MIC-1 is strongly induced by transactivation of p53 (Kannan et al., 2000; Li et al., 2000; Tan et al., 2000). A well-studied function of p53 is its activity as a transcription factor, regulating genes whose products are involved in a variety of cellular processes including growth arrest, apoptosis, senescence and genome stability (Ko et al., 1996). Loss-of-function of DNA target recognition by mutated p53 is an important step in cell transformation, and over 50% of human cancers contain various inactivated p53 mutants (Hollstein et al., 1994). This indicates that some of the genes that are transactivated by p53, including MIC-1, may play an important role in the development of cancers. Furthermore, two studies by Tan et al. (Tan et al., 2000) and Li et al. (Li et al., 2000) both show that MIC-1 inhibits tumour cell growth through the
Prostate Cancer and Inflammatory Genes

TGF-β signalling pathway following activation by p53.

More direct evidence for links between MIC-1 and cancer, have been obtained from serial analysis of gene expression, which has indicated that MIC-1 is highly expressed in both prostate cancer and benign prostatic hyperplasia tissue compared to normal prostate tissue, with a higher expression in cancer tissue than benign prostatic hyperplasia tissue (Welsh et al., 2001, 2003; Nakamura et al., 2003). A recently published exploratory proteomic analysis of prostate cancer tissue and benign prostatic hyperplasia tissue originating from the same prostate tissue block supports this expression pattern (Hood et al., 2005). Elevated MIC-1 expression is also coupled with a number of other cancers, including colon, breast, and pancreas cancers (Buckhaults et al., 2001; Koopmann et al., 2004; Wollmann et al., 2005).

Analyses of serum samples of patients have indicated that there is a relationship between elevated serum MIC-1 and the metastatic progression of colorectal, prostate and breast (Welsh et al., 2003) (Brown et al., 2003). MIC-1 levels are also elevated in the serum of patients with pancreatic ductal cancer (Koopmann et al., 2004). These findings have led to the suggestion that measurement of MIC-1 levels may be useful for the diagnosis and surveillance of cancer. Serum MIC-1 levels can be markedly elevated in metastatic cancer and seem to parallel the stage and extent of disease, particularly in colorectal cancer (Brown et al., 2003). Furthermore, despite the relationship between elevated serum MIC-1 levels and metastatic progression, a number of studies have shown MIC-1 to have an antitumorigenic function, since it induces apoptosis and inhibits proliferation of several tumour cell lines. Liu et al. demonstrated that overexpression of MIC-1 in prostate cancer cell lines reduces cell adhesion and induces apoptosis (Liu et al., 2003). These findings are supported by observations of increased rates of apoptotic death in mammary carcinoma cells where MIC-1 is overexpressed (Li et al., 2000; Graichen et al., 2002). Moreover, Back et al. showed that NSAIDs that inhibit tumour development also induce colon cancer cells to undergo apoptosis, mediated by autocrine/paracrine induction of MIC-1 (Back et al., 2001).

Apart from playing an important role in inhibiting tumour progression, MIC-1 has been shown to exert anti-inflammatory effects by inhibiting the late phase of macrophage activation (Boochoov et al., 1997). Since MIC-1 is a potent p53 target gene and has anti-inflammatory activity, it has been suggested that increased p53 expression in inflammatory tissues may reflect a tissue defence mechanism that triggers a signalling pathway, leading to activation of MIC-1 and ultimately inhibition of inflammation (Tan et al., 2000). Understanding of the biological function of MIC-1 in the prostate may help delineate its role in prostatic physiology and pathobiology.

IL-1RN

The interleukin-1 (IL-1) family consists of three different cytokines; the two proinflammatory cytokines IL-1α and IL-1β, and the IL-1 inhibitor, IL-1 receptor antagonist (IL-1RN) (Dinarello, 1994). The IL-1RN gene is located on the long arm of human chromosome 2 at band 2q14.2 in close proximity to the genes coding for IL-1α and IL-1β, distributed over 430 kb (Steinkasserer et al., 1992). IL-1α, IL-1β and IL-1RN are all produced in a wide variety of
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cells - including monocytes, macrophages, neutrophils and epithelial cells of many organs (Arend et al., 1998) – and they bind to the same IL-1 receptors. IL-1α and IL-1β are pro-inflammatory cytokines, and their binding to the receptors initiates a cascade of events leading to the recruitment and activation of macrophages and neutrophils, vascular dilation and fever, and a potent pro-inflammatory immune response (Dinarello, 1988). When the anti-inflammatory cytokine IL-1RN binds to the same receptors the activity of IL-1α/β is blocked. Consequently, the biological function of IL-1α and IL-1β cytokines is neutralized in both physiological and pathophysiological immune and inflammatory responses. The relative levels of IL-1RN, IL-1α and IL-1β at an inflammatory site determine whether a pro-inflammatory response will be initiated and persist or will be terminated (McIntyre et al., 1991). Typically, the level of IL-1RN increases in the later stages of inflammatory events, thereby promoting their termination and limiting the risks that inflammation will become chronic and cause damage to healthy cells (Granovitz et al., 1991). The central role of the IL-1 system is protection against many different types of lesion, ranging from microbial colonisation to infection and malignant transformation (Witkin et al., 2002).

In the second intron of IL1-RN, there is a variable number of tandem repeat (VNTR), consisting of two to six copies of a 86-bp sequence (Tarlow et al., 1993). The frequency of the individual alleles varies among different ethnic and geographical populations, but allele 1 (containing four repeats) is always the most common allele, while the less common allele 2 (containing two repeats) has been associated with a variety of human diseases. Several studies have shown that people with allele 2 of the VNTR have a more prolonged and severe pro-inflammatory immune response than usual, and that carriers of this genotype more efficiently combat microbial infection or colonization (Hu et al., 2005). The majority of studies relating IL-1RN gene polymorphisms to disease have focused on patients with autoimmune diseases or disorders associated with chronic inflammation, including inflammatory bowel disease, psoriasis, lichen sclerosus, and multiple sclerosis (Witkin et al., 2002). A number of studies have evaluated the presence of the VNTR in malignant diseases, and the results have been conflicting rather than conclusive. Studies from Caucasian populations have shown that homozygous carriage of allele 2 is associated with an increased risk of gastric cancer (El-Omar et al., 2000; Machado et al., 2001; Glas et al., 2004), however this association was not replicated in Asian populations (Wu et al., 2003; Zeng et al., 2003; Chang et al., 2005). In addition to gastric cancer, a few studies have evaluated possible links between VNTR and risks of other kinds of cancers. Recently, Sehouli et al. found in a case-control study of 162 women with ovarian cancer and 121 controls that patients who were heterozygous for allele 2 had a significantly higher risk for ovarian cancer (Sehouli et al., 2003).

Although the role of genetic variation in IL-1RN has been investigated in many types of cancers, the role of IL-1RN polymorphisms in prostate cancer pathogenesis has not been examined.
Aims

The specific aims of the work underlying this thesis were:

- To investigate whether mutations in the Macrophage Scavenger Receptor 1 gene influence the risk of prostate cancer in both Swedish families with hereditary prostate cancer and in a cohort of unselected prostate cancer.

- To evaluate the possible role of genetic sequence variants in the Interleukin-1 Receptor Antagonist gene on prostate cancer risk in a Swedish population.

- To evaluate the possible role of genetic sequence variants in the Macrophage Inhibitory Cytokine-1 gene on prostate cancer risk in a Swedish population.

- To study the serum levels of MIC-1 in relation to prostate cancer and MIC-1 genotypes and whether MIC-1 levels are associated with prostate cancer risk.
Material and methods

Hereditary prostate cancer families used for mutation screening (Study I)

Since 1995 our research group has been identifying (mainly on the basis of referrals from urologists and oncologists) and collecting information on Swedish families with a history of HPC. In the analysis of the Macrophage Scavenger Receptor I gene we examined material from 83 of these families.

The 83 families included in this study had an average of 4.5 affected men/family and the mean age of diagnosis was 67 years. Nearly two-thirds of the cases were diagnosed before PSA measurement was introduced as a diagnostic tool in Sweden. The majority of the cases (79%) had clinical symptoms at diagnosis and 60% of the cases were diagnosed with advanced or metastatic disease.

Patients with prostate cancer and controls (Studies I, II, III, IV)

The Northern Sweden Health and Disease Cohort

In study I, we used a study population from Northern Sweden Health and Disease Cohort (NSHDC) to test for associations between eight selected sequence variants and prostate cancer risk. The design of the NSHDC has been presented in detail before (Stattin et al., 2000). For this study 215 incident cases of prostate cancer were identified by cross-reference to cancer and all-cause mortality registries. Data on patient and tumour characteristics were obtained through medical records. Two control subjects were selected for each case subject from all members who were alive and free of cancer at the time of diagnosis of the case in each subcohort, and matched the index case subject in terms of sex, age (± 6 months), and date (± 2 months) at recruitment. Controls were randomly selected from each group of eligible subjects if more than two subjects matched the identified case. If fewer suitable control subjects were found by this procedure, less than two control subjects was accepted. We identified 425 eligible control subjects, with an average age of 66 years at the time of recruitment in this study. All participants were residents of the county of Västerbotten.

