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THE INFECTIOUS PATHOGENESIS OF PROSTATE CANCER

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Accumulating evidence points to a role of chronic inflammation in the pathogenesis and progression of cancers, including prostate. Infections are important agents in the genesis of inflammation. For prostate cancer, several lines of evidence point to a role of infections as important agents, although no specific infection has consistently been identified. In this project, we are examining two specific infectious agents with respect to prostate cancer: <i>T vaginalis</i> , the most common non-viral sexually transmitted infection, and the recently identified retrovirus XMRV. The aims of this study are 1-) To assess the role of the newly identified XMRV virus in prostate carcinogenesis and progression; 2-) To characterize the role of the infectious protozoa <i>T. vaginalis</i> in prostate carcinogenesis and progression. The current study is nested within the Swedish Watchful Waiting Cohort, a population-based cohort of 1,256 Swedish men diagnosed with localized prostate cancer. During 28 years of follow-up, 320 men have died of cancer, and thus this is a powerful population in which to examine determinants of prostate cancer progression. A tumor repository from archival tissue specimens have been collected from all men in the cohort and will be used to assay for presence of the infections.					
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# Table of Contents

	<u>Page</u>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>5-7</b>
<b>Key Research Accomplishments.....</b>	<b>8</b>
<b>Reportable Outcomes.....</b>	<b>9</b>
<b>Conclusion.....</b>	<b>10</b>
<b>References.....</b>	<b>11</b>
<b>Appendices.....</b>	<b>12-26</b>
<b>Supporting Data.....</b>	<b>27-29</b>

## INTRODUCTION

Prostate cancer has considerable biologic heterogeneity, such that some men experience an aggressive course while many have a slow growing or indolent disease (1-3). Thus, central issues in prostate cancer research are to identify mechanisms which are amenable to prevention and treatment, and to understand pathways that lead to aggressive cancer.

A growing body of epidemiologic, genetic and molecular pathological data points to the role of chronic inflammation in the pathogenesis and progression of prostate cancer (4). The pathways involved in chronic inflammation induce cellular damage and compensatory cellular proliferation (5). Clinical prostatitis, which occurs in approximately 9% of men between the ages of 40 and 79, has been linked to prostate cancer in several epidemiologic studies (6). Moreover, surgical prostate tumor specimens often exhibit histological evidence of prostatitis, although the determinants of this prostatic inflammation are unclear.

Perhaps of greater importance, data also suggests that the degree of inflammation may be a predictor of more aggressive disease. In a study of 161 men undergoing radical prostatectomy (7), 5-year recurrence-free survival was significantly lower among patients with high-grade inflammation in malignant tissue (27%) than in patients with low-grade or no evidence of inflammation (65%), independent of Gleason grade, preoperative PSA level, and pathologic stage.

Infectious agents are likely targets involved in the initiation and exacerbation of chronic inflammation, and infections can lead to increased risk of several cancers (8). Indeed, an estimated 15% of malignancies globally are thought to have an infectious etiology (9, 10). Infectious agents may also have direct effects on carcinogenesis through the transformation of cells via incorporation of active oncogenes into the host genome, inhibition of tumor suppressors, stimulation of proliferation signals, or through immune suppression. Known oncogenic infections are typically highly prevalent within the host population, persistent within the host, and require a variety of co-factors for malignant transformation.

Two papers (11, 12) provide emerging evidence suggesting involvement of the newly identified murine-like retrovirus XMRV and the protozoan *T. vaginalis* in prostate cancer; these are the focus of the proposed study. The objective of the proposed study is to evaluate and extend the initial findings on *T. vaginalis* and XMRV, and to more fully characterize the potential role of these infections in the pathogenesis and progression of prostate cancer in large population-based cohorts of men with prostate cancer who have been followed prospectively for more than two decades.

## BODY

### **Aim I. To assess the role of the newly identified XMRV virus in prostate carcinogenesis and progression.**

A major effort of this year has been the assay development for the XMRV virus to apply on our TURP and prostatectomy cohorts. Our initial approach had been to use immunohistochemistry on our constructed tissue microarrays to characterize for presence or absence of the XMRV virus, and an antibody has been raised against XMRV by a group at Columbia (13). However, protein expression was noted in the prostate tumor epithelium, rather than in the stroma which had been seen in the initial publication (12). As such, we have concerns about the specificity of the protein expression. Thus, we have decided to use RTPCR on DNA extracted from the tissue specimens. We have been developing the protocol for the experimental conditions, and the extraction is scheduled to take place during the early summer 2010.

The pathologist has also completed review of cases for circling areas of benign tissue on the tumor blocks for extraction of DNA for the characterization of RNASEL genotype. In preliminary work, we have shown excellent yields of DNA (100-200 ng) from 3 cores of benign tissue, which will be more than sufficient for our genotyping assays. DNA has been extracted from the cases, and the genotyping will be undertaken using a sequenom platform.

We have completed two biomarker studies on the tumor tissue microarrays that are critical to this study: 1-) Tumor apoptosis using a TUNEL assay to assess extent of tumor tissue undergoing apoptosis, and 2-) cellular proliferation assessed by immunohistochemistry with antibody to *ki67*. The data on these two markers have been cleaned and outputted as SAS databases.

Our pathologist has undertaken and completed a comprehensive review of all cases for evidence of atrophy lesions and chronic and acute inflammation. He reviewed all available H&E slides from the 680 men. We have characterize for presence of simple atrophy (SA), simple atrophy with cyst formation (SACF), post atrophic hyperplasia (PAH) and partial atrophy (PA); data on evidence of high grade PIN and perineural invasion, as well as for evidence of acute and chronic inflammation (none, mild, moderate/severe). The data were entered into an ACCESS database, indicated by a Research ID, have been reviewed for quality control, and are now linked together with the clinical database (Gleason grade, T stage, tumor extent, body mass index, date of diagnosis, date and cause of death) as a SAS database.

