EPIDEMIOLOGIC APPROACHES TO UNDERSTANDING ADVANCED PROSTATE CANCER: CHEMOPREVENTION, GENETICS AND RESPONSE TO THERAPY

by

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DEDICATION

To George: For your endless support, love and encouragement. I am truly blessed to have you in my life.

To Michael &Miranda: For unknowingly giving me the encouragement to keep going throughout this process and for being a constant source of joy.

To my wonderful parents, Bahgat and Aida: I thank you for instilling in me the importance of education, learning, and growth. I truly wish my father were with me today.

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Abbreviation	Definition
ADT	Androgen deprivation therapy
AIM	Ancestral informative marker
AR	Androgen receptor
BCR	Biochemical recurrence
BRCA1	Breast cancer
CI	Confidence interval
cM	Centimorgans
DHT	Dihydrotestosterone
ESP	Exome Sequencing Project
EVS	Exome Variant Server
FPC	Familial prostate cancer
HPC	Hereditary prostate cancer
HR	Hazard ratio
IBD	Identity by descent
JHU	Johns Hopkins University
LD	Linkage disequilibrium
LOD	Logarithm of the odds
MAF	Minor allele frequency
NSAIDs	Non-steroidal anti-inflammatory drugs
OR	Odds ratio
PCa	Prostate cancer
PCGP	Prostate Cancer Genetics Project
PSA	Prostate-specific antigen
RRP	Retropubic radical prostatectomy
SD	Standard deviation
SNP	Single nucleotide polymorphism

ABSTRACT

Prostate cancer is the most commonly diagnosed non-cutaneous cancer among men and is the subject of debate with regards to screening practices, overdiagnosis and overtreatment. Prostate cancer is a highly heterogeneous disease ranging from clinically indolent tumors to metastatic prostate cancer, the cause of prostate cancer mortality. In this dissertation, epidemiologic methods are applied to further understand advanced forms of prostate cancer in order to increase our understanding of the biologic mechanisms of the most relevant forms of prostate cancer.

Statin medications are widely used cholesterol-lowering agents. Stain use has been associated with a decreased risk of advanced prostate cancer and inconsistently associated with risk of recurrence after prostate cancer diagnosis and treatment. I investigated the association between statin use and risk of biochemical recurrence (BCR) after radical prostatectomy (RRP) in men with inherited forms of prostate cancer enrolled in the University of Michigan Prostate Cancer Genetics Project (PCGP). BCR was defined as an increase in prostate-specific antigen (PSA) level to $\geq$0.4 ng/mL after treatment. Of 539 men surveyed, 115 (21%) participants experienced BCR after RRP and 47.9% used statins. Ever-statin use was not associated with risk of recurrence in crude models (HR=1.04, 95% CI=0.72-1.49, *p* value =0.86) or in models adjusted for clinical characteristics (HR=1.06, 95% CI=0.68-1.64, *p* value =0.81). Men with a family history of prostate cancer represent a subgroup of men who may benefit from further study of statin medication use to slow or prevent BCR.

Genetic linkage studies have consistently identified the 17q21-22 chromosomal region as a potential prostate cancer susceptibility locus. To date, few genes have been associated with prostate cancer and those genes do not explain a large portion of the familial clustering observed in prostate cancer. Three genes in 17q21-22 are potential candidates based on plausible biologic

function as well as location within this linkage peak. Mitogen-activated protein kinase kinase kinase 14 (*MAP3k14*) encodes a serine-threonine protein kinase that stimulates NF-κβ activity. The *ARHGAP27* gene codes for a Rho-like small GTPase-activating protein and *RND2* is part of the Rho GTPase family implicated in endosomal trafficking. Using next-generation sequencing techniques, all exons within the 17q21-22 were interrogated in 94 probands from hereditary prostate cancer (HPC) families that have exhibited linkage to the 17q21-22 region. Participants were from the PCGP and Johns Hopkins University. Confirmation of mutation status among family members (both affected and unaffected) was performed using Sanger sequencing. No deleterious truncating mutations were detected in any of these genes and five missense variants were identified in the *MAP3K14* and *ARHGAP27* genes. Four of the five variants are novel (not described in dbSNP) and four out of the five variants are predicted to be damaging on protein function by SIFT and/or PolyPhen. *ARHGAP27* demonstrated evidence of cosegregation within families and was associated with prostate cancer in the presence of linkage (Z Score=2.87, *p* value=0.004). Further research should investigate these variants in larger studies of HPC families.

Androgen deprivation therapy (ADT) is the standard treatment for metastatic prostate cancer. Despite an initial response, most men on ADT progress and develop castrate-resistant prostate cancer (CRPC). There are few established prognostic factors to predict success on ADT and studies have suggested that germline variation in hormone synthesis/metabolism genes may be associated with response to ADT. Using data from the SWOG-9346 randomized controlled trial of men receiving ADT, I assessed the role of germline variation in candidate genes involved in androgen synthesis/metabolism in response to ADT. DNA samples from 210 metastatic patients, including 24 African-American participants, were genotyped on a 1,536 single nucleotide polymorphism (SNP) Illumina GoldenGate genotyping platform. A total of 15 SNPs in *SLCO1B1*, *SRD5A1*, *SRD5A2*, *CYP19A1* and *ESR2* were associated with response to ADT, after correction for ancestry. These associations did not remain significant after correction for multiple testing. Among the associated SNPs is rs2306283, a

missense variant (OR = 0.50, 95% C.I.: 0.30-0.89, nominal *p* value =0.02) in *SLCO1B1*. Findings from this study may inform *a priori* prediction of those metastatic patients that are more or less likely to achieve better treatment outcomes.