CAPS

For the association analyses in Studies II, III, IV we utilized men included in the population-based CAPS (CAncer of the Prostate in Sweden) case-control study. The
case participants, diagnosed for prostate cancer between January 2001 and September 2002, were recruited from four of the six regional cancer registries that cover the entire population of Sweden. Each of these registries serves one health care region (Northern, Central, Stockholm, and South Eastern) and they collectively encompass approximately 6 million inhabitants (67% of Sweden’s population). The source-person-time was divided into two age-specific study groups. The first group included men 35-65 years of age, living in all regions mentioned above. The second study base included men 66-79 years of age at the time of the study entry, living only in the central and northern region. Swedish law requires both the attending physician and pathologist to report newly diagnosed cancer cases to the cancer registries. Therefore, the registries include records of almost 100% of all cancers diagnosed in Sweden. The cases were linked to the National Prostate Cancer Registry and clinical information on aspects such as TNM (tumour-node-metastasis) stage, Gleason sum, PSA level at the time of diagnosis, methods of diagnosis and primary treatment were obtained for 95.3% of the cases. The cases were then classified as either

Localised (T1-2, N0/NX, M0/MX, Grade I-II/Gleason sum 2-7, and PSA<100) or
Advanced (having or being prone to progressive disease; T3/4 or N+ or M+ or Grade III or Gleason sum 8-10 or PSA>100). For cases where at least one reported family member with prostate cancer a more detailed family history of prostate cancer was obtained through additional questionnaires and record linkage to the Swedish Cancer Registry or medical records. The families were subsequently classified as hereditary prostate cancer families, where three or more relatives had prostate cancer (52 cases), or familial prostate cancer families: where two relatives had prostate cancer (130 cases). In total 1961 prostate cancer cases were invited for participation in the CAPS study, of those 1444 (73.6%) approved to participate by donating a blood sample and answering the questionnaire. At the time of the studies in this thesis DNA was available for 1383 of the cases who accepted to participate.

Control subjects were randomly selected from the continuously updated Swedish Population Registry, frequency matched according to the expected age distribution (within five years), geographic origin of the cases and sex. Of the 1697 randomly selected controls that were invited for participation in the study, 866 (52.0%) accepted to participate with blood donation and questionnaire. At the time of the studies in this thesis DNA was available for 780 of the controls that approved to participate. Mean age (age at diagnosis for case patients and age at inclusion for control subjects) for the cases and controls were 66.6 and 67.9 years, respectively. Blood (4 x 10 ml) was collected from all cases and controls and separated into serum, plasma, and buffy coat. A detailed description of the study sample is presented in Study III. Clinical characteristics of the prostate cancer cases in the study are summarized in Table 1.
Table 1. Clinical characteristics of the 1383 cases with prostate cancer included in the CAPS study.

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Genetic analysis

Genomic PCR (Studies I and II)

For the mutation screening in Study I and genotyping of the VNTR in IL1-RN (Study II), intron complementary primer pairs that could amplify all exons, exon-intron junctions, the promoter region, and 3’-UTR in the MSR1 and the VNTR in IL-1RN were used for amplification from genomic DNA.

Mutation screening (Study I)

The analysis of the MSR-1 gene was started by screening the entire coding sequence - including exon-intron junctions, promoter regions and 5’- and 3’-untranslated regions - for mutations by Temperature Modulated Heteroduplex Analysis (TMHA) using High Performance Liquid Chromatography (HPLC) and a DNA-binding column (DNAsep™). This technique is based on the principle that heteroduplexes formed between mutated DNA and normal DNA molecules can be distinguished from homoduplexes consisting entirely of normal DNA molecules, by differences in their elution profiles at a given optimized temperature. The experimental procedure includes two main steps:

First, the nucleic acids of interest (for example PCR-products) are denatured at 95°C for 5 minutes, then gradually reannealed by reducing the temperature to 20°C over 50 minutes, a process that enables formation of mismatched DNA (heteroduplexes). The samples are subsequently bound to the column using trietylammomium acetate (TEAA). The binding strength of nucleic acids to the column is proportional to the number of ion pairs formed between the negatively charged nucleic acids and the positively charged TEAA ions absorbed to the stationary phase.

The second step is elution of the nucleic acids bound to the column using a linear gradient of Acetonitrile, which weakens the bonding between the column and TEAA by competitive hydrophobic interactions and thus releases the nucleic acids. The helical nature of the duplex is disrupted in the heteroduplexes due to the presence of incorrect base pairing at the site of the mutation, which reduces the number of ion-pairing bonds with the column and thus results in the heteroduplex eluting earlier (at a lower acetonitrile concentration) than the homoduplex (Figure 2). This approach to detecting heteroduplexes is most suitable when screening for rare mutations and mutations, for which homozygotes of the rare allele are unlikely to be present. Since this was our aim, we did not modify the method to enable common polymorphisms (for which homozygotes of both alleles are likely to present in the study population) to be detected. A detailed description of the method is presented elsewhere (Xiao et al., 2001).

The sequence variants identified using the HPLC analyses were confirmed by direct sequencing. The amplicon of interest was amplified from corresponding genomic DNA. PCR products were purified and then sequenced with a dye terminator kit. To ensure high sequence quality, all fragments were sequenced from both directions. Primers used for amplification of the PCR product were used as sequencing primers.
Common sequence variants of the \textit{MSR-1} gene (Study I)

Five common sequence variants identified of the \textit{MSR-1} gene together with three rare mutations were further evaluated for possible association with prostate cancer risk. For the genotyping of the five common SNPs (PRO3, INDEL1, IVS5-57, P275A, and INDEL7) and the rare nonsense mutation (R293X), the MassARRAY system was used (SEQUENOM, Inc. Valencia, CA). PCR reactions were performed in a total volume of 5 µl with 10 ng of genomic DNA, 2.5 mM of MgCl₂, 0.1 U of HotStarTaq polymerase, (QIAGEN Inc.Valencia, CA), 200 µM of each dNTP and 200 nM of each primer. The PCR conditions were as follows: 95°C for 15 minutes followed by 45 cycles of 95°C for 20 seconds, 50°C for 30 seconds and 72°C for 1 minute with a final extension of 72°C for 3 minutes. The hME reactions were performed in a total volume of 9 µl with 50 µM of each d/ddNTP, 0.063 U/µl of Thermo Sequenase (both from SEQUENOM, Inc.) and 600 nM of extension primer. The cycling conditions were 94°C for 2 minutes followed by 55 cycles of 94°C for 5 seconds, 52°C for 5 seconds and 72°C for 5 seconds. After cleaning up the hME reaction products with SpectroCLEAN the products were transferred to a SpectroCHIP using SpectroPOINT, and then scanned through SpectroREADER. Genotyping was done using SpectroTYPER (all from SEQUENOM, Inc.).

In addition to the MassARRAY genotyping system we utilized HPLC analyses (described above) to genotype the two splice site mutations located adjacent to exon 4. Since both were rare among the HPC families and we did not expect any homozygotes of the rare allele HPLC was a suitable method.
Association of MIC-1 sequence variants and prostate cancer (Study III)

To fully elucidate possible associations between prostate cancer risk and SNPs in the inflammatory gene MIC-1, the following approach was used. First, the target region for selection of SNPs was defined as 2 kb of the promoter plus all exons, introns, and the 3’UTR. The SNP information for this region was obtained from public databases (NCBI dbSNP, and SNPer) or from data obtained by re-sequencing samples from 24 control subjects when detailed SNP information was not publicly available. Two criteria were used to select SNPs for further analyses:

1) the minor allele frequency had to be at least 5%, at a resolution of 1 SNP per kb of DNA across the genome region of MIC-1. The density of the selected SNPs was chosen to ensure that they sufficiently reflected the haplotype block pattern throughout the genome (Wall et al., 2003).

2) all SNPs that lead to an amino acid substitution, since such changes are more likely to have an effect on prostate cancer susceptibility and pathogenesis.

From the public database, six SNPs (Exon1+25, Exon1+142, IVS1+904, IVS1+1809, Exon2+2423, 3’-UTR+2816) located in the exons, introns and 3’UTR were selected. In addition, two SNPs (MIC1-1576, and MIC1-893) located in the promoter region that fulfilled the criteria were selected by the re-sequencing samples from 24 randomly selected CAPS controls (Figure 3).

DNA from 94 control subjects selected at random from CAPS was genotyped with respect to the eight chosen SNPs using a 5’ nuclease assay with TaqMan MGB probes. The SNP genotyping assays were designed using Applied Biosystems’ Assay-by-Design service (Applied Biosystems Inc., Foster City, CA). All reactions were performed in 25 µl mixtures consisting of 10 ng genomic DNA, 900 nM of each primer, 200 nM of each probe and 12.5 µl of TaqMan universal master-mix. PCR cycling conditions were as follows, 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. The samples were analyzed using an ABI 7700 sequence detection system. For quality control, two CEPH DNA samples and additional water blanks were included on each 96-well plate.