In preliminary statistical analysis of these data, we have found substantial evidence of atrophy, with 73% of cases showing at least one atrophic lesions, including 20% with evidence of the proliferative atrophic lesion PAH. Both chronic (26%) and acute (14%) atrophy were also commonly evident in the tissue specimens. Among 680 men, 220 died of prostate cancer. **(Supporting Data, Table 1)** We found no overall evidence of PAH and prostate cancer-specific mortality (Odds ratio 1.3, 95% confidence interval 0.8-1.7). However, men who had both evidence of PAH and moderate to severe inflammation were significantly more likely to die of prostate cancer (Odds ratio 2.5, 95% confidence interval 1.3-3.8) suggesting a joint effect of atrophy and inflammation in prostate cancer progression. The data on inflammation and atrophy will play a critical role in our understanding of the infectious pathogenesis of prostate cancer.

### **Aim II. To characterize the role of the infectious protozoa *T. vaginalis* in prostate carcinogenesis and progression.**

Much of the preliminary work summarized in Aim I is directly relevant to Aim II of the project, including the clinical data review, tissue retrieval, histologic evaluation, TMA construction, and atrophy/inflammation assessment.

## Related work

**Serologic evidence of *T vaginalis*.** Related to this project, we have published a study of *T vaginalis* serostatus measured in prediagnostic blood and prostate cancer risk and progression in the Physicians' Health Study among 673 incident prostate cancer cases and 673 matched controls. The bloods were collected and stored prospectively, and cases were diagnosed from 1982-2000. The men have been followed prospectively through 2009 for cancer-specific and overall mortality. We found a suggestion of *T. vaginalis* seropositivity and overall prostate cancer risk (Odds ratio 1.23; 95% confidence interval (CI): 0.94-1.61). Moreover, seropositive men had a 2.2-fold increase in risk of extraprostatic prostate cancer (95% CI: 1.08-4.37) and a 2.7-fold increase in risk (95% CI: 1.37-5.28) of cancer that would ultimately progress to distant metastases or prostate cancer-specific death. These data provide compelling evidence of a role of this infectious protozoan in the progression of prostate cancer. The data were presented as an Oral Session in the American Association for Cancer Research Frontiers in Cancer Prevention meeting, and a manuscript of these data was published in *Journal of the National Cancer Institute* in 2009 and appears in this report as **Appendix 1**.

**Correlates of Atrophy/inflammation.** Using an identical approach to characterizing atrophy and inflammation in the Swedish cases, our pathology team is now reviewing men with prostate cancer who are participants in the Health Professionals Follow-up Study and Physicians' Health Study. In line with the Swedish cases, we found that one-third of cases exhibited moderate-severe chronic inflammation; one-quarter of the cases had evidence of post-atrophic hyperplasia. The prevalence of both PAH and simple atrophy were higher among specimens that also contained substantial chronic inflammation (**Supporting Data, Figure 1**). Two of the atrophic lesions, simple atrophy (SA) and simple atrophy with cyst formation (SACF) were positively associated with older age at diagnosis, while the PAH lesions were positively associated with tumor proliferation. (**Supporting Data, Figure 2**). The frequency of PAH lesions was substantially higher among men with a greater adiposity, as determined by waist to hip ratio.

**RNASEL.** *RNASEL* located at chromosome 1q25 encodes ribonuclease L, part of the interferon-mediated immune response to viral infection. The initial publication on XMRV identified the virus through comparing differential viral probes according to variation in *RNASEL*. We investigated the association between variation in *RNASEL* and prostate cancer risk and progression in a study of 1286 cases and 1264 controls nested within the prospective Physicians' Health Study. Eleven SNPs were selected using the web-based *Tagger* in the HapMap CEPH panel. Unconditional logistic regression models assessed the relationship between each SNP and incident, advanced stage (T3/T4, T0-T4/M1, lethal disease), and high Gleason grade ( $\geq 7$ ) prostate cancer. Further analyses were stratified by calendar year of diagnosis. Cox proportional hazards models examined the relationship between genotype and prostate cancer-specific survival. We also explored associations between genotype and serum inflammatory biomarkers interleukin-6 (IL-6), C-reactive protein (CRP), and TNFR2 using linear regression. Individuals homozygous for the variant allele of rs12757998 had an increased risk of prostate cancer (AA vs. GG; OR: 1.63, 95% CI: 1.18-2.25), and more specifically, high grade tumors (OR: 1.90, 95% CI: 1.25-2.89) (**Supporting Data, Table 2**). The same genotype was associated with increased CRP ( $p=0.02$ ) and IL-6 ( $p=0.05$ ) levels. Missense mutations R462Q and D541E were associated with an increased risk of advanced stage disease only in the pre-PSA era. There were no significant associations with survival. The results of this study support a link between *RNASEL* and prostate cancer, and suggest the association may be mediated through inflammation. These data were presented at the American Association for Cancer Research meeting in 2009, and a manuscript summarizing the data is under submission.

**mRNA signature of Gleason grade.** Gleason grade is a measure of prostate tumor differentiation, and we have previously shown is a strong predictor of prostate cancer survival. We reasoned that distinct sets of genes or pathways affect or are affected by the de-differentiation process and sought to identify an mRNA signature that distinguishes high from low Gleason grade. We measured the mRNA expression of 6,100 genes in prostate tumor tissue from patients in the Swedish Watchful Waiting cohort (N=358) and Physicians' Health Study (PHS, N=109). Comparing individuals with Gleason  $\leq 6$  to those with Gleason  $\geq 8$ , we built a 157-gene signature using Prediction Analysis of Microarrays in the Swedish data with good discriminatory ability; when

this signature was applied to PHS the discriminatory ability remained high. Applying the signature to men with Gleason 7, the probability of being high grade improved a model's ability to predict lethal disease beyond knowing whether the Gleason score was 4+3 or 3+4 ( $p=0.01$ ). Our expression signature may enhance our understanding of the de-differentiation process of prostate tumors and may have clinical applications for men with Gleason 7, improving their classification of a high or low risk of dying from cancer and guiding therapy decisions. A manuscript summarizing these data has been submitted for peer review.