CHAPTER 1

Introduction

1.1 Heterogeneity of prostate cancer

Prostate cancer is the most commonly diagnosed non-cutaneous cancer among American men and the second most common cause of cancer deaths in men. An estimated 241,740 men will be diagnosed with prostate cancer in the U.S. during 2012 and 28,170 men will die of the disease in the same year. (1) The established risk factors for prostate cancer are increasing age, African-American race and a positive family history for the disease. The potential for overdiagnosis and over treatment in prostate cancer has been the subject of much debate and interest. The use of serum prostate-specific antigen (PSA) measurement as a screening test for prostate cancer became widespread during the early 1990s. The introduction of PSA screening has resulted in more than 1 million additional men being diagnosed and treated for prostate cancer in the U.S. and a substantial part of this increase is associated with overdiagnosis in that many of these cancers are not clinically significant and will not result in compromised function or health.(2) These data are coupled by findings from autopsy studies which estimate that the prevalence of undiagnosed prostate cancer in U.S. men over the age of 70 is 81-83%.(3) Several large studies were conducted to address whether screening was

associated with a decrease in mortality. The only randomized controlled trial conducted in the U.S., the Prostate, Lung, Colorectal, Ovarian (PLCO) cancer screening trial, showed a non-significant increase in prostate cancer specific mortality associated with screening.(4) Men often experience adverse effects associated with treatment including erectile dysfunction, urinary symptoms or incontinence, and bowel dysfunction, among others. The majority of men will experience at least one adverse effect of treatment, and these adverse effects can reduce quality of life.(5) The majority of men diagnosed prostate cancer are found to have low-risk disease defined as stage ≤ T2a, PSA level < 10 ng/mL and Gleason grade ≤ 6; low-risk disease is associated with an approximately 6% risk of prostate cancer death within 15 years after diagnosis.(6) Neither PSA screening nor any other screening tool have the ability to discriminate between tumors that require treatment and tumors that are clinically insignificant and pose no threat to longevity. Given the financial and quality-of-life costs of prostate cancer treatment, attention is focused on how to limit the potential for overtreatment and how to address the need to better differentiate between clinically significant and insignificant tumors prior to progression. Identifying genes and variants involved in advanced prostate cancer may uncover the biologic mechanisms underlying the various types of prostate cancer. This knowledge may also provide important prognostic and predictive information relevant to more effective clinical management through chemopreventive strategies, determining genetic predictors of response to therapy and genetic predictors of disease risk.

This dissertation incorporates traditional epidemiologic methods, techniques in identifying genetic variants, including linkage and genetic association studies to identify the molecular pathways involved in the development, progression and treatment of advanced prostate cancer. The studies herein focus on narrow definitions of prostate cancer and this approach is relevant to moving forward in our understanding of the heterogeneous nature of this disease.

This research is motivated by the desire to better understand various forms of prostate cancer and focus on sub-populations of men who would most benefits from improved chemoprevention, diagnosis risk prediction and better prediction of therapeutic outcome and novel therapeutic targets. The conflicting results among prostate cancer studies may be due to genetic and phenotypic heterogeneity in study populations. Gains in our understanding of genetic susceptibility, chemoprevention and personalized therapies will be best achieved when studies focus on homogeneous groups of subjects. My dissertation research focuses on various aspects and stages of advanced prostate cancer. Men at increased risk for prostate cancer due to family history, men at risk for recurrence for prostate cancer and men with metastatic disease represent populations with clinically relevant forms of prostate cancer. Identifying chemopreventive strategies, genetic risk markers and prognostic markers may aid in understanding and defining subtypes of prostate cancer.

1.2 Risk reduction strategies in men with a family history of prostate cancer

Prostate cancer is a commonly occurring cancer, yet our understanding of risk factors outside of age, race and family history, all non-modifiable factors, is limited. Given the high number of prostate cancer diagnoses every year, preventive strategies to reduce prostate cancer incidence, recurrence and mortality are of paramount importance. To date, few preventive strategies in prostate cancer have gained widespread use. Five-alpha reductase inhibitors (finasteride and dutasteride) have been associated with decreased prostate cancer risk (7), but concern over the potential increase in high-grade cancer diagnoses and their negative side effect profiles have limited their acceptance by men at risk for prostate cancer diagnosis/recurrence. Statin medications, 3-hydroxy-3-methyl-glutaryl-coenzyme reductase inhibitors, are widely used cholesterol-lowering agents. Due to their widespread use and safe toxicity profiles, interest has increased in studying statins as potential prostate chemopreventive agents. Laboratory and *in vitro* studies have shown that the statins may exhibit cholesterol and non-cholesterol mediated effects that may inhibit prostate cancer initiation, growth and progression.(8) Although epidemiologic studies have been conflicting with regard to the effects of statins on overall prostate cancer risk, the most consistent results demonstrate that statins may reduce the risk of advanced prostate cancer.(9) Several studies have shown that statins may confer a decreased risk of advanced prostate cancer defined as high Gleason grade prostate cancer, prostate cancer related mortality, or prostate cancer recurrence. A subgroup of men who may benefit from the identification of prostate chemopreventive agents are men with a family history of prostate cancer. Due to their increased risk, men

with a family history of prostate cancer have reported a high rate of vitamin and supplement usage but very low rates of finateride/dutasteride use.(10) No studies to date have considered the association between statin use and risk of prostate cancer recurrence in men with inherited forms of prostate cancer. Using data from University of Michigan Prostate Cancer Genetics Project (PCGP), a family-based study of inherited forms of prostate cancer, I investigated the association between statin use and risk of biochemical recurrence in men who have previously been diagnosed with prostate cancer and treated with radical prostatectomy. Currently no chemopreventive guidelines exist for men with a family history of prostate cancer. In the second chapter of my dissertation, I will use data from both medical records and a health survey administered to PCGP participants to assess the association between long-term statin medication use (10 years) and risk of recurrence in men treated with a radical prostatectomy.