Following the first genotyping, the SNP MIC1-1576 was excluded from further analysis since it deviated significantly from HWE (p=0.003). Haplotype frequencies for the remaining seven SNPs were estimated using the statistical method proposed by Stephens et al. (2001), as implemented in the computer program PHASE (http://www.stats.ox.ac.uk/mathgen/software.html). A subset of “Haplotype Tagging SNPs” that could uniquely represent at least 95% of the haplotype information observed among the 94 control individuals was selected using the computer software htSNP2 (http://www.gene.cimr.cam.ac.uk/clayton/software/stata ). Four SNPs (Exon1 +25, Exon1 +142, IVS1 +1809, and Exon2 +2423 (H6D)) that captured 98.6% of the haplotype variation among the 94 controls were selected as haplotype tagging SNPs. Each of these four SNPs was in HWE among both the cases and controls. These four htSNPs were genotyped for all 1383 cases and 780 controls from the CAPS study. The genotyping was performed using the MassARRAY system (SEQUENOM Inc., Valencia, CA), described in detail above.
The DNA samples were labelled blindly and shipped from Umeå University, Sweden, to the core genotyping laboratory in the Center for Human Genomics, Wake Forest University, USA. In total, 37 controls from the CEPH foundation (1331-01, 1331-02), and 29 blind repeats were spread among the DNA samples. In addition, every DNA plate contained two water blanks.

**Association of Interleukin-1 receptor antagonist haplotype with prostate cancer risk (Study II)**

The procedure described above for selecting MIC-1 sequence variants for analysis was also used to select SNPs of the IL1-RN gene. In total, 16 SNPs and two repeats (the 86-bp VNTR and an 8-bp repeat) were identified by scrutinizing public databases (NCBI dbSNP, and SNPper) (Figure 3). Three different methods were used for genotyping.

First, the 16 identified SNPs and the 8-bp repeat were genotyped in 94 control subjects using a 5’ nuclease assay with TaqMan MGB probes. The SNP genotyping assays were designed using the Assay-by-Design service (Applied Biosystems Inc., Foster City, CA) and the samples were analyzed using an ABI 7700 sequence detection system. Since it was not possible to design a 5’ nuclease assay for the 86-bp VNTR in intron 2, it was genotyped by temperature modulated heteroduplex analysis using a High Performance Liquid Chromatography system, performed as described above (Study I). Haplotypes of these SNPs were estimated using a Markov Chain Monte Carlo approach as implemented in the PHASE software package (http://www.stats.ox.ac.uk/mathgen/software.html). Haplotype-tagging SNPs, which captured at least 95% of the haplotype variation among the 94 controls, were selected using the htSNP2 computer program (www-gene.cimr.cam.ac.uk/clayton/software/stat). Four htSNPs were identified and they were genotyped in all 1383 cases and 779 controls. The htSNPs (rs878972, rs315934, rs3087263, rs315951) were then genotyped using the MassARRAY system (SEQUENOM, Inc. Valencia, CA).
Figure 3. A) Single nucleotide polymorphisms (SNPs) of the Macrophage Inhibitory Cyokine-1 gene and Interleukin-1 Receptor Antagonist gene. The diagrams of the genes show exons (marked in red), the location of selected SNPs (relative to the transcriptional start site) and SNPs that result in an amino acid substitution. Arrows represent transcription start sites. B) Location of the haplotype-tagging SNPs that were selected to represent at least 95% of the haplotype information.
Determination of MIC-1 serum levels (Study IV)

MIC-1 serum levels were determined by a MIC-1 sandwich ELISA, using the mouse monoclonal antibody (MAb) 13C4H3 for antigen capture and a sheep polyclonal antibody (PAb), 233B3-P, for detection. The optimum concentration of both antibodies was determined and then used for all subsequent studies. Ninety-six-well Maxisorp ELISA plates were coated with MAb 13C4H3 supernatant diluted 1:5 (final concentration, approximately 20 ng/mL) in coating buffer at 4°C for 24 hours. The plates were then washed three times with 300 μL/well 1% (wt/vol) BSA in PBS for 2 h at 37°C. rhMIC-1 standards, tissue culture supernatant and patient serum were then added to the plates (100 μL/well) and incubated for 1 h at 37°C. The plates were washed three times, 100 μL/well of the sheep PAb 233B3-P diluted 1:5000 in antibody diluent (Ab dil) was added and they were incubated for 1 h at 37°C. The plates were then washed three times, 100 μL/well of biotinylated donkey antisheep IgG diluted 1:5000 in Ab dil was added and they were incubated for 1 h at 37°C. The plates were washed four times, followed by the addition of 100 μL/well of peroxidase substrate (1 mg/mL o-phenylenediamine dihydrochloride from Sigma) in 0.05 mol/L phosphate-citrate buffer containing 0.014% H2O2, pH 5.0 (Sigma). Colour development was allowed to proceed for 5-15 min and was terminated by the addition of 100 μL/well of 4N H2SO4. The absorbance was measured at 490 nm in a microplate reader. The concentration of hMIC-1 in the samples was determined by comparison with an rhMIC-1 standard curve constructed using standard curve-fitting software supplied with the microplate reader (Pasteur Diagnostics). The level of rhMIC-1 in the standard curve was determined on the basis of a comparison of this standard to a master standard of highly purified recombinant MIC-1. The master standard protein concentration was determined by averaging eight estimates of total amino acid composition. All samples were assayed in triplicate.

The serum samples were labelled blindly and shipped from Umeå University, Sweden, to the Centre for Immunology, St Vincent's Hospital & University of New South Wales, Australia, where the serum concentrations were determined.

Statistical analysis

Tests for Hardy-Weinberg Equilibrium (Studies I, II, III)

Hardy-Weinberg Equilibrium (HWE) tests for each sequence variant and pair-wise linkage disequilibrium (LD) tests for all sequence variants were performed using a replication method, as described by (Weir, 1996). For each test, 10,000 permutations were performed and the Fisher probability test statistic for each replicate was calculated from the new corresponding multilocus table. Empirical p-values for each test were estimated as the proportion of replicate data
sets found to be as probable as, or less probable than, the observed data set, as implemented in the software package Genetic Data Analysis (GDA).

**Association analysis (Studies I, II, III, IV)**

Associations between genotypes/MIC-1 serum and prostate cancer risk were assessed by the score tests in conditional logistic regression of a covariate equal to the number of rare alleles (0, 1, 2) (Studies II and III) and by dichotomized MIC-1 serum levels (with a cut-off value of 1000 pg/ml, based on the median level among unaffected controls) (study IV). Genotype specific risks were estimated as Odds Ratios (OR) with associated 95% confidence intervals by conditional logistic regression. Both when testing for association and estimating ORs, the conditional logistic regression was stratified by each combination of age (5-year age groups) and geographical region (the northern part of Sweden vs. the south eastern part of Sweden and the Stockholm area) to adjust for the matching conducted in collecting control subjects. Besides age and geographical region, no other factors were included in the regression analysis. In Study I unconditional logistic regression was used to test for association between genotypes and affection status. We adjusted for age and geographical region using indicator variables representing each combination of age of onset (5-year age groups) and geographical region.

**Haplotype analysis (Studies II, III)**

Tests of association between haplotypes and prostate cancer risk were performed using a score test developed by Schaid et al. (Schaid et al., 2002), as implemented in the software HAPLO.STAT for the R programming language. This method, based on the generalized linear model framework, allows adjustment for possible confounding variables and provides both global and haplotype-specific tests. In these analyses, age and geographic region were adjusted for using indicator variables representing each combination of age category (5-year age groups) and geographical region was adjusted as described earlier. Haplotypes with estimated frequencies less than 0.005 were pooled into a single group. Empirical P-values, based on 10,000 simulations, were computed for the global score test and each of the haplotype-specific score tests.

**Assessment of serum levels (Study IV)**

Serum MIC-1 levels of prostate cancer cases and controls were presented as means +/- standard deviation (SD). Formal comparison of MIC-1 levels between different subject groups were conducted using analysis of variance (ANOVA) methods. To evaluate the diagnostic value of MIC-1 serum levels, nonparametric receiver operating characteristic (ROC) analysis was performed.
Results and comments

Genetic analysis of MSR1 (Study I)

In order to evaluate the role of MSR1 gene mutations in prostate cancer in the Swedish population we carried out a genetic analysis in a large number of subjects from two different study populations in Sweden. We initially screened a set of DNA samples representing one affected individual from each of 83 Swedish families affected by HPC. Among these individuals we identified 18 sequence variants in total, including two exonic variants, four intronic and nine variants located in the 5'- or 3'-uncoding regions. A nonsense mutation at codon 293 (R293X) and five common sequence variants (PRO3, INDEL1, IVS5-57, P275, INDEL7) identified in this study, were also reported by Xu et al. (Xu et al., 2002).

The truncating mutation R293X results in a deletion of most of the collagen-like domain, including the ligand-binding region and the cysteine-rich domain. Experimental studies have demonstrated that an MSR1 mutant harbouring a similar truncating mutation to R293X has a dominant-negative phenotype when expressed in vitro (Dejager et al., 1993). R293X was found in two probands from two different families among the 83 HPC families (2.4%). One family had one affected (the proband) and three unaffected R293X carriers, while the other family had one affected (the proband) and two unaffected R293X carriers. However, due to the low numbers of affected men in these two families, it was not possible to determine whether the mutation segregated with the disease.