## KEY RESEARCH ACCOMPLISHMENTS

- Completed pathologic review of cohort on extent of inflammation, atrophy, high grade PIN, and perineural invasion on all cases
- Completed biomarker studies on tumor tissue microarrays to assess extent of tumor apoptosis and cellular proliferation
- Created a merged clinical and tissue SAS database, including clinical information, atrophy and inflammation data, and biomarker data for statistical analyses
- Completed review of tissue specimens for indentifying histologically normal tissue for DNA extraction; extracted DNA from benign tissue in pilot study of 92 cases and found excellent DNA yields
- Undertook statistical analyses linking data on inflammation and atrophy in relation to prostate cancer-specific mortality
- Finalized protocol for assessment of XMRV on tissue specimens
- Accepted manuscript on serostatus for *T vaginalis* and prostate cancer risk and progression based on the Physicians' Health Study which was accepted in *Journal of the National Cancer Institute*
- Identified and validated gene signature of Gleason grade, that discriminates lethal outcomes on Gleason 7 tumors, and submitted manuscript summarizing the results
- Identified novel SNP in RNASEL that is associated with high grade prostate cancer, as well as circulating levels of the inflammatory markers; submitted manuscript summarizing the results



## REPORTABLE OUTCOMES

- Post-doctoral fellow working on this project (Jennifer Stark) was promoted to Instructor
- New student working on this project (Mara Meyer) will defend her thesis this month, and will be appointed as Post-doctoral fellow
- Dr. Stark received a 2<sup>nd</sup> year of funding for her Career Development Award from the Dana Farber/Harvard Cancer Center Prostate Cancer SPORE based on an extension of this project
- Dr. Mucci was named the Outstanding Young Investigator from the Prostate Cancer Foundation based on experience supported by this award
- Development of prostate tumor tissue repository of TURP specimens and clinical-tumor database
- Submitted related manuscripts to peer-reviewed journal
- Accepted manuscript in Journal of the National Cancer Institute

## CONCLUSION

We have demonstrated our ability to undertake this large cohort and collect archival tumor specimens from 680 men. We have demonstrated a proven working relationship with the pathology team, as shown by completion of the construction of the tissue microarrays, standardized Gleason grading, evaluation of atrophy and inflammation, and successful completion of biomarkers on the tissue microarrays. Moreover, our preliminary statistical analyses on atrophy-inflammation and prostate cancer-specific mortality, combined with the findings of serologic evidence of *T vaginalis* and prostate cancer mortality, as well as the SNP in RNASEL provide supportive evidence for the study hypothesis.

During the next year, we will complete the XMRV assessment on the tissue specimens and also proceed on the analyses related to atrophy and inflammation. Ultimately, this project has the potential to provide strong evidence (for or against) in assessing the role of infectious agents and inflammation in prostate pathogenesis. Moreover, the tumor tissue repository we are establishing is a unique resource in which to test future hypothesis. Given the substantial biologic heterogeneity of prostate cancer, the proposed project would ultimately have exciting implications for prevention and potentially treatment of prostate cancer.

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## APPENDICES

Publication in JNCI on T vaginalis and prostate cancer by Stark JR et al.

***Trichomonas vaginalis* infection and prostate cancer incidence and mortality: a prospective study in the Physicians' Health Study**

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**Running title:** *T. vaginalis* and Prostate Cancer

**Key words:** prostate cancer, inflammation, sexually transmitted infection, *Trichomonas vaginalis*

## Abstract

**Background:** A recent nested case-control study found that the presence of antibodies against *Trichomonas vaginalis*, a common non-viral sexually transmitted infection, was positively associated with subsequent incidence of prostate cancer. We sought to confirm these findings in an independent population, as well as to relate serostatus to prostate cancer progression.

**Methods:** We conducted a case-control study nested within the Physicians' Health Study that included 673 prostate cancer cases and 673 individually matched controls. We used conditional logistic regression to estimate the odds ratio of incident prostate cancer and Cox proportional hazards regression to estimate hazard ratios of prostate cancer death/development of bony metastases among cases.

**Results:** Although not statistically significant, the magnitude of the association between *T. vaginalis* seropositivity and overall prostate cancer risk (OR: 1.23; 95% CI: 0.94, 1.61) was similar to the original study. Further, seropositive men had a 2.2-fold increase in risk of extraprostatic prostate cancer (95% CI: 1.08, 4.37) and a 2.7-fold increase in risk (95% CI: 1.37, 5.28) of cancer that would ultimately progress to distant metastases or prostate cancer-specific death. Cases with serologic evidence of infection prior to cancer diagnosis had a 51% greater rate (95% CI: 1.03, 2.20) of progressing to bony metastases or dying from prostate cancer.

**Conclusions:** In this large prospective case-control study, we confirmed the previously reported association between anti-*T. vaginalis* antibodies and prostate cancer risk and found that *T. vaginalis* infection was principally associated with clinically relevant, potentially lethal prostate cancer.