1.3 Novel approaches to discovering inherited prostate cancer susceptibility

In general, familial prostate cancer (FPC) and hereditary prostate cancer (HPC) has not been consistently associated with more aggressive or clinically relevant cancer as compared to sporadic prostate cancer cases. However, FPC/HPC is associated with early disease onset and is diagnosed, on average, 6 to 7 years earlier than sporadic prostate cancer.(11) An earlier age of onset for men with inherited forms of prostate cancer is meaningful in that men will experience longer post-treatment periods and they have an increased need for accurate risk prediction. Genetic risk prediction can be used in a clinical setting to more

accurately inform screening of men with a family history of prostate cancer. The utility of genetic variants in risk prediction has been long investigated, but the identification of high penetrant mutations involved in hereditary prostate cancer has been elusive. Despite clear evidence of familial clustering in prostate cancer, pedigree-based analyses have not been highly successful in identifying susceptibility genes/variants. Even within pedigrees, there are few features to distinguish between men who have a hereditary form of prostate cancer from a sporadic cancer that occurred in hereditary prostate cancer family. Further, susceptibility may lie in a gene without known prostate cancer function or may be due to multiple genes/variants. Genome-wide association studies (GWAS) results have discovered over 30 polymorphisms associated with prostate cancer but these variants are associated with low magnitudes of risk and do not likely account for familial clustering in prostate cancer. (12) Prostate cancer linkage studies have suggested the existence of a prostate cancer susceptibility gene on chromosome 17q21-22.(13-15) The chromosome 17q21-22 region has been associated with strong linkage signals in families with hereditary prostate cancer. Using data from the PCGP, a genome-wide scan was conducted on 175 pedigrees, the majority containing three or more individuals diagnosed with prostate cancer. The genome-wide scan detected suggestive evidence for linkage on chromosome 17q (LOD=2.36).(13) However, mutation screening in this region in prostate cancer families with evidence of linkage to 17q21-22 failed to identify deleterious mutations accounting for this linkage signal.(16) In the third chapter of my dissertation, I present a study utilizing next generation targeted sequencing

techniques to interrogate exons of three candidate genes located within the 17q21-22 region, *MAP3K14*, *RND2* and *ARHGAP27,* to identify potential disease-causing mutations using hereditary prostate cancer cases. Our study population chosen for sequencing is enriched for men with early-onset disease. Segregation analyses have revealed that even among men with family history there is likely a different mode of inheritance between early age at onset and late age at onset (17). Increasing the likelihood of identifying prostate cancer susceptibility genes and variants requires study of distinct subpopulations of men who are more likely to have prostate cancer due to an inherited germline mutation. This study employs epidemiologic methods coupled with novel sequencing techniques to better understand the underlying genetic structure of 17q21-22 chromosomal region in HPC cases.

1.4 The role of inherited variation in response to therapy in men with metastatic disease

Although the majority of men with metastatic prostate cancer will initially respond to androgen deprivation therapy (ADT), the development of castrate-resistant prostate cancer (CRPC) almost always occurs. CRPC is characterized by poor prognosis and is the most lethal form of prostate cancer. Several clinical factors can be predictive of the response to ADT including Gleason grade, pretreatment PSA, visceral metastases and presence of distant lymphadenopathy, however they predictors are not highly accurate at *a priori* prediction of response and resistance to ADT often occurs in patients. (6) Men who die of prostate cancer die of metastatic disease. A need exists for more effective and well-

tolerated therapies in CRPC, as well as for better outcome prediction for men prior to treatment initiation. Germline variants may represent potential prognostic markers, which can predict response to therapy. In a study of over 500 men receiving ADT (including non-metastatic cases), three single nucleotide polymorphisms (SNPs) were found to be significantly associated with either improved or worsened response to ADT. The first SNP is 5 kb upstream of *CYP19A1*, which encodes for the aromatase protein, and is involved in the conversion of testosterone to estrogen, a SNP 13 kb upstream of *HSD3B1* (encodes for proteins involved in deactivating dihydrotestosterone) and a SNP within an intron in *HSD17B4* (involved in regulating the production of dehydroepiandrosterone). However, none of these SNPs are in coding regions.(18) Another study found a variant in *SLCO1B3* associated with time to progression while on ADT. *SLCO1B3* is a gene that codes for proteins involved in testosterone transport. Although, these findings were not replicated in further studies they indicate that germline variants may be associated with improved/worsening outcomes while on ADT and these findings in genes with biologic plausibility in the androgen milieu are potential targets for prognosticating clinical course. The success of ADT is dependent on the ability to reduce androgen levels, making genes involved in androgen synthesis and metabolism compelling candidate genes that may be associated with response to therapy. In the last chapter of my dissertation, I utilized DNA samples from men with metastatic prostate cancer enrolled in a randomized clinical trial of intermittent versus continuous ADT conducted by SWOG to test the hypothesis

that there exist variants within genes involved in androgen synthesis and metabolism that differentially alter the response (i.e. ability to reach a decline in PSA levels) to ADT. This study includes both Caucasian and African-American men. Previous studies may be somewhat limited given that they included all men taking ADT whether metastatic or non-metastatic disease and considered only Caucasian subjects. Further, identifying genetic variants involved in the response to ADT may offer clinically relevant prognostic markers will elucidate the underlying biologic mechanisms of metastatic prostate cancer disease, which are not well understood. These findings are relevant to the development of tailored and personalized cancer therapies.