In agreement with our findings, R293X mutation was observed in the same frequency (3.2%) among Caucasian HPC families in the study from the United States, (Xu et al., 2002). In addition, Seppälä et al. reported the same frequency (2.5%) among 120 families affected with HPC (Seppälä et al., 2003), as well as Wang et al. among 163 families affected with familial prostate cancer (3.1%) (Wang et al., 2003) (Table 2).

We further evaluated the association between this mutation and prostate cancer by screening a group of 215 unrelated men with prostate cancer and 425 age-matched controls. The R293X mutation was found more frequently in men with prostate cancer (10 individuals, 4.9%) than their matched controls (10 individuals, 2.7%), however this difference was not statistically significant (P=0.16) (Table 2). In contrast, Xu et al. reported a significant difference in frequencies of the R293X mutation between men with non-HPC and unaffected men (2.5% versus 0.39%, P=0.047) (Xu et al., 2002). The choice of control population might explain some of the differences between our results and the previous report. The men in the control population in the present study were on average 66 years old at recruitment, an age at which only 15% of all prostate cancers have been diagnosed. Thus, some of the unaffected R293X carriers are likely to develop prostate cancer eventually. Another difference is that Xu et al. only included men with PSA values <4 and a normal prostate examination.
Two other studies that investigated the possible association between the R293X mutation and prostate cancer did not find any statistically significant difference between unselected prostate cancer cases and controls (Seppälä et al., 2003; Wang et al., 2003) (Table 2). If the R293X mutation increases the risk for prostate cancer by a factor of two and the frequency is ~3% in the general population, as our results suggest, a much larger case-control study would be needed to significantly verify this.

In addition, we detected two novel potential pathogenic mutations, IVS3-4 A>G and IVS4+3 A>G, respectively located in the splice donor region and splice site acceptor region of exon 4. Both of these mutations were observed in single families, and neither of them was detected when screening the group of unselected men with prostate cancer or age-matched healthy controls. Point mutations which alter a conserved sequence in the splice donor region, the splice acceptor region or a region nearby may cause aberrant splicing of a gene. Several studies have shown that mutations located in similar positions to IVS3-4 and IVS4+3 can lead to exon skipping, intron retention or insertions and deletions due to utilization of cryptic splice sites (Margaglione et al., 2000; Attanasio et al., 2001, 2003). Further functional studies are needed to assess the functional significance, if any, of these mutations.

Besides the three rare mutations described above, we decided to elucidate the importance of five common sequence variants found in the mutation screening of MSR1: an SNP in the promoter region (PRO3), a 15-bp insertion/deletion of “GAATGCTTTATTGTA” in intron 1 (INDEL1), an SNP in intron 5 (IVS5-57), a SNP in Exon 6 (P275A), and a 3-bp insertion/deletion of “TTA” in intron 7 (INDEL7). The initial study evaluating the role of the five sequence variants in the MSR1 gene, reported a significant difference in the allele frequencies of each of the five variants between patients with non-HPC and unaffected control subjects, which led to the suggestion that these common MSR1 variants are associated with prostate cancer.

### Table 2. Reported studies of the association between the MSR1 nonsense mutation R293X and prostate cancer risk

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of carriers/total (frequency)</th>
<th>Families with HPC</th>
<th>Cases with unselected prostate cancer</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xu et al. 2002</td>
<td></td>
<td>6/190 (3.2%)</td>
<td>8/317 (2.52%)</td>
<td>1/256</td>
<td>0.047*</td>
</tr>
<tr>
<td>Seppälä et al. 2003</td>
<td></td>
<td>3/120 (2.5%)</td>
<td>6/537 (1.1%)</td>
<td>5/480</td>
<td>0.91</td>
</tr>
<tr>
<td>Wang et al. 2003</td>
<td></td>
<td>5/163 (3.1%)</td>
<td>14/496 (2.8%)</td>
<td>16/492</td>
<td>0.69</td>
</tr>
<tr>
<td>Lindmark et al. 2004</td>
<td></td>
<td>2/83 (2.4%)</td>
<td>10/215 (4.9%)</td>
<td>10/425</td>
<td>0.16*</td>
</tr>
</tbody>
</table>

* Fisher exact test (two-sided) between cases and controls.
risk in the general population (Xu et al., 2003). To investigate whether these variants are associated with prostate cancer risk we decided to genotype them in 215 unrelated patients with prostate cancer and 425 age-matched controls. None of the variants were found to be associated with prostate cancer (Table 3).

### Table 3. Allele frequencies of common MSR1 sequence variants in unselected prostate cancer cases and unaffected control subjects

<table>
<thead>
<tr>
<th>Sequence variant</th>
<th>Allele frequencies (%)</th>
<th>Allele frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls Cases P-values*</td>
<td>Controls Cases P-values*</td>
</tr>
<tr>
<td>PRO3 G</td>
<td>6.1 6.0 1.00</td>
<td>7.6 12.3 0.01</td>
</tr>
<tr>
<td>INDEL1 +</td>
<td>5.9 5.9 0.94</td>
<td>7.9 11.8 0.04</td>
</tr>
<tr>
<td>IVS5-57 A</td>
<td>3.2 2.9 0.80</td>
<td>3.6 7.0 0.02</td>
</tr>
<tr>
<td>P275A C</td>
<td>96.9 96.3 0.55</td>
<td>91.2 95.0 0.01</td>
</tr>
<tr>
<td>INDEL7 -</td>
<td>97.2 96.7 0.62</td>
<td>91.3 94.4 0.04</td>
</tr>
</tbody>
</table>

* $\chi^2$-test for the allele

Two other studies have evaluated the common sequence variant in exon 6 (P275) for its role in prostate cancer risk in the general population. Carrier frequencies of the P275A variant were compared between unselected prostate cancer cases and controls. Their data are consistent with our results because they found no statistically significant difference in the carrier frequencies between the different sample groups (Seppälä et al., 2003; Wang et al., 2003).

It is not known how MSR1 could contribute to the development of prostate cancer. However, MSR1 is induced in macrophages by oxidative stress and may modify the amounts of reactive oxygen intermediates. The inflammation and proliferative regeneration of prostate epithelium in the presence of increased oxidative stress that are associated with MSR1 expression may have a role in the development of prostate cancer (Xu et al., 2002).

In conclusion, we found no evidence to support the hypothesis that the sequence variants in the MSR1 gene play a role in the development of hereditary or sporadic prostate cancer.
**Genetic analysis of IL-1RN (Study II)**

Several studies have shown that IL1-RN acts as an important regulator of the inflammatory response by inhibiting the action of IL-1α and IL-1β. Since chronic inflammation in the prostate appears to be a co-factor in the pathogenesis of prostate cancer, we hypothesized that functional polymorphisms in inflammation-related genes, such as IL-1RN may be associated with prostate cancer risk. To test this hypothesis, we performed genotype analyses for four IL1-RN gene polymorphisms in a large population-based case-control study of 1383 prostate cancer case patients and 779 control subjects.

Quality control of the genotype results (based on included CEPH controls, repeated study samples and water blanks) provided an estimated error rate of 0%. Except for the SNP rs315951, which significantly deviated from HWE in cases \((P=0.04)\), but not controls \((P=0.34)\), the genotype data for the remaining three SNPs were consistent with HWE (all \(P>0.05)\). The significant deviation of the genotype frequencies of SNP rs315951 from expected proportions among the prostate cancer subjects may be a result of genotyping errors, population stratification, selection, or statistical fluctuations. However, given the high quality of the genotyping results and the ethnic homogeneity of the Swedish population, it seems most plausible that chance alone was responsible for the observed departure from HWE. Since the departure from HWE was in the direction of excessive homozygosity, the error in haplotype estimations (based on the EM algorithm) was not increased (Fallin et al., 2000). Moreover, all statistical inferences regarding haplotypes were based on simulated P-values, which are expected to be less sensitive to departure from HWE than the asymptotically distributed score statistic.

The SNP analysis showed that there were no significant differences in proportions of any of the four htSNPs between the controls and prostate cancer cases. Assuming that the SNPs had a dominant or recessive allelic effect on prostate cancer risk did not alter these findings. Furthermore, stratified analyses based on age (<65 or ≥65), tumour stage (localized or locally advanced) and family history (sporadic or familial/hereditary) detected no significant differences in genotype frequencies between cases and controls.

We also performed a haplotype analysis of the four htSNPs, which identified the presence of eight major haplotypes. Global tests for association between haplotypes and prostate cancer risk all yielded non-significant results. However, individual haplotype analyses revealed that one haplotype was statistically significantly associated with prostate cancer risk (Table 4). The frequency of the ATGC haplotype of SNPs rs878972, rs315934, rs3087263, and rs315951 was significantly higher among cases (38.7%) compared to controls (33.5%) (haplotype-specific \(P=0.009)\). Furthermore, in the stratified analysis the frequency of the “ATGC” haplotype was higher in sporadic (39.3%) than in familial (34.8%) prostate cancer cases. Likewise, cases with advanced disease had a higher prevalence (40.0%) than patients with localised disease (37.5%). The fact that the association was strengthened in cases with advanced disease is particularly noteworthy. While it is important to identify disease susceptibility genes, it is equally or
probably even more important to identify genes that play roles in disease progression, especially for prostate cancer since the disease has a late average age of onset and is life threatening in a relatively small proportion of cases (aggressive cases). Polymorphisms in genes mediating progression of prostate cancer could function as genetic markers predicting an increased risk of progressive disease.