## Introduction

A number of inflammation-related factors have been implicated in prostate cancer risk and progression, but the origin of inflammation is unclear (1). Infections are one possible source. *Trichomonas vaginalis* is a common non-viral sexually transmitted infection (STI), with an estimated 174 million annual infections globally (2). Prevalence in American men ranges from approximately 3% among young men in the general population (3) to 65% among military personnel with nongonococcal urethritis (4). Little is known about the prevalence of infection in older men, but in contrast to other common STIs, the infection has been observed to be more prevalent among men in their late twenties or thirties than in 18-20 year old men (3, 5). Urethral symptoms associated with *T. vaginalis* tend to be less severe than other common STIs such as *Chlamydia trachomatis* or *Neisseria gonorrhoeae* (6). Further, *T. vaginalis* is frequently associated with asymptomatic infections in approximately half to three-quarters of cases (7, 8). Consequently, many men are unaware that they are infected with the parasite.

Men infected with *T. vaginalis* often experience spontaneous resolution, as evidenced by decreasing rates of infection with time since last sexual contact with an infected partner (9) and a natural history study, where 5 of 14 individuals cleared the infection without treatment within approximately two weeks (7). Nevertheless, a smaller proportion of men experience long-term asymptomatic infection (7). *T. vaginalis* can ascend the urethra to the prostate and infect prostate epithelium (10, 11), where it is associated with evidence of acute and chronic inflammation (10). Among men with prostatitis, *T. vaginalis* has been isolated in a wide range of cases depending on the study, but was found in up to 85% of men with antimicrobial response failure or long-term symptoms (12). As such, chronic prostatic infection with *T. vaginalis* may initiate an inflammatory response that could increase the risk of developing prostate cancer (10), and potentiate disease progression.

A recent case-control study nested in the Health Professionals Follow-up Study (HPFS) found that seroprevalence of *T. vaginalis* infection was positively associated with subsequent prostate cancer risk, with a suggestion of the greatest risk for more aggressive disease defined by high Gleason grade disease (13). To follow up on the positive finding between *T. vaginalis* serostatus and prostate cancer risk, we conducted a large nested case-control within the Physicians' Health Study (PHS). Chronic inflammation may potentiate

prostate cancer progression, and thus we also evaluated potential associations between antibody status and time to lethal prostate cancer.

## Methods

**Study Population.** The PHS I (14, 15) was initiated in 1982 as a randomized, double-blind, placebo-controlled trial of aspirin and  $\beta$ -carotene for the primary prevention of cardiovascular disease and cancer. The study included 22,071 healthy U.S. male physicians age 40 to 84 years at baseline. Prior to randomization, 14,916 of the men (68%) provided a blood sample (16). These participants comprise the study base for the nested case-control study.

We included 673 cases diagnosed with prostate cancer up to 18 years after blood draw (1982 – 2000) who had available plasma. Controls were selected from the population at risk at the time of the case's diagnosis, i.e., those who had provided blood, had not had a prostatectomy, and had not reported a diagnosis of prostate cancer at the time the case developed. For statistical efficiency, controls were individually matched to cases by age (within 1 year), smoking status (never, former, or current), and follow-up time.

**Laboratory Assessment.** Plasma from prospectively collected samples from each case and his matched control subject (stored at  $-80^{\circ}\text{C}$ ) was thawed and assayed for anti-*T. vaginalis* antibodies with an assay that detects IgG antibodies against purified, recombinant *T. vaginalis*  $\alpha$ -actinin protein. ELISA reactions were optimized with known negative and positive pooled sera of uninfected individuals and patients with trichomonosis, respectively, that gave reproducible readings after incubation with microtiter wells containing immobilized  $\alpha$ -actinin. In this study, paired prostate cancer case and control plasma samples were diluted at 1:10 (v/v) in PBS-Tween 20 containing 5% skim milk, and 100  $\mu\text{L}$  of diluted plasma was added to each well. After incubation for 3 hours at  $37^{\circ}\text{C}$ , the plates were washed thrice with PBS-Tween 20 followed by the addition of 100  $\mu\text{L}$ /well of secondary goat anti-human IgG (Fc-specific) conjugated to horseradish peroxidase at a 1:1,500 dilution in PBS-Tween 20 containing 5% skim milk. Plates were incubated again for 1 hour at  $37^{\circ}\text{C}$  and then washed thrice with PBS-Tween 20. Color development was done by adding 100  $\mu\text{L}$ /well of substrate solution (ABTS; phosphate-citrate buffer with 0.03% sodium perborate, Sigma Chemical Co., St. Louis, MO) as



per manufacturer's recommendations, and plates were incubated at room temperature for 10 minutes.

Absorbance values were then obtained by examining the supernatants spectrophotometrically at  $A_{405}$  using an ELISA reader (Bio-TEK instruments, Inc, Winooski, VT).

Case-control pairs were assayed in adjoining wells, with blinding of laboratory personnel as to case-control status. All samples were tested in duplicate. Absorbance scores were based on the reproducibility of ELISA readings compared to known positive and negative controls, and assigned to each sample based on the mean duplicate absorbance value (0, absorbance < 0.24; 1+,  $0.241 \leq$  absorbance < 0.371; 2+,  $0.371 \leq$  absorbance < 0.441; 3+,  $0.441 \leq$  absorbance < 0.542; 4+, absorbance  $\geq$  0.542). Samples with absorbance scores of 3+ or 4+ were considered positive for history of trichomoniasis. We included 29 QC pairs/trios randomly distributed across plates. Concordance in serostatus was achieved for 90% of the QC pairs/trios; 17 of 22 the concordant pairs/trios were seropositive.

**Statistical Analysis.** We used conditional logistic regression to analyze prostate cancer risk according to serostatus adjusting for matching factors. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated comparing men who were *T. vaginalis* seropositive vs. seronegative at baseline. We additionally controlled for randomization to aspirin assignment and body mass index (continuous), and evaluated risk within subgroups of stage and grade at diagnosis.