1.5 Public Health Relevance

This research has the potential to impact public health through its focus on populations of men at risk and men with forms of prostate cancer that are clinically relevant. Given that over 240,000 men are diagnosed with prostate cancer annually, a chemopreventive agent that can reduce prostate cancer diagnosis and recurrence risk is of enormous public health significance. Considering the overdiagnosis and overtreatment that characterizes prostate cancer, even a small reduction in incidence/recurrence is of profound importance. Using next-generation sequencing to potentially identify variants associated with hereditary prostate cancer can elucidate variants for clinical risk prediction in men with a family history of prostate cancer. Lastly, identifying genetic variants involved in response to therapy in men with metastatic disease may allow for better outcome prediction, which can reduce the toxicities associated with

treatment. Further, identification of variants may elucidate the poorly understood biologic and molecular mechanisms of metastatic and CRPC. The findings of this study may inform our understanding of distinct subtypes of prostate cancer.

1.6 Conclusions

The motivation behind my dissertation is to investigate associations in unique cohorts of men who demonstrate what may be considered advanced, clinically relevant forms of prostate cancer using epidemiologic approaches in order to understand the heterogeneous nature of prostate cancer. I demonstrate that statin medication use is high in men with inherited forms of prostate cancer. Although the findings from this study did not support the hypothesis that statin medication use is associated with a modified risk of BCR in men treated with RRP, men with a family history of prostate cancer, due to their high risk for the disease, represent a group in need of chemopreventive agents to address both primary and secondary prevention of prostate cancer. I also present five variants detected in genes with biologic plausibility in hereditary prostate cancer families using next-generation targeted sequencing methods. The gene *ARHGAP27* was found to be associated with prostate cancer in the presence of linkage in family-based association tests. Variants found to be associated with HPC can have utility as predictive markers of disease risk. Finally, in the fourth chapter of this dissertation, I identified several SNPs associated with response to ADT in men with metastatic prostate cancer, including rs2306283 a nonsynonymous missense

variant in the *SLCO1B1* gene. S*LCO1B1* encodes for proteins involved in prostate cancer and in drug transport and influx.

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Chapter 2

The association between statin medication use and the risk of recurrence after radical prostatectomy in a cohort of men with inherited/early-onset forms of prostate cancer

2.1 Introduction

The use of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) inhibitors, or statin medications, in the treatment of hypercholesterolemia was introduced in 1987 and their effectiveness is well documented. As of 2004, 24 million Americans were using statins and 36% of US adults with elevated low-density lipoprotein cholesterol (LDL-C) levels were statin users.(1) The remarkable prevention of cardiovascular disease (CVD), the relative safety of statin drugs, and their widespread use in the last two decades and have led to interest in statins as potential chemopreventive agents. The association between statin use and prostate cancer risk has been studied extensively with conflicting results. A meta-analysis of thirteen observational studies and six randomized controlled trial demonstrated that long-term statin use did not significantly affect the overall prostate cancer risk (RR=0.93, 95% CI:0.77-1.13). In contrast, five studies that had specifically examined statin use and the risk of advanced prostate cancer indicated a protective association between statin use and advanced prostate

cancer (RR=0.77; 95% CI:0.64-0.93).(2) The definitions of advanced prostate cancer varied between studies and included stage III (cancer that has spread beyond the prostate), invasive prostate cancer, cancer with positive lymph node involvement, metastatic cancer or prostate cancer death.

Radical retropubic prostatectomy (RRP) is the most common primary treatment for prostate cancer. Although radical prostatectomy is curative for most patients with localized prostate cancer, approximately 20–30% will experience disease recurrence by 10 years post-surgery.(3) Statin medications are also hypothesized to alter the risk of recurrence after treatment for prostate cancer; however, the findings from these studies have been conflicting. Primary chemoprevention aims to prevent the occurrence of cancer in individuals at high risk for developing the disease, while secondary chemoprevention aims to prevent recurrence of caner in patients who have been diagnosed with cancer and received curative treatments. Statin use was an independent predictor of biochemical recurrence (BCR) after RRP and statin users were found to have a lower 5-year BCR-free survival compared with non-statin users (75% vs 84%, p value < 0.05).(4) In a hospital-based cohort of men treated with RRP, the hazard ratio for recurrence among men who used a statin for at least one year as compared to nonusers was 0.77 (95% CI 0.41-1.42).(5) In another cohort of RRP patients, statin use was associated with a 30% lower risk of PSA recurrence and the association occurred in a dose-dependent manner. (6) Statin use at the time of

therapy was associated with improved freedom from biochemical progression in men undergoing external beam radiotherapy(7).