The observation that the most common haplotype was associated with prostate cancer risk but none of the individual htSNPs, highlights the advantages of studying all variations in a gene with the use of a haplotype-tagging approach instead of testing a limited number of single variants. Two different scenarios may explain this observation. First, the association may be a result of two or more sequence variants in the region that do not individually confer a detectably increased risk for prostate cancer, but do confer a statistically significant risk in combination, which can be detected using the haplotype-association strategy. For example, there may be two SNPs in the gene that only alter protein function significantly when they occur together. Second, the ATGC-haplotype may be in strong linkage disequilibrium (LD) with a risk-conferring sequence variant in IL1-RN, or in close vicinity to the gene, while the degree of LD between this risk-conferring sequence variant and each of the four htSNPs is much weaker.

In this study the overall haplotype test gave a non-significant result, while the haplotype-specific score was significant for the most common haplotype. However, the estimated odds ratios show significantly increased risk only for homozygous carriers (OR = 1.6; 95% CI, 1.2-2.2) of the “ATGC” haplotype and not for heterozygous carriers (OR = 1.0; 95% CI, 0.8-1.2). Since the proportion of individuals that are homozygous for this haplotype is considerably lower than the overall frequency of the haplotype, the power of the global test will be considerably lower than if heterozygous carriers were also at increased risk.
Table 4. Estimated haplotype frequencies in the IL1-RN gene in controls, patients with sporadic prostate cancer (SPC) and patients with advanced prostate cancer.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Controls</th>
<th>Cases SPC</th>
<th>Advanced PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs878972</td>
<td>0.338</td>
<td>0.387</td>
<td>0.26</td>
</tr>
<tr>
<td>rs315934</td>
<td>0.176</td>
<td>0.155</td>
<td>-1.01</td>
</tr>
<tr>
<td>rs3087263</td>
<td>0.175</td>
<td>0.153</td>
<td>-1.16</td>
</tr>
<tr>
<td>rs315951</td>
<td>0.146</td>
<td>0.135</td>
<td>-0.58</td>
</tr>
<tr>
<td>rs878972</td>
<td>0.084</td>
<td>0.077</td>
<td>-0.80</td>
</tr>
<tr>
<td>rs3087263</td>
<td>0.058</td>
<td>0.054</td>
<td>-0.38</td>
</tr>
<tr>
<td>rs315951</td>
<td>0.018</td>
<td>0.027</td>
<td>0.97</td>
</tr>
<tr>
<td>rs878972</td>
<td>0.006</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

Score test statistics for association between haplotype and prostate cancer risk.

<table>
<thead>
<tr>
<th>Overall</th>
<th>C</th>
<th>A</th>
<th>T</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Empirical P values based on 10,000 replications.
Several studies have evaluated the role of IL-1RN in a number of malignancies by investigating the frequency of the 86-bp VNTR in various study populations. However, since the VNTR only covers a minor part of the variance in the gene, it is not enough to solely analyze the repeat to evaluate the role of IL-1RN. Therefore, we used another approach, in which we studied a set of tagging SNPs that captured almost all the genetic information in the entire gene. We genotyped four htSNPs in the study population, which explained >98% of diversity in the gene. The VNTR was not included in the genotyping, but it was almost equivalent to the htSNP rs878972 (Pearson correlation coefficient = 0.99). The htSNP rs878072 was not associated with prostate cancer risk, thus it is unlikely that the VNTR in intron 2 by itself contributes significantly to prostate cancer risk.

The IL-1RN gene belongs to the IL-1 gene cluster, which spans a 360-kb region of chromosome 2 (Steinkasserer et al., 1992). This cluster of genes contains several pro- and anti-inflammatory cytokine genes that are expressed in both physiological and pathological conditions and play a key role in the inflammatory immune response. The high density of IL-1 genes in this region, which share similar functional characteristics, raises the possibility that an association between a haplotype in IL-1RN and prostate cancer may in fact be due to an association between another gene or genes in the cluster and prostate cancer. Knowledge of the degree of LD across this region is vital to our understanding of the combinations of genotypes that are important in disease. Several analyses have been performed to determine the degree of LD across this region, all of which have found moderate LD in this gene cluster that is not strictly correlated with distances between markers (Cox et al., 1998; Bensen et al., 2003). Thus, additional SNPs in this region need to be identified and analyzed to determine whether apparent associations between IL-1RN and prostate cancer reflect a causative relationship, or are due to the effects of another gene in the cluster.

Our results suggest that (a) prostate cancer risk variant(s) may be located somewhere in the region of IL1-RN. The exact location and biological function of this/these sequence variant/variants remain to be identified. Further studies are needed to replicate our findings in other populations and to elucidate the biological function of the IL-1RN haplotype in relation to prostate cancer risk.

**Genetic analysis of MIC-1 (Study III)**

Due to the potential importance of MIC-1 in tumour development and regulation of inflammation responses, we decided to further elucidate the role of MIC-1 by investigating possible associations between sequence variants in the gene and prostate cancer risk.

As a first step, we genotyped four common SNPs (Exon1+25, Exon1+142, IVS1+1809, and Exon2+2423 [H6D]) in a large population-based case-control study of 1383 prostate cancer patients and 780 control subjects. With these four SNPs it was possible to capture >98% of the
haplotype variation in the gene, and each of them were in HWE among both cases and controls (all P>0.05). These SNPs were in strong LD, as most of the pair-wise D' estimates were 1.0, with the lowest at 0.94.

Testing for genotype frequency differences between cases and controls revealed one SNP (H6D) for which there was a significant difference between cases and controls (P=0.006); proportions of homozygotes for the common CC genotype encoding the wild-type protein being higher among prostate cancer cases than among controls (53.0% versus 48.5%). In an assessment of genotype-specific risk we found that the OR for carriers of the GC or GG genotype, which encodes the H6D mutant, was lower than the OR for the CC genotype carriers (OR=0.83; 95% CI, 0.69-0.99). The decreased risk for carriers of the GC or GG genotype compared with that of the CC genotype carriers was further accentuated both in patients with advanced disease (OR=0.79; 95% CI, 0.63-0.99) and in patients with a positive family history of prostate cancer (OR=0.68; 95% CI, 0.48-0.96) (Table 5). The observation that the association was further strengthened in patients with a positive family history is consistent with the hypothesis that risk genotypes may co-segregate with unknown gene alterations with low penetrance within families. In summary, the findings that an association between the H6D variant and prostate cancer is present in sporadic cases and further accentuated in familial cases are consistent with current knowledge.

Although this study provided strong evidence for an association between the H6D sequence variant in the MIC-1 gene and prostate cancer, the underlying mechanisms involved remain unclear. The substituted amino acid at position six is located adjacent to the cysteine at position seven, which has been indicated to be important for the stability of MIC-1 (Fairlie et al., 2001). Because of the different biochemical properties of aspartic acid and histidine, the H6D polymorphism may alter MIC-1's stability and function. If it abolishes or reduces MIC-1 activity, inflammation in the prostate may go unchecked in carriers with the risk genotype, leading to an increased risk for tumour development. On the other hand, expression of a MIC-1 protein with increased activity or stability may lead to a reduced pro-inflammatory response, which in turn could reduce the capacity to eradicate certain pathogens effectively. This is in line with the infectious aetiology of prostate cancer that has been proposed (Dennis et al., 2002a). Furthermore, altered functionality of MIC-1 due to the H6D polymorphism may also lead to decreased tumour cell growth inhibition mediated through the TGF-β signalling pathway. Other types of investigations, such as in vitro and in vivo functional studies of the H6D variant, are needed to address these possibilities.

In conclusion, this is the first published study evaluating the possible association between sequence variants in the MIC-1 gene and prostate cancer risk. Our study provided evidence for such an association, but more studies are needed to confirm or refute this finding in independent populations and to elucidate the mechanism whereby the H6D sequence variant affects the expression and function of MIC-1 in the signalling pathways that seem to control macrophage regulation and tumour growth.
Table 5. Association between prostate cancer and the H6D sequence variant of the MIC-1 gene in a large population-based study (CAPS).