To determine the association of *T. vaginalis* seroprevalence on prostate cancer progression/death, we conducted time-to-event analyses among the 673 cases. We followed men from date of cancer diagnosis to development of bony metastases or prostate cancer death, or censored at time of death from other causes or end of study follow-up (March 1, 2008). Cox proportional hazards models that adjusted for age at diagnosis (continuous), tumor aggressiveness (dichotomous, "1" if stage T3/T4/N1/M1 or Gleason 8-10 at diagnosis, and "0" otherwise), body mass index (continuous), randomized aspirin assignment, and smoking status (current vs. ever vs. never) were used to estimate hazard ratios (HR) and 95% CI. All p-values were two-tailed and  $\alpha=0.05$  was used to determine statistical significance.

## Results

On average, cases were 68.7 years ( $\pm 7.4$ ) at diagnosis. Most cases were diagnosed with well differentiated tumors (54% Gleason 2-6) at a localized stage (83% T1/T2). Mean time between blood draw and prostate cancer diagnosis was 9.3 years (range 0.3 – 17.9 years). The seroprevalence of *T. vaginalis* infection was 21% in controls and 25% in cases (Table 1). *T. vaginalis* absorbance scores were not associated with age or baseline PSA levels in cases or controls.

*T. vaginalis* seropositivity was not statistically significantly associated with prostate cancer risk overall or by grade. However, serologic evidence of *T. vaginalis* infection was associated with a significant 2.2-fold increase in risk of diagnosis of advanced stage prostate cancer and a significant 2.7-fold increase in risk of cancer that would ultimately progress to distant metastases or cancer-specific death (Table 1). We also found that the association between *T. vaginalis* and prostate cancer was stronger for men diagnosed more proximally to blood draw (Table 1). Compared to cases overall, the 94 cases diagnosed within 5 years of blood draw tended to be somewhat older at diagnosis (>65 years: 77% vs. 66%), more advanced (T3/T4/N1/M1: 25% vs. 15%), and were all diagnosed in the pre-PSA era (prior to 1992: 100% vs. 38%). However, cross-classifying men on these characteristics suggested that the time scale that most influenced effect estimates was duration between blood draw and diagnosis. Given the observed increased risk for cancer soon after blood draw, we explored the association between *T. vaginalis* serostatus and lethal prostate cancer according to years from blood draw to diagnosis. Among the 39 men diagnosed with lethal cancer within 5 years of blood draw and their matched controls, men positive for history of trichomonosis (n=15) were 6.4 times more likely to develop lethal prostate cancer compared to men without serological evidence of infection (95% CI: 1.5, 27.9).

During an average of 9 years of follow-up, 131 men with prostate cancer developed distant metastases or died of their disease. In survival analyses among the prostate cancer cases, we found a significant association between *T. vaginalis* seropositivity and time to prostate cancer progression/death (Figure 1). Seropositive men who had their blood drawn on average 9 years prior to cancer diagnosis were 51% more likely to develop lethal prostate cancer (95% CI: 1.03, 2.20) compared to seronegative cases.

## Discussion

In this large nested case-control study, we provide further evidence supporting the previously reported association between *T. vaginalis* seropositivity and prostate cancer risk (13). The magnitude of the overall association with incidence in our study, though not statistically significant, was similar to that observed in the original case-control study nested in the HPFS (OR: 1.43; 95% CI: 1.00, 2.03). The HPFS study found a suggestion that infection was primarily associated with more aggressive disease as evidenced by higher Gleason score at diagnosis, but small numbers prohibited a subgroup analysis among men with advanced disease. In the present analysis, we found that a history of *T. vaginalis* infection was primarily associated with clinically relevant prostate cancer. Compared to men without anti-*T. vaginalis* antibodies, men with serologic evidence of infection prior to cancer diagnosis had more than double the risk of developing prostate cancer diagnosed at an advanced stage. Moreover, using more than two decades of follow-up of prostate cancer cases, our results suggest that *T. vaginalis* infection is associated with progression to distant metastases and prostate cancer death, independent of BMI, smoking status, aspirin randomization, age of diagnosis, and tumor stage and grade. We found no evidence of a stronger association with higher Gleason grade, but believe that this finding should be considered in the context of the subjectivity of Gleason grading and the shift in scores over time (17-19).

Because all men provided blood samples in 1982 and all of the *T. vaginalis* assays were completed in 2008, the sensitivity and specificity of the assay should not be differentially influenced by specimen quality according to date of cancer diagnosis. The unknown period of time between infection and blood draw, however, could influence the sensitivity and specificity of the assay. Presumably, men who were infected closer to the time of blood draw in 1982 would be more likely to have detectable levels of antibodies. As cases and controls were matched on age (range 40-84 years at blood draw) and timing of infection is more likely to be related to age than calendar time, this misclassification would likely be non-differential with respect to case/control status and thus lead us to underestimate our effect estimate.

We acknowledge that there are alternative explanations for the positive association observed between *T. vaginalis* infection and prostate cancer risk. To address the possibility that cases with *T. vaginalis* infection were more likely to be diagnosed with prostate cancer, we related antibody levels to baseline PSA levels but found no association. We found that the association between *T. vaginalis* and incidence of prostate cancer

was stronger among men diagnosed within 5 years of blood draw. Biomarkers more strongly associated with early occurring disease typically raise concerns about reverse causation, *i.e.*, early pre-clinical disease influencing levels of the measured biomarker. In this study, however, the stronger association in the early years of follow-up must also be interpreted within the context of the introduction of the PSA test, which occurred 1986. Consequently, cases diagnosed earlier in follow-up are more likely to be clinically relevant. To account for our study findings, the carcinogenic process would have to lead to either higher levels of detectable antibodies. While there is no data to support or contest the assumption that anti- *T. vaginalis* antibody levels increase during cancer development, tumorigenesis has been demonstrated to alter adaptive immune response (20). Conservatively, serologic history of infection with *T-vaginalis* may be a marker of clinically relevant disease, as suggested by the association between infection and time to progression to distant metastases and prostate cancer death.