The hypothesized chemopreventive properties of statins are supported by laboratory data demonstrating that statins can inhibit the proliferation of prostate cancer cells and induce tumor specific apoptosis. Specifically in prostate cancer, statins have anti-inflammatory properties that may reduce prostate cancer risk as chronic inflammation may contribute to prostate carcinogenesis.(8) A study of lovastatin and simvastatin, two commonly prescribed statins, demonstrate the induction of apoptosis and cell growth arrest by these statins in prostate cancer cell lines and the underlying mechanism may be mediated through inactivation of Ras homolog gene family, member A (RhoA), which is overexpressed in many cancers and is associated with cell cycle and transcriptional control.(9)

Statins are hypothesized to have additional properties that influence prostate cancer initiation and/or development including anti-inflammatory effects, evidenced by reduced C-reactive protein (CRP) levels. (10) Although the role of chronic inflammation in prostate cancer is not completely understood, inflammation may create atrophic lesions, which may be precursors to adenocarcinomas. (8) Further, statins inhibit the synthesis of cholesterol, a precursor to androgens. Steroid sex hormones (both androgens and estrogens) are important in prostate cancer. (11) Circulating levels of androgens have been found to be unchanged in statin users (12), however circulating levels of androgens may

be less important than intraprostatic hormone levels in prostate carcinogenesis. In addition, prostate cancer cells are known to exhibit cholesterol dysregulation and recent studies show that Akt (protein kinase B) cell signaling, which plays a role in apoptosis and cell survival, is cholesterol-sensitive, therefore lowering cholesterol could influence prostate carcinogenesis. (13) Men with desirable cholesterol levels (< 200 mg/dl) were less likely to develop high-grade prostate cancer. (14) Other anti-cancer mechanisms of statins include their ability to inhibit angiogenesis, inhibit tumor formation (through induction of apoptosis), stimulate cellular immunity as well as the attenuate metastatic potential. (15) Some of the potential mechanisms by which statins may influence prostate carcinogenesis are depicted in **Figure 2.1**.

BCR is measured primarily through PSA testing, aiding in the detection of recurrent disease months to years before its clinical appearance. (16, 17) A successful RRP results in an undetectable PSA level and a recurrence occurs if subsequent increase in PSA occurs. To date, the predictors of BCR are high Gleason score at time of initial diagnosis (18), increasing age (19, 20) and pre-diagnostic PSA levels of greater than 20 ng/mL (21). Prognostic tools based on these and other clinical and pathologic variables are imperfect in their risk prediction accuracy due to heterogeneous biologic nature of prostate cancer tumors.(22)

A positive family history, increasing age and African-American race are the only established risk factors for prostate cancer. Prostate cancer risk increases

to approximately 5-fold for those with ≥ 2 affected first-degree family members. Risk increases when a first-degree relative is diagnosed at an earlier age. In addition, risk of early-onset disease (before age 65) is influenced by family history. Studies have also consistently shown an increased risk for those with an affected brother, compared with those with an affected father: 3.37-fold versus 2.17-fold in a meta-analysis.(23) Further, prostate cancer diagnosis concordance among monozygotic twins is 27% and significantly higher than among dizygotic twins.(24) Despite the increased risk of prostate cancer, there are currently no proven chemopreventive strategies for men with a family history of prostate cancer, either in the primary or secondary prevention setting. 5-alpha-reductase inhibitors (5-ARIs) are inhibitors of the enzyme that converts testosterone to dihydrotestosterone (DHT) and have been shown to reduce incident prostate cancer risk. However the prevalence of use among men with a family history is low (25) and conflicting results as to the possible increased risk of high-grade prostate cancer in men taking 5-ARIs may limit the potential for their widespread use. (26, 27) If the actions of statin medications in prostate cancer or the subgroup of men that can benefit from their use could be elucidated, statin medications offer a potential chemopreventive agent with few side effects. Prostate cancer is an attractive target for primary chemoprevention strategies because of its high incidence, prevalence, treatment-related morbidity and mortality. To date, no studies of statin medication use have been performed in a cohort enriched with men with inherited forms of prostate cancer. These men with high-risk profiles may benefit greatly from chemopreventive agents.

The objective of this study is to assess the association between statin medication use and risk of BCR after RRP men among men with inherited and early-onset forms of prostate cancer.

2.2 Materials and methods

2.2.1 Study subjects

Study subjects come from the University of Michigan Prostate Cancer Genetic Project (PCGP). Since its inception in 1995, the goal of the PCGP has been to identify genes predisposing to inherited forms of prostate cancer. Enrollment into the PCGP is restricted to (*a*) families with two or more living members with prostate cancer in a first- or second-degree relationship or (*b*) men diagnosed with prostate cancer at ≤55 years of age without a known family history of the disease. All participants are asked to provide a blood sample, extended family history information, and access to medical records. Family members, both affected and unaffected, are also recruited to the study and asked to provide DNA samples and medical history information. The majority of the PCGP families were recruited directly from the University of Michigan Comprehensive Cancer Center; other sources included direct patient or physician referrals. Prostate cancer diagnoses are confirmed by medical record whenever possible. Independent confirmation of the diagnosis by at least two family members is required. Upon enrollment, prostate cancer cases completed questionnaires that collected information on family history of prostate and other

cancers, medical history, and demographic factors. In addition, detailed clinical information relating to the diagnosis and treatment of prostate cancer, including Gleason score from biopsy and RRP, tumor stage, pre-operative PSA level, date of diagnosis and pre-diagnostic and post-operative PSA, and age at diagnosis are obtained from medical records. Participants are asked to self-report race, ethnicity, income, education and other demographic information through a structured phone interview. All study procedures were approved by the University of Michigan-Institutional Review Board and written informed consent was obtained from all study participants prior to participation and for medical record release. In addition to data collected at enrollment, a biannual health update survey (HUS) is mailed to PCGP participants. A HUS was mailed to 2,483 participants in June 2009 to obtain updates on information relevant to prostate cancer, overall health and statin medication use. A total of 843 participants returned the survey by mail (n=744) or completed the survey on-line (n=99). Of 2,483 participants who were mailed a HUS, 1,362 were eligible for inclusion in this study. The overall response rate among participants was 34% and the response rate among participants who are eligible for this study (i.e. were diagnosed with prostate cancer diagnosis and had RRP as their primary treatment) was 41.7%. Men who are enrolled in PCGP, have been diagnosed with prostate cancer and treated with RRP and completed the 2009 health update survey providing information as to their statin use were eligible for inclusion in our study (n=556). Ten participants were excluded due to missing statin medication use data and 7 participants were excluded because their RRP was performed after the

survey date or because the participant experienced a recurrence within 6 months of their RRP.