<table>
<thead>
<tr>
<th>Study subset</th>
<th>No. of subjects</th>
<th>GC vs. CC (OR, 95% CI)</th>
<th>GG vs. CC (OR, 95% CI)</th>
<th>GC or GG vs. CC (OR, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All PC</td>
<td>1340</td>
<td>0.77 (0.64, 0.93)</td>
<td>1.22 (0.84, 1.75)</td>
<td>0.83 (0.69, 0.99)</td>
</tr>
<tr>
<td>Advanced PC</td>
<td></td>
<td>0.75 (0.59, 0.95)</td>
<td>1.11 (0.70, 1.74)</td>
<td>0.79 (0.63, 0.99)</td>
</tr>
<tr>
<td>FPC and HPC†</td>
<td>159</td>
<td>0.61 (0.42, 0.89)</td>
<td>1.18 (0.61, 2.29)</td>
<td>0.68 (0.48, 0.96)</td>
</tr>
<tr>
<td>Advanced PC</td>
<td>57</td>
<td>0.44 (0.24, 0.81)</td>
<td>0.62 (0.18, 2.05)</td>
<td>0.47 (0.26, 0.82)</td>
</tr>
</tbody>
</table>

*Conditional logistic regression stratified by age and geographical region.
†FPC= Familial Prostate Cancer, HPC= Hereditary Prostate Cancer.

Analysis of MIC-1 serum levels (Study IV)

The development of sensitive immunoassays for MIC-1 has made it possible to study MIC-1 protein levels in human serum and other body fluids. Brown et al. found the MIC-1 serum concentration to be higher in patients with colorectal cancer than in controls, and production of the protein was also up-regulated in tumour tissues compared to its normal counterparts (Brown et al., 2003). The same pattern was also observed in a small study in patients with breast and prostate cancer (Buckhaults et al., 2001). Based on these findings, together with the results from study III, we decided to determine the serum MIC-1 concentration in the CAPS population to elucidate whether the circulating MIC-1 levels are related to prostate cancer and MIC-1 genotype and whether MIC-1 levels are associated with prostate cancer risk. At the time of the study sera from 620 controls and 1116 cases from the CAPS study were available for analyses. 19 prostate cancer cases were excluded from further statistical analyses since clinical data were missing for these subjects.

Examination of serum MIC-1 levels among control subjects revealed that MIC-1 concentrations increased with increasing age (P<0.001) (Figure 4). This is an interesting and important finding since it influences the interpretation of the serum analyses. A number of investigations of potential serum markers for cancer (including MIC-1), have used a small number of control subjects, drawn from volunteers or blood donors, limiting the scope to identify possible correlations between serum levels and age. Further, using a control group that differs significantly in mean age from the case subjects could result in significant difference in serum concentration between cases and controls that are solely due to the differences in mean age between the two
groups. The reason for the relationship between age and serum MIC-1 levels found among the controls in this study is unclear, but may be related to an increased general inflammation burden.

The mean serum MIC-1 level among all prostate cancer patients analysed was 1357 pg/ml, SD=1534, which was significantly higher (P= 0.004) than the MIC-1 level among the control subjects (1190 pg/ml, SD=942) (Table 6) In addition, MIC-1 levels were significantly higher (P<0.001) among patients with advanced disease than among patients with localised prostate cancer, (mean 1666 pg/ml, SD=2138 and 1121 pg/ml, SD=733, respectively (Table 6). The prostate cancer group was further divided into subgroups depending on the treatment (watchful waiting, hormonal treatment, radical prostatectomy and radiotherapy) they received. For 43 patients, treatment was not specified so these patients could not be classified. Significant differences in serum MIC-1 level between the different treatment options were observed (P<0.001). However, multiple ANOVA analyses of MIC-1 levels with age, disease stage and treatment as independent factors revealed significant effects of age and disease stage, while no significant effect was observed for treatment.
Table 6. Serum MIC-1 level of all prostate cancer patients and controls according to stage and treatment

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>Mean ± S.D</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MIC-1 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td>620</td>
<td>1190±942</td>
<td></td>
</tr>
<tr>
<td><strong>Prostate cancer cases</strong> (all)</td>
<td>1097</td>
<td>1357±1534</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>622</td>
<td>1121±733</td>
<td></td>
</tr>
<tr>
<td>Advanced</td>
<td>475</td>
<td>1666±2138</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Watchful waiting</td>
<td>245</td>
<td>1278±773</td>
<td></td>
</tr>
<tr>
<td>Radical prostatectomy</td>
<td>219</td>
<td>1059±929</td>
<td></td>
</tr>
<tr>
<td>Hormonal treatment</td>
<td>506</td>
<td>1575±2057</td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>84</td>
<td>1153±573</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Analysis of variance (ANOVA)

To further refine the correlation between serum MIC-1 levels and disease stage, the prostate cancer group was stratified based on the TNM classification. The stratified analysis revealed a significant increase in serum MIC-1 levels with increasing TNM-stage (P<0.001, age adjusted)(Figure 5). The same trend was observed when only untreated cases (n=245) were included in the analysis (P=0.029) (data not shown), clearly indicating that this phenomenon is not related to treatment effects. All together these results clearly demonstrate a relationship between MIC-1 serum level and tumour burden. These data are consistent with previous reports that patients with metastatic cancer had significantly elevated serum MIC-1 levels compared with other cancer patients (Brown et al., 2003). However, if the elevation is a secondary effect of the tumour growth or whether MIC-1 plays an active role in the disease progression, where an increase in serum concentration enhances tumour growth, is still needed to be investigated.
A possible association between serum levels and prostate cancer risk were also assessed. Using logistic regression analysis, a serum MIC-1 level above the median of the controls is indicated to be a significant predictor for development of prostate cancer. Thus, considering a cut-off serum level of > 1000 pg/ml, the relative risk for development of prostate cancer was 1.5 (95% CI, 1.1-2.0). To test the ability of the serum assay to differentiate prostate cancer cases from control subjects, ROC analyses were performed. The ROC curve clearly showed that there is no diagnostic usefulness MIC-1 (area under ROC curve: 0.56) (Figure 6). On the other hand, the clear relation between clinical stage and serum MIC-1 levels indicates that MIC-1 may be useful as a prognostic factor, where high serum concentrations at diagnosis of prostate cancer are associated with a poor prognosis. For this purpose, serial measurements of MIC-1 among diagnosed cancer patients would probably be of greater value than a single observation.
Finally, to test for possible association between MIC-1 genotype and serum levels, we compared serum levels between control subjects with different genotypes according to the H6D polymorphism. No significant differences in MIC-1 serum levels between the genotypes was observed ($P=0.41$) (Figure 7). We therefore suggest that the H6D polymorphism may alter the function or stability may of the protein, for example the D allele may be more biologically active or function in a different way compared to the H allele and thereby alter the risk for prostate cancer development.

Another important question needed to be answered, but that is beyond the result from this study, involves the role that MIC-1 may play in the development of prostate cancer. The increase in serum MIC-1 level associated with the presence of prostate cancer implies that an inflammatory process in the prostate may be involved in the development or progression of prostate cancer, since in vitro studies have shown that MIC-1 may function as a suppressor of
macrophage-mediated proinflammatory activity. However, studies of several tumour cell lines have also shown that MIC-1 has proapoptotic and antitumorigenic activities (Bauskin et al., 2005). Brown et al. proposed the following explanation for this dual role of MIC-1 in cancer progression. As the prostate cancer tumour progresses, MIC-1 secretion may rise, leading to an inhibitory effect on tumour growth. However, this inhibitory effect may be negated by the decrease in local tumour immunity, mediated by the immune suppressor effect of MIC-1, and thus providing a mechanism for tumour escape (Brown et al., 2003).

Another explanation for the incongruous indications of both proapoptotic and antitumorigenic functions for MIC-1 – together with the striking correlation between elevated MIC-1 levels and metastatic progression of prostate, breast and colorectal cancer – is related to MIC-1’s similarity to the TGF-β protein. It has been reported that MIC-1, like TGF-β, requires an intact signaling pathway mediated by type I and type II TGF-β receptors, as well as receptor-activated Smad4 to exert its effects (Tan et al., 2000). TGF-β is considered to act as a tumor supressor during the early stages of cancer (by inhibiting cell proliferation), and as a promoter of growth and metastasis in later stages, thereby enhancing angiogenesis, immunosupression and synthesis of extracellular matrix (Dumont et al., 2003).

![Figure 7. Correlation between MIC-1 genotypes and serum level in 620 controls without prostate cancer. No significant difference in MIC-1 serum levels among control subjects with different genotypes was observed (P=0.41).](image)

H6D genotype

**MIC-1 (pg/ml)**

- CC
- CG
- GG

**Figure 7. Correlation between MIC-1 genotypes and serum level in 620 controls without prostate cancer. No significant difference in MIC-1 serum levels among control subjects with different genotypes was observed (P=0.41).**
In conclusion, a clear association between disease stages and serum MIC-1 levels was found, indicating that MIC-1 may be involved in prostate cancer tumorigenesis. Moreover, our results indicate that serum MIC-1 levels are associated with an increased risk for prostate cancer. We also assessed the clinical value of the MIC-1 protein as a serum marker. Our data revealed that the differences in serum MIC-1 levels between cases and controls are not large enough to provide a discriminatory test for the presence of prostate cancer. However, the clear relation between clinical stage and serum MIC-1 levels indicates that MIC-1 may be useful as a prognostic factor, high serum concentrations being associated with a poor prognosis.
Discussion

Association studies

Association studies offer a potentially powerful approach to identify genetic variants that influence susceptibility to complex diseases, but are afflicted by the impression that they are not consistently reproducible. Nevertheless, a number of complex diseases have been consistently shown to be associated with specific genetic variants, including thrombophilia, Alzheimer’s disease, and diabetes types 1 and type 2 (Hattersley et al., 2005). The susceptibility variants identified to date either common, and carry a modest risk, or uncommon and carry a substantial relative risk. However, few of the many possible associations investigated in published cancer-genetic association studies have been subsequently established beyond reasonable doubt (Pharoah et al., 2004). In other cases, the results of further investigations have generally been conflicting, for several possible reasons, including population stratification, small sample sizes, and misclassification of study subjects.