Disease heterogeneity, an important consideration in studies of prostate cancer, could largely explain the apparent discrepancy between our findings and those of a recent study using data from 616 cases and 616 matched controls sampled from the Prostate Cancer Prevention Trial (PCPT), a randomized trial of finasteride in 18,882 men, which found no association between *T. vaginalis* seropositivity and prostate cancer incidence (21). We found that *T. vaginalis* was principally associated with aggressive, potentially lethal disease, but the majority of prostate cancer diagnosed in the PCPT was early-stage disease, as it was most often diagnosed as a result of annual PSA screening and end-of-study prostate biopsy (22). Accumulating evidence suggests that the risk factors for lethal and indolent prostate cancer may differ (23).

The proportion of cases and controls with high seropositivity for anti-*T. vaginalis* antibodies was somewhat higher in the present study (24.5% of cases; 21.4% of controls) than in the PCPT (15.2% of cases; 15.0% of controls) or the HPFS (13% of cases; 9% of controls) (13). Assays for all three studies were prepared under the direction of the same laboratory colleague (J.F.A.) using an ELISA to detect antibodies against  $\alpha$ -actinin protein. The same positive and negative controls were used to assign absorbance score cut-points in both the PCPT and the PHS. In the HPFS study, however, absorbance score cutpoints were based on previous serological findings (24, 25), as sera from positive and negative controls were not available. Further, absolute readings from the ELISA in all three studies could be influence by the specific technician conducting

the assay and the fact that the lab relocated. Thus, differences in assay sensitivity may account for some of the variation in distribution of *T. vaginalis* seropositivity across studies, especially given that demographic characteristics do not appear to explain the observed variability. All three studies included men from across the U.S. Though African American race and lower socioeconomic status are generally associated with higher rates of STIs (26), including *T. vaginalis* (3), the study with the greatest proportion of men seropositive for *T. vaginalis*, the PHS, has the smallest proportion of African Americans (<1%) and a relatively high SES, as all participants are physicians. Further, the mean age at blood draw in all three studies was similar: 66 years in HPFS, 64 years in PCPT, and 59 years in PHS.

As other STIs occur concurrently with *T. vaginalis*, we cannot rule out the possibility that *T. vaginalis* is acting as a marker for another infection. However, studies report concomitant infection with other common STIs, including *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, to occur only in 10-20% of cases (6, 8). Further, the previous study in the HPFS investigated other common STIs including *N. gonorrhoeae*, *C. trachomatis*, *Treponema pallidum*, and human papillomavirus (HPV) and found no association with prostate cancer, except for an inverse association for human herpesvirus type 8 (HHV-8) infection (27, 28). Nested case-control studies utilizing data from the Nordic biobank consortium found no association between prostate cancer risk and HPV-16/18/33 (29), HSV-2 or HHV-8 (30), but observed a statistically significant inverse association with serologic evidence of *C. trachomatis* infection (31). A study nested within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (21) found that seroprevalence of *C. trachomatis*, HPV-16 and -18, herpes simplex virus-2, cytomegalovirus, and HHV-8 were not individually associated with prostate cancer risk among Caucasian men. Men with one or more STIs, however, had a modest increase in risk of developing prostate cancer (OR: 1.3; 95% CI: 1.0, 1.6), indicating that the measured infections could perhaps be serving as proxies for another infection such as *T. vaginalis*.

While our study may elucidate one mechanism by which local prostatic inflammation could arise and lead to downstream events that influence prostate cancer development and progression, studies that focus on local response to infection in the prostate are needed to determine whether *T. vaginalis* is a causal agent. Nonetheless, in light of the limited understanding of factors that lead to lethal prostate cancer, our finding of an association between *T. vaginalis* serostatus and aggressive prostate cancer is noteworthy. If our findings are

confirmed, *T. vaginalis* could serve as a marker for adverse outcomes in patients for prostate cancer, or more optimistically, a target for secondary chemoprevention.