2.2.2 Ascertainment of recurrence

Whether a participant experienced a recurrence was determined from one of three sources. A participant was considered to have experienced BCR if they reported a PSA test value of 0.4 ng/mL or greater on the 2009 HUS. A PSA value cut-off of 0.4 ng/mL was used instead of a less stringent 0.2 ng/mL cut-off as there is 72% probability that a single PSA test value of 0.4 ng/mL or higher will predict continuing PSA progression or secondary treatment.(28) Participants were also asked to report whether a physician had told the participant that he experienced a recurrence or if the participant were treated for a recurrence. In the latter case, participants were asked to report recurrence date, recurrence treatment, and treatment dates. Further, recurrence was assessed from the PCGP existing database where participants may have provided this information in the past or the information was taken from medical records. In order to be considered to have had a biochemical recurrence, the PSA test value of >0.4 must occurred at least 6 months after the RRP surgery date in order not to be considered adjuvant therapy. (29)

2.2.3 Ascertainment of statin medication use

Statin medication was only asked on the 2009 HUS, our study population was limited to men who responded either by mail or through the internet survey to 2009 HUS. The survey asked participants to report all statin medication use data

(name, start dates of use and end dates of use) over the last 10 years (1999-2009). A list of both brand names and generic names of the nine most common statin medications was provided on the survey to aid recall. However, no statin dose information was obtained. In addition, statins were grouped into two classes 1) lipophilic or fat soluble statins including lovastatin (Altocor, Altoprev, Mevacor, Simcor), atrorvastatin (Lipitor, Caduet), simvastatin (Vytorin, Zocor), cerivastatin (Baycol, Lipobay) and 2) hydrophilic statins including pravastatin (Parachol), rosuvastatin (Crestor), and fluvastatin (Lescol). Lipophilicity of the statins is considered to be quite important since the hepatoselectivity of the statins is related to their lipophilicity. The more lipophilic statins tend to achieve higher levels of exposure in non-hepatic tissues, while the hydrophilic statins tend to be more hepatoselective.(30) In cases where a participant reported a history of both lipophilic and hydrophilic statins (n=26), the most recently used statin medication was used to determine whether the individual was a lipophilic or hydrophilic statin user. Duration of statin use was the aggregate of all periods of statin use reported.

2.2.4 Covariates

Current NSAID use was included as a covariate due to the high concordance of NSAID and statin medication use and possible synergistic effects of NSAIDS with statin medication, particularly in anti-inflammatory properties.(31) BMI was measured from self-reported height and weight (at time of initial prostate cancer diagnosis) and was calculated as BMI (kg/m^2) =

(Weight in Pounds /(Height in inches x Height in inches)) x 703. The decade in which RRP was performed (1990–1999 vs 2000– present) and pre-diagnostic PSA, the PSA test value which led to biopsy, were also included as covariates.

Covariates included in multivariable analysis were daily NSAID use at time of survey (y/n), BMI, kg/m^2, at time of diagnosis (<25, 25-29.9, >=30), decade of surgery (1990s, 2000s), Gleason grade at RRP (>=6, 7=3+4, 7=4+3 - 10), natural-log transformed pre-diagnostic PSA (continuous), age at time of surgery (continuous) and pathologic stage (T2a, T2b and T3). A directed acyclic graph describing the relationships among variables is presented **Figure 2.2.** In cases where Gleason Grade, pre-diagnostic PSA and clinical stage were missing, medical records were reviewed to extract missing data. In a subset of patients, Gleason grade at RRP was unavailable and Gleason grade at biopsy was used instead (n= 22).

2.2.5 Statistical Analysis

Descriptive analysis was performed with contingency tables and chi-square tests for categorical variables and with Student's T tests were used to compare means of continuous variables. Both crude (unadjusted) and covariate-adjusted Cox proportional hazard models were constructed to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for the risk of BCR among ever-statin users compared with the referent group, never-statin users. Statin users were categorized as 1) current-users vs. non-current users and 2) ever-users vs. never-users. I categorized statin medication use as a time-dependent variable initially defined in 1999 and updated annually until 2009 using the Lexis function

implemented in the R Statistical Programming Package 2.14.1 (http://www.r-project.org/) to split exposure time into annual increments.(32) In addition, separate stratified analyses were performed for lipophilic and hydrophilic statin users. The follow-up period was calculated from the time of surgery to the time of survey or recurrence. Adjusted models for prostate cancer recurrence among statin users compared with non-users were adjusted for all the covariates mentioned above. Effect modification was investigated among the covariates that demonstrated evidence of association with BCR in crude analysis.

The association between statin use and time to BCR adjusted for covariates was obtained using Kaplan–Meier survival analysis to compare the BCR-free survival estimates for ever-statin users vs non-users. Kaplan–Meier survival analysis was also performed within comparing statin users who reported statin use for 5 years or more as compared to statin users who reported statin use of less than 5 years.