One of the major factors hindering association studies of prostate cancer are that prostate cancer is often detected by PSA measurements in cases where there are no clinically distinguishing features. A further complication is that the presence of prostate cancer is also frequently undetected among controls, partly due to the fact that prostate cancer is commonly a late age of onset disease, and there are often long periods in which it is present but there are no obvious symptoms. Evidence from autopsies indicate that over 50% of men harbour tumours in the prostate by the time they are 60 years old, which means that a larger proportion of men die with prostate cancer rather than from it (Sakr et al., 1994). This makes it difficult to find case-control populations where cases and controls differ significantly in the phenotype of interest, thus reducing the probability of detecting cancer-associated factors.

Another problem that arises when performing association studies of prostate cancer, as well as other complex diseases, is the heterogeneous nature of these diseases. Prostate cancer patients may be predisposed to the condition, due to the inheritance of different sequence variants of the same or different genes from different founders. This causes two major problems. First, inherited susceptibility could vary significantly among ethnic groups. Second, different distributions of ethnicity among cases and controls could lead to population stratification, so observed differences in genotype frequencies of a variant may partially reflect differences in the genetic background of cases and controls. However, if cases and controls are well matched, differences in the frequency of genotypes should only be seen at predisposition loci.

A further substantial problem is that a large number of genes and genetic variants may affect the risk for prostate cancer through different mechanisms, and the effect of each given gene, or variant of a gene, is likely to be minor in the general population. Such polygenic effects could apply even in a homogenous population.
This phenomenon is observed in other complex diseases, as reported in a recent meta-analysis of genetic association studies in complex diseases (Lohmueller et al., 2003). After evaluating 301 published studies that attempted to replicate reported disease associations for 25 different genes, the study confirmed the associations for only eight of the 25 genes. Strikingly, seven of these eight genes were associated with modest estimated genetic effects (ORs between 1.07 and 1.76 in the pooled analyses). This highlights the need to use studies with large and well characterised populations to identify such effects convincingly. For example, in order to detect a sequence variant with a frequency of 20% in the population conferring a relative risk of 1.5 (50% increased risk) a study of approximately 1000 cases and 1000 controls will be needed (Pharoah et al., 2004). Historically, the majority of genetic association studies have analyzed a relatively small number of study subjects, leading to both false positive and false negative reports.

The genotyping quality is an important factor for a successful and reliable association study (Hattersley et al., 2005). Robust quality control needs to be implemented in the genotyping procedure, including both positive and negative controls and at least 5% repeated study samples. To assure reliable results both cases and control samples should be included on the same plate and, furthermore, the case-control status of each sample should be blinded during genotyping. Most reported deviations from Hardy-Weinberg equilibrium originate from problems with genotyping methods.

Another issue highlighting a problem in interpreting the findings of an association study is the risk of type I error due to multiple testing. As a result of a large number of possible candidate genes and multiple sequence variants with in a gene, there is the substantial problem of multiple tests. To overcome this problem, correction methods have been suggested that compensate for multiple testing. However, in analysis of variance, the multiple testing is further complicated by the fact that the comparisons are not independent. Due to linkage disequilibrium among closely spaced sequence variants the different tests of the variants within a gene or other defined region will not be independent. At present there is a lack of appropriate methods to adjust for multiple, dependent tests (Cordell et al., 2005).

In addition, a candidate variant that segregates with a specific condition may not be causally linked to it, but could be in linkage disequilibrium (LD) with the causal variant or variants. A number of variants that are in LD with a specific variant of interest are likely to be associated with a disease even if there is only one underlying variant that causes the risk. Systematically studying a set of hSNPs that may or may not be functionally relevant to a disease, but capture all the sequence variation in the entire genes, makes it possible to assess whether sequence variants in the genes are associated with a particular disease (Johnson et al., 2001). If the association is confirmed in an independent population, the region that is represented by the associated hSNP or hSNPs needs to be sequenced. After identifying all genetic variants within the region, \textit{in vivo} and \textit{in vitro} functional studies of the identified sequence variants will be needed to finally establish the identity of the causal variant.

Finally, performing an association study with one candidate SNP also has another major drawback when no association is found, since the lack of association does not
exclude the possibility that there may be another important variant in the same gene. By using the tagging SNP approach, in which the variance in the entire gene is evaluated, it is easier to obtain evidence that could exclude the possibility that a candidate gene plays a role in prostate cancer pathogenesis (Pharoah et al., 2004).

We have performed association studies to evaluate the role of sequence variants in three different genes in prostate cancer susceptibility, and have therefore taken into account the issues described above. The criteria needed to perform a well-designed association study have been fulfilled with varying degrees in the three studies.

In study I, we used a homogenous population with minimal risks for population stratification drawn from the NSHDC. Each case had two controls matched in terms of sex, age, date at recruitment and geographical residency. However, the NSHDC case-control study sample is too small to detect modest genetic risks (OR 1.5-2.0) such as those expected for the five common sequence variants evaluated (PRO3, INDEL1, IVS5-57, P275A, INDEL7). For instance, to evaluate convincingly the effects of the rare mutation R293X, which is present at a frequency of 3% in the general population, according to our observations, and increases prostate cancer risk by a factor of two (OR ~2.0), a much larger case-control study would be needed.

In Studies II and III we used 780 controls and 1383 cases drawn from the CAPS study, a population-based case-control study that recruited subjects from 70% of the Swedish population. The genetic background of the Swedish population is homogenous, which significantly reduces the risk for population stratification. In addition, the population-based study was carefully designed, and almost all patients who met the inclusion criteria enrolled in the study. Control subjects were frequently matched to case subjects on the basis of residence area, sex and age. Further, the full clinical spectrum of prostate cancer was well represented, with over 45% of cases having advanced disease. The large number of study subjects increases the statistical power to detect a modestly altered risk for prostate cancer. However, it should be noted that the statistical power to detect a risk genotype for an allele present at low frequencies is still limited unless the study is very large. For example, to have 80% power to detect a risk genotype that confers an OR of 1.4 (at a 5% significance level according to two-sided tests), the risk genotype need to be present in 5% of the population. If the risk genotype only confers an OR of 1.2, it needs to be present in 20% of the population to have 80% power to detect the genotype (Figure 8). Finally, a careful quality control was implemented in the genotyping procedure by including both cases and controls (including CEPH samples, blinded repeats and water blanks on the same DNA plates). No indication of genotyping error was observed in Studies II and III, the genotype consistency was 100% for the CEPH control DNA samples, and results from duplicated samples were 100% concordant, giving an estimated error rate of 0%.
Cytokine polymorphisms in prostate cancer

In 1863, Virchow first suggested that cancer originates at sites of chronic inflammation (Balkwill et al., 2001). Chronic inflammation has also been proposed recently to be an important factor in prostate cancer pathogenesis (Platz et al., 2004). Inflammatory processes involve the action of a number of mediators, including metabolites of arachidonic acid, cytokines, chemokines, and free radicals. The balance between the effects of pro-inflammatory and anti-inflammatory cytokines strongly influences the outcome of inflammatory events. Cytokine genes are highly polymorphic and the stability and function of the cytokines that specific variants encode, which in turn can affect the inflammation response, depend on the location and nature of the polymorphisms.
The role of sequence variants in two genes encoding cytokines (MIC-1, IL-1RN) was evaluated in the studies underlying this thesis. Both of these cytokines are important regulators of the inflammatory response, although MIC-1 has also been documented to act as an inhibitor of tumour growth. The haplotype-tagging approaches used enabled us to elucidate most of the genetic variation with a limited number of SNP genotypes.

The anti-inflammatory cytokine IL-1RN is well known for its role as a competitive inhibitor of the pro-inflammatory cytokines IL-1α/β, and thus as a “neutralizer” of the physiological and pathophysiological inflammatory responses. Our results revealed that the most common haplotype of IL-1RN was associated with an increased risk of prostate cancer. The genetic analysis of the MIC-1 gene showed that a non-synonymous polymorphism in exon two was associated with prostate cancer. The polymorphism alters an amino acid in the mature protein, but although this variant has been previously reported, no functional studies have evaluated its biological role. Further studies of other types are needed to elucidate the biological rationale for the association between sequence variants in these two genes and prostate cancer, but it is tempting to speculate that these variations could be linked to an imbalance in the inflammatory process.