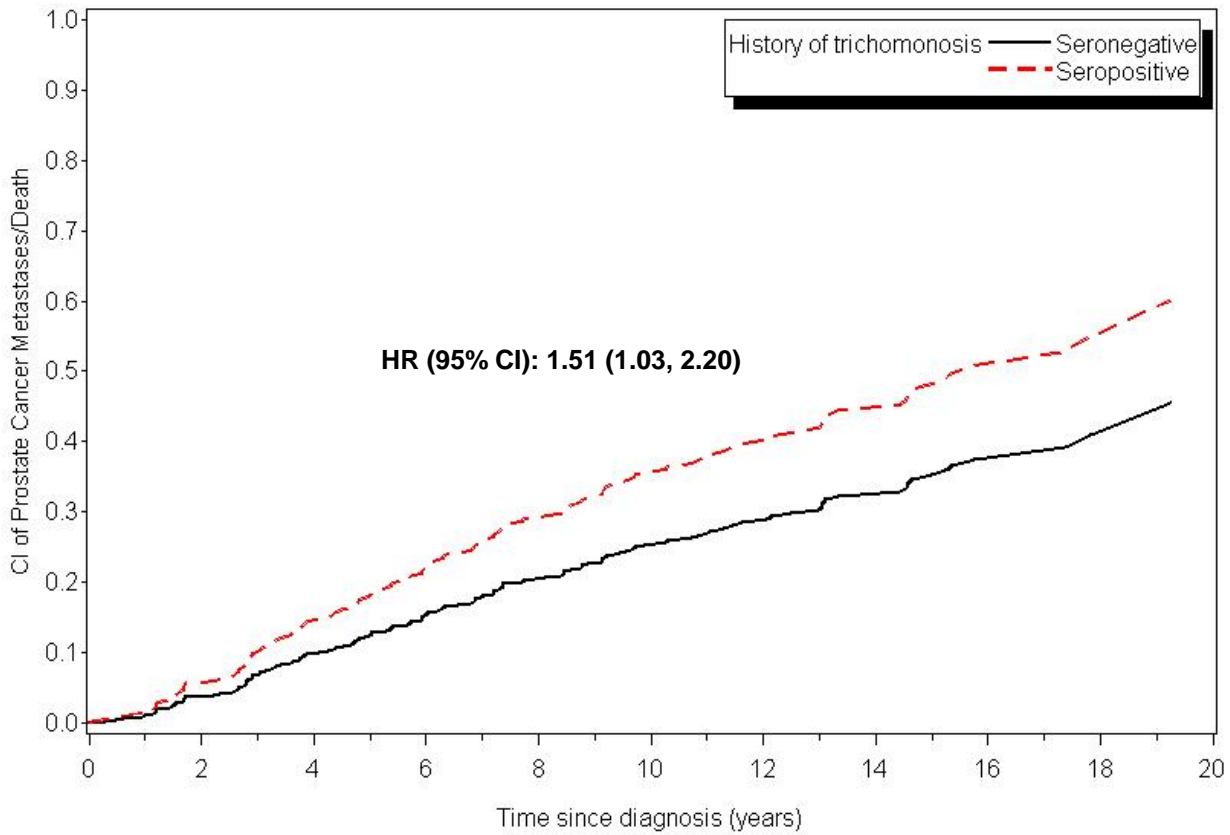
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**Table 1. Odds ratios (95% CI)\* of prostate cancer for *T. vaginalis* antibody serostatus in 673 matched pairs nested in the Physicians' Health Study, 1982 - 2000**

	<i>T. vaginalis</i> Serostatus	
	Negative	Positive
Controls, N (%)	529 (78.6)	144 (21.4)
All prostate cancer		
Cases, N (%)	508 (75.5)	165 (24.5)
OR (95% CI)	1.00 (Ref)	1.23 (0.94, 1.61)
Tumor grade: Gleason 2-6		
Cases, N (%)	238 (76.3)	74 (23.7)
OR (95% CI)	1.00 (Ref)	1.16 (0.77, 1.74)
Tumor grade: Gleason 7-10		
Cases, N (%)	204 (76.7)	62 (23.3)
OR (95% CI)	1.00 (Ref)	1.10 (0.72, 1.68)
Tumor stage: Localized (T1/T2)		
Cases, N (%)	406 (76.6)	124 (23.4)
OR (95% CI)	1.00 (Ref)	1.10 (0.81, 1.49)
Tumor stage: Extra-prostatic (T3/T4/N1/M1)		
Cases, N (%)	70 (66.7)	35 (33.3)
OR (95% CI)	1.00 (Ref)	2.17 (1.08, 4.37)
Non-lethal cancer		
Cases, N (%)	416 (76.7)	126 (23.3)
OR (95% CI)	1.00 (Ref)	1.01 (0.75, 1.37)
Lethal cancer/bony metastases		
Cases, N (%)	92 (70.2)	39 (29.8)
OR (95% CI)	1.00 (Ref)	2.69 (1.37, 5.28)
Age of diagnosis: <65 years		
Cases, N (%)	169 (76.5)	52 (23.5)
OR (95% CI)	1.00 (Ref)	1.41 (0.86, 2.31)
Age of diagnosis: ≥65 years		
Cases, N (%)	339 (75.0)	113 (25.0)
OR (95% CI)	1.00 (Ref)	1.12 (0.81, 1.56)
Time from blood draw to dx: ≤ 5 years		
Cases, N (%)	64 (68.1)	30 (31.9)
OR (95% CI)	1.00 (Ref)	2.86 (1.27, 6.47)
Time from blood draw to dx: > 5 years		
Cases, N (%)	444 (76.7)	135 (23.3)
OR (95% CI)	1.00 (Ref)	1.09 (0.81, 1.46)
PSA era: Dx pre-1992		
Cases, N (%)	233 (74.0)	82 (26.0)
OR (95% CI)	1.00 (Ref)	1.35 (0.92, 1.98)
PSA era: Dx 1992 or later		
Cases, N (%)	275 (76.8)	83 (23.2)
OR (95% CI)	1.00 (Ref)	1.14 (0.77, 1.68)

\* From logistic regression conditioned on age and smoking and additionally adjusted for randomized aspirin assignment and body mass index



**Figure 1.** Cumulative incidence of prostate cancer metastasis/death among 673 prostate cancer cases according to *T. vaginalis* serostatus, Physicians' Health Study 1982-2008. Cumulative incidence curves estimated from proportional hazards models controlling for age at diagnosis (65 years), tumor aggressiveness (Gleason Score 8-10 or T3/T4/N1), baseline BMI (23 kg/m<sup>2</sup>), baseline smoking status (never), and aspirin randomization arm (placebo).