The assumption of proportionality was tested using hypothesis testing of exposure variables multiplied by time. Martingdale residuals were reviewed to check for outliers and assess model fit.

Given that PCPG is a family-based cohort, the study population included 470 families with only 1 participant from the family and 60 families with more than one member included in our study (54 families (11.5%) with 2 participants, 3 families (0.6%) with 3 participants and 3 families (0.6%) with 4 participants). In order to account for the potential clustering of outcome among families while

using a Cox proportional hazards model, frailty models were used to model correlated observations in survival analysis. Frailty modeling introduces a random effect, i.e. an unobserved random proportionality factor that modifies the hazard function of an individual. I chose a correlated gamma distribution for the model given the large number of clusters. The degree of dependence between family membership and the association between statin use and recurrence was analyzed using Kendall's Tau $\tau = \theta /(\theta +2)$ where Θ indexes the degree of dependence within the cluster. Frailty is an unmeasured subject covariate ξi (with mean 1 and variance Θ for the ith subject in each cluster) and the hazard function is given ξ_i is $\Lambda(t|\xi) = \Lambda^c(t)\, \xi_i$ When Theta = 0, data are independent and beta coefficients can be obtained from a Cox proportional hazards model with no frailty estimate. Clusters with large number of failures, the higher the resulting frailty. Frailty models were fitted in R Statistical Package 2.14.1 using the frailty package in Coxph. Except where otherwise mentioned, statistical analyses were performed using SAS 9.2 (Cary, NC).

2.3 Results

A total of 539 observations were available for analysis. The overall mean (SD) age at surgery was 56.5 (7.6) years. Based on self-report, 258 subjects were classified as ever-statin users and 281 subjects were never-statin users. Statin users were older at time of surgery as compared to non-statin users (58.0 y and 55.2 y, respectively; *p* value <0.001), had longer follow up times (101.5 months and 88.8 months, *p* value =0.009), and had higher BMI values (27.4 kg/m2 and 26.5 kg/m2, *p* value=0.004). Statin users were more likely to be NSAID users and

were more likely to have had an RRP prior to 2000 as compared to non-statin users. (Table 2.1) Statin users and non-statin users did not differ with respect to clinical characteristics including Gleason grade, clinical stage and pre-diagnostic PSA. The mean duration of overall statin use among ever-users was 86.5 months. Recurrences were reported by 115 (21.3%) subjects during the follow up period. The percentage of ever-statin users reporting BCR was 23% as compared to 19.6% of and non-statin users reporting BCR (p value =0.30). **(Table 2.1)**

Of the 115 subjects who experienced recurrence, 60 (52.2%) were statin users and 55 (47.8%) were non-statin users. In Cox proportional hazard models, the assumption of proportionality was tested using time-adjusted covariates and no significant departures from the proportional hazards assumptions were observed among covariates of interest. Overall, the HR of BCR among ever-statin users, modeled as a time-dependent covariate, was 0.959 (95% CI= 0.664-1.386, p =0.82) in crude analyses. This association between ever-statin use and BCR was similar after adjustment for age at surgery (years), BMI, Gleason, pre-diagnostic PSA, clinical stage and decade of surgery (HR=1.06, 95% CI=0.680-1.641, p value=0.81). **(Table 2.2)** In adjusted analysis, current NSAID use, Gleason 7=3+4 pattern (compared to referent Gleason ≤ 6), Gleason 7=4+3 and 8-10 pattern (as compared to Gleason ≤ 6) and pre-diagnostic PSA (log transformed), as well as having RRP after 2000 (as compared to prior to 2000) were each associated with BCR. Further, there was no association between duration of statin use and recurrence. For subjects using statins for 5 years or more (n=149) as compared to

subjects whose total statin use duration was less than 5 years (n=100), no association between statin use and BCR was observed (HR=0.932, 95% CI=0.53-1.63, *p* value =0.932). **(Table 2.3)**

I also investigated the association between statin use and BCR among lipophilic and hydrophilic statin users separately. Comparing lipophilic users (n=196) to non-statin users, in adjusted models, lipophilic statin users were found to have a HR of 1.20 of BCR (95% CI= 0.80-1.81, *p* value =0.43). In adjusted models, among hydrophilic statin users (n=81) the HR of BCR was 0.735 (95% CI= 0.35-1.54, *p* value =0.42). **[Table 2.4]**

In order to assess whether the association between statin medication use and BCR may be specific to advanced prostate cancers, I compared men with high Gleason grade cancers to men with low Gleason grade cancer. **[Table 2.5].** Among men with Gleason grade of 7=4+3 and 8-10 pattern cancers (n=87) (as compared to Gleason grade 6 or less cancers), statin use was not associated with BCR. Among men with Gleason grade 7=3+4 pattern cancers (n=255), statin use likewise was not associated with BCR (HR=1.15, 95% CI=0.717-1.833, *P* value=0.57).

In Kaplan-Meier survival analysis, ever-statin use was not associated with BCR free estimates **(**Wilcoxon test p value =0.85) **[Figure 2.3]**. In Kaplan-Meier survival analysis comparing duration among statin-users, duration of statin use (5

years or more as compared to participants who used statins for 5 years or less) was not associated with BCR free estimates (Wilcoxon test p value=0.54) **[Figure 2.4].**

In **Table 4.6**, I present results from Cox proportional hazards models adjusted for frailty using a gamma distribution. These results indicate that accounting for clustering within the 60 families with more than one subject included in this study did not materially change the hazard ratios or *p* values for statin use (both in crude and adjusted models). The values of Θ (dependence between clusters) extracted from these models were 0.36 and 0.432 from crude and adjusted models, respectively. Based on these values of dependence, the correlation between familial clustering and outcome is minimal, 0.15 and 0.19, in crude and adjusted models, respectively. The stability of the beta coefficients observed between frailty adjusted models and unadjusted Cox proportional hazards models and the minimal correlation value indicate that a Cox model without frailty is suitable to test the association between statin use and BCR in this study population.