Polymorphisms affecting susceptibility to complex disorders, like prostate cancer, are likely to be fairly common in the population, and therefore should not be deleterious for the carrier. They are unlikely therefore to disrupt gene activity or protein function completely, but instead affect gene expression levels, mRNA stability, protein folding or the affinity of the encoded proteins to their receptors or substrates. These polymorphisms might be beneficial in some situations and harmful in others. For example, decreased stability of the mRNA or protein encoded by the MIC-1 gene could lead to a prolonged inflammatory response, which could be advantageous for combating infectious agents. However, chronic exposure of inflammatory mediators leads to increased cell proliferation, mutagenesis, oncogene activation, and angiogenesis - the ultimate results of which will be proliferation of cells that have lost normal growth control (Shafer et al., 2002).

Another interesting observation regarding the association between these two genes and prostate cancer risk is the fact that both genes are expressed in macrophages. Macrophages, together with lymphocytes, are the predominant cell types in areas of chronic inflammation, a condition that is extremely common in the prostate, but are rarely found in tumour tissues. Furthermore, increased macrophage activity and infiltration of lymphocytes in the tumour has been found to be related to poor prognosis in prostate cancer (Irani et al., 1999; Lisbrant et al., 2000; McArdle et al., 2004). Both MIC-1 and IL1-RN are anti-inflammatory cytokines; MIC-1 has been shown to down-regulate the overall activity of macrophages, whereas IL1-RN is known to inhibit the function of IL1-α/β, and thereby decrease the pro-inflammatory activity of the macrophage. Since macrophages produce a number of mediators that strongly influence cell proliferation and differentiation under both physiological and pathological circumstances, as well as being the key cells regulating reactions leading to and driving chronic inflammation, increases in their activity could enhance tumour initiation and progression. Sequence variants in these two genes could be the causes of such increases in activity.
In collaboration with a US research group at Wake Forest University we systematically evaluated associations between sequence variants of 20 inflammatory genes and prostate cancer. The genes were selected based on their roles in inflammation and the results of gene expression profiling studies in prostate cancer tissue. Sequence variants of eight of these genes – MIC-1, IL1-RN, TLR-1, TLR-4, TLR-6, TLR-10, IL-10, and COX-2 (Lindmark et al., 2004, 2005; Zheng et al., 2004; Sun et al., 2005) – were found to be associated with prostate cancer risk. The high frequency of positive results obtained for this group of genes clearly indicate that genes related to inflammation processes may play an important role in the development of prostate cancer.

So far, most studies evaluating possible associations between sequence variants and prostate cancer risk have tended to focus on one gene at a time, even though the aetiology of prostate cancer cannot be explained by genetic variability in a single gene. One reason for the focus on single genes is probably that most studies lack the statistical power to evaluate the combined effect of several genetic polymorphisms. However, there is a need to assess the effects of multiple genes simultaneously, as clearly demonstrated by considering how cytokines function. Cytokines act in a highly complex coordinated network in which they induce or repress their own synthesis as well as that of other cytokines and cytokine receptors (Howell et al., 2002). Furthermore, there is often considerable overlap and redundancy between the functions of individual cytokines. Due to their highly interrelated functions, it is very important to simultaneously evaluate polymorphisms in the genes encoding cytokines, their receptors and downstream effectors. In an attempt to do so, Xu et al. explored the joint effects of 20 genes (mentioned above) involved in the inflammation pathway on prostate cancer risk. Using a data-mining method (MDR), they found that the interactions of four SNPs in these inflammatory genes (one SNP each from IL-10, IL-1RN, TIRAP, and TLR5) are significant indicators of prostate cancer risk (Xu et al., 2005).
Future perspectives

Many questions arise from the studies appended to this thesis, but the overall findings are in concordance with the growing body of evidence that chronic inflammation plays an important role in the development of prostate cancer. In the two association studies reported in Papers II and III we detected sequence variations in two inflammatory genes that alter the risk for developing prostate cancer. Since these findings are novel, there is a need for large-scale, independent confirmatory studies. However, if the results are corroborated, the next steps would be to identify the causal variants in these regions and to elucidate the underlying mechanism(s) responsible for these associations. Since the cytokine pathways are highly interrelated it is possible that polymorphisms in several related cytokines are needed to cause disease. Furthermore, the effect of variations in the ability to influence an inflammatory response may not be manifest unless exposure to certain infectious agents or oxidants occurs. These issues may have to be addressed in order to identify the casual variant or variants in the cytokine genes.

The results of Study IV clearly showed a positive correlation between serum MIC-1 levels and disease stages. Among the group of patients with TNM-stage IV there were large variations in MIC-1 values. A recent study found that mice transfected with prostate cancer cell line DU145 expressing human MIC-1 dramatically lost weight, and the degree of weight loss was proportional to serum levels of tumour-derived human MIC-1. This effect could be reversed in a dose-dependent manner by injecting the mice with a monoclonal antibody specific for human MIC-1 (personal communication, Samuel Breit). Collectively, these observations raise the possibility that the large variations in MIC-1 values seen among stage IV patients could influence the outcome for these patients. Performing a follow-up study in which clinical parameters and disease-progression-data are collected regarding the cases included in the CAPS study, could help assess this possibility.

MIC-1 has been reported to be expressed in both epithelial cells and macrophages, two types of cells that are abundant in tissues in, and adjacent to, prostate tumours. To further elucidate the role of MIC-1 in cancer pathogenesis, it would be valuable to determine how much MIC-1 (if any) these cell types produce in normal prostate tissue and the various prostate cancer stages. This could be done by analyzing protein expression in prostate tissue sections from a selected number of cases and controls drawn from the CAPS study. Another approach would be to use sections of prostate tissue with various disease stages from TRAMP mice to examine the localisation and expression pattern of the MIC-1 protein.

In summary, although multiple pieces of evidence clearly indicate that prostatic inflammation plays an important role in the development of prostate cancer, much work remains to be done to elucidate the underlying mechanisms.
Conclusions

Based on the findings in Paper I-IV, the following conclusions can be made:

- Genetic analysis in a large number of subjects from two different study populations in Sweden failed to support a role of sequence variants in the MSR1 gene in the development of hereditary or sporadic prostate cancer.

- A comprehensive evaluation of a possible association between sequence variants of IL1-RN gene and prostate cancer susceptibility revealed that the most common haplotype in the IL1-RN gene confers an increase risk for prostate cancer. These findings highlight the advantages of studying all variation in a gene with the use of a haplotype-tagging approach instead of testing a limited number of single sequence variants.

- A large population-based association study provided strong genetic data supporting an association between the nonsynonymous polymorphism H6D in the MIC-1 gene and prostate cancer risk. More studies are needed to understand the mechanism by which sequence variation in this gene affect the function of the MIC-1 protein which in turn confers an altered prostate cancer risk.

- MIC-1 serum levels are elevated in prostate cancer patients compared to control subjects. In addition, MIC-1 serum levels were significantly associated with age and disease stage. The differences in serum concentration between prostate cancer patients and healthy individuals are not large enough to provide a discriminatory diagnostic test for the presence of this disease. However, serial measurements of MIC-1 may be useful in the management of prostate cancer.

- The findings of the studies underlying this thesis are in overall concordance with the growing evidence from epidemiological, molecular, and histopathological studies that chronic inflammation plays an important role in the development of prostate cancer. The results indicate that further research is required on the possible roles of genetic variation in genes involved in the inflammatory process and on how this variation may affect susceptibility to, and development of, prostate cancer.
Acknowledgements

The work for this thesis was performed at the department of Radiation Sciences, Oncology, Umeå University. I hereby wish to sincerely thank all of those involved in helping me to complete it. In particular I would like to thank:

**Henrik Grönberg**, my supervisor, for excellent scientific guidance and for helpful discussions throughout my research studies. Also thanks for your great understanding in how I have organized the research studies together with my medical studies.

**Jianfeng Xu** and **Lilly Zheng**, for your excellent collaboration and assistance in experiments and for stimulating the scientific discussions.

**All co-authors**, for contributing to my work.

All the people in the research group **Björn-Anders J** for friendship and for all interesting discussions in many different areas, and for always being there with a helping hand, **Fredrik W** for your invaluable and comprehensive statistical help and for given me self-confidence at the squash court, **Ingela G** for truly skilful laboratory assistance and **Lena L** for keeping track on everything and helping me when I have run out of time, **Monica E**, **Elisabeth S** and **Karin A** for all your work with the study populations, **Kristina C**, for god company during the writing procedure, and **Camilla T**, **Beatrice M**, **Sara L**, **Stina** for valuable comments and enthusiasm in my research.

**Mark Hunter**, for introducing me into the tricky business of MIC-1 ELISA.

**Anders Bergh**, for all interesting discussions and for valuable comments on the final draft of this thesis.

All the colleagues at the Department of Medical Biosciences, for providing a nice working-atmosphere.

**Carina Ahlgren** and **Anna Wernblom** for excellent help no matter what kind of problems I have brought to you.

To all I have forgotten to mention **Gun-Brith** and **Royne**, my parents in law, for all your support both of my family and me.

**My parents**, **Monica** and **Jörgen** for devoted support and for always believing in me.

**Margareta** and **Freddie** for being part of the family and for your support.

**Benjamin** and **Tuva**, our wonderful kids, for always helping me keep things in perspective and for bringing so much joy into our lives.

**Anna**, my love, for sharing your life with me, and for your endless support.
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