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## SUPPORTING DATA

**Table 1. Prevalence of atrophy and inflammation in the Swedish Watchful Waiting Cohort, overall and by Gleason grade**

	Gleason grade				
	Overall N=619	2-6 N=288	3+4 N=121	4+3 N=90	8-10 N=120
<b>Focal Prostate Atrophy</b>					
Proliferative atrophic hyperplasia	125 (20.2%)	18.4%	25.6%	21.1%	18.3%
Simple atrophy	367 (59.3%)	61.8%	59.5%	56.7%	55.0%
Simple atrophy with cyst formation	38 (6.1%)	7.6%	1.7%	8.9%	5.0%
Partial atrophy	11 (1.8%)	2.8%	2.5%	0%	0%
<b>Chronic inflammation</b>					
None	164 (26.5%)	24.3%	22.3%	28.9%	34.2%
Mild	296 (47.8%)	51.0%	52.1%	40.0%	41.7%
Moderate/Severe	159 (25.7%)	24.7%	15.6%	31.1%	24.2%
<b>Acute inflammation</b>	84 (13.6%)	16.7%	14.1%	12.2%	6.7%
<b>Perineural invasion</b>	43 (7.0%)	0.7%	3.3%	10.0%	23.3%
<b>High grade PIN</b>	81 (13.1%)	6.6%	16.5%	21.1%	19.2%

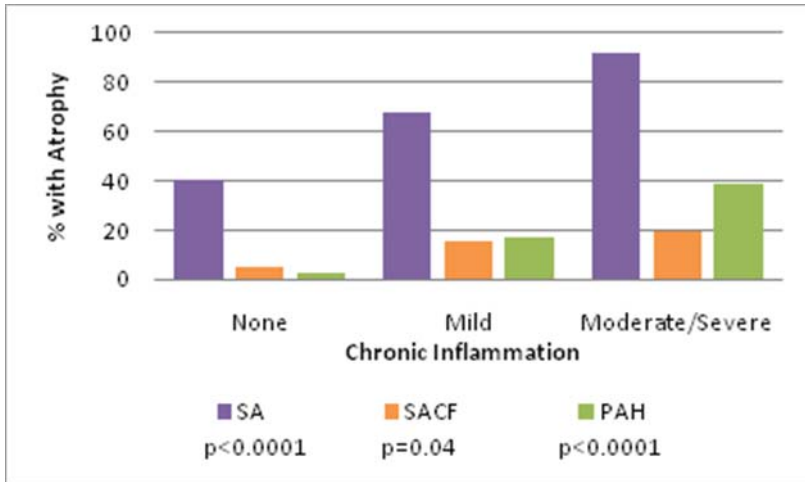
**Table 2. Association of *RNASEL* SNPs with high grade and advanced stage disease in the**

SNP*	Genotype	High Grade disease (Gleason $\geq$ 7)				Advanced stage (T3/T4/N1/M1, or PCa mortality)			
		cases (n)	OR†	95% CI	p‡	cases (n)	OR†	95% CI	p‡
rs682585	GG (ref)	173	1.00	ref	0.24	93	1.00	ref	0.12
	GA	203	1.03	(0.81, 1.31)		106	0.97	(0.71, 1.33)	
	AA	61	0.78	(0.56, 1.10)		28	0.63	(0.40, 1.01)	
rs486907 (R462Q)	GG (ref)	190	1.00	ref	0.72	86	1.00		0.17
	GA	185	0.91	(0.71, 1.15)		100	1.05	(0.76, 1.45)	
	AA	56	0.95	(0.67, 1.36)		39	1.51	(0.98, 2.34)	
rs627928 (D541E)	TT (ref)	90	1.00	ref	0.56	39	1.00	ref	0.18
	TG	200	1.17	(0.88, 1.57)		108	1.43	(0.95, 2.15)	
	GG	132	1.10	(0.80, 1.50)		73	1.42	(0.92, 2.19)	
rs533259	CC (ref)	377	1.00	ref	0.62	202	1.00	ref	0.91
	CT/TT	57	1.09	(0.78, 1.51)		26	1.03	(0.65, 1.63)	
rs11807829	AA (ref)	204	1.00	ref	0.34	103	1.00	ref	0.23
	AG	175	0.87	(0.68, 1.10)		82	0.76	(0.55, 1.05)	
	GG	43	0.80	(0.55, 1.17)		25	0.98	(0.60, 1.60)	
rs627839	CC (ref)	125	1.00	ref	0.61	63	1.00	ref	0.78
	CA	214	1.12	(0.86, 1.45)		113	1.13	(0.80, 1.60)	
	AA	98	1.00	(0.73, 1.36)		50	1.09	(0.72, 1.66)	
rs10911099	AA (ref)	330	1.00	ref	0.26	165	1.00	ref	0.20
	AG/GG	109	1.16	(0.90, 1.50)		60	1.25	(0.89, 1.77)	
rs12729828	GG (ref)	317	1.00	ref	0.43	172	1.00	ref	0.99
	GA/AA	99	1.12	(0.85, 1.46)		46	1.00	(0.70, 1.45)	
rs635261	GG (ref)	177	1.00	ref	0.45	90	1.00	ref	0.39
	CG	187	0.89	(0.70, 1.13)		92	0.80	(0.57, 1.10)	
	CC	65	0.83	(0.59, 1.15)		38	0.89	(0.58, 1.36)	
rs12757998	GG (ref)	208	1.00	ref	0.003	109	1.00	ref	0.22
	GA	161	1.02	(0.80, 1.30)		72	0.84	(0.60, 1.17)	
	AA	43	1.90	(1.25, 2.89)		16	1.44	(0.79, 2.63)	
rs12034888	CC (ref)	353	1.00	ref	0.42	182	1.00	ref	0.25
	CT/TT	74	1.13	(0.84, 1.53)		42	1.26	(0.86, 1.85)	

\* SNPs listed by genomic position

† Odds ratios adjusted for age, smoking status, follow-up time, ‡ p-values from likelihood ratio test

**Figure 1. Frequency of focal atrophy lesions by chronic inflammation grade in the Physicians' Health Study and Health Professionals Follow-up Study, 1982-2009**



**Figure 2. Direction of association between presence of focal atrophy/ chronic inflammation by clinical and tumor characteristics in the Physicians' Health Study and Health Professionals Follow-up Study, 1982-2009**