In separate analysis (not presented in tables), observations were limited to those to subjects that had a prostatectomy after the year 1999 (during the same period as the exposure measurement). For ever-statin users as compared to non-users, limited to observations who had complete statin exposure data during the period post-RRP, no association was observed between ever-statin use and BCR

was HR= 1.034, 95% CI=0.716-1.492, *p* value =0.859. Further, I created a variable to account for participants who were using a statin medication at the time of prostatectomy (n=218). No association was observed between statin use at the time of surgery and BCR in either crude analysis (HR=1.294, 95%C.I.= 0.85-1.98, *p* value=0.2366) nor in adjusted analysis (HR= 1.367, 95% C.I.= 0.859-2.176, *p* value=0.1870).

Power was calculated for Cox proportional hazards regression models taking into account the correlation between covariates. The power to detect an association between statin use and risk of BCR using a two-sided hypothesis test at a 5% significance level (α =0.05) was limited. In this study population of 539 individuals with approximately 21% experiencing a recurrence, I had 20.2% power to detect a hazard ratio of 0.8, 33% power to detect a hazard ratio of 0.7 and 58.7% to detect a hazard ratio of 0.6. **[Figure 2.5]** In other words, if the effect of statin use of the risk of BCR among men treated with prostatectomy is modest (i.e. HR > 0.8), this study had insufficient power to detect this effect.

2.4 Discussion

Among 539 men in this retrospective cohort study with inherited forms of prostate cancer, self-reported statin use was not associated with BCR among men treated with radical prostatectomy. Furthermore, there was no association observed between duration of statin use (>5 years of use as compared to ≤ 5 years of statin use) and BCR. A 21% rate of recurrence was observed in this study and is comparable to the rate of recurrence expected with 10 years of RRP.

Previous studies of the association between statin use and risk of recurrence have been inconsistent in their findings. For 691 men treated with radiation therapy, statin use (median follow-up time of 50 months) was associated with improved freedom from biochemical failure and improved survival.(7) However, Ritch et al.(4) found an increased risk of BCR after post-operative statin use after a median follow-up time of six months. In 1,319 men undergoing RRP, Hamilton et al. (6) found that statin use was associated with a 30% decreased risk of biochemical recurrence and the associations occurred in a dose-dependent manner. Since we did not collect data on statin medication dose, we were prohibited from observing such a relationship. Further, a recent meta-analysis of 19 studies concluded that statins are not associated with the risk of prostate cancer incidence, but may be protective with respect to advanced prostate cancer.(2) Our study found that recurrence was strongly associated with pre-diagnostic PSA and high Gleason grade (7=4+3, 8-10), which are consistent predictors of biochemical recurrence.(21, 33)

In this study, BMI was not associated with the risk of BCR in crude analysis or in an analysis adjusted for other clinical covariates. The association between BMI and prostate cancer has not been consistently reported in the literature. BMI has been hypothesized to decrease PSA levels and therefore, may complicate diagnosis.(34) In a study of over 3,000 patients who underwent RRP, obesity (BMI $\geq$30 kg/m^2) was associated with increased disease grade, PSA levels

and rates of positive surgical margins, however, these unfavorable prognostic factors did not translate into decreased biochemical recurrence-free survival.(35)

Among study participants in this study, a low percentage of high Gleason grade cancers defined as Gleason 8-10 (17.4%) was found. In both crude and adjusted models Gleason grade of 7=4+3 pattern and higher was significantly associated with BCR. In stratified models, comparing participants with high Gleason grade cancers with participants with low Gleason grade cancers (Gleason grade 6 and below), we did not observe any significant associations between statin use and the risk of BCR. However, if the true effect of statin use in reducing prostate cancer risk is specific to high-grade cancers, we would have been underpowered to detect such an association. The inconsistent results among studies of statin use and recurrence could be linked to residual confounding after attempting to control for clinical or pathologic characteristics that may be indicative of advanced prostate cancer. As none of the studies of prostate cancer recurrence and statins have been performed prospectively, we can not conclude that the associations between statin use and prostate cancer risk or recurrence are not being driven by high-grade cancers with poorer pathologic features.

However, despite the conflicting results from observational and cohort studies, the findings from *in vitro* studies that statins may inhibit cancer cell growth, induce apoptosis(36) and cell cycle arrest (37) continue to fuel interest in exploring statin therapy as a potential chemopreventive agent in prostate cancer.

Currently there are no guidelines as to the use of statin medications in cancer risk or prevention. Studying these agents in men with a family history may provide a motivated group of men to focus preventative strategies. Men with a family history of prostate cancer have demonstrated their willingness to use chemopreventive agents to decrease risk. (25) Further,

BMI may be mediating the relationship between prostate cancer and statin use. This study may also be difficult to interpret in light of a study assessing men entering RRP on statins and found that pre-operative PSA was lower in statin users and compared to non-statin users. (38) Whether the observed decrease in PSA measurements leads to a detection bias or to actual changes in cancer progression is unknown. However, if the effect of statin medication use was solely to lower PSA but not to decrease risk, we would expect studies to find increased prostate cancer-specific or cancer-specific mortality and after a long-follow period. However, in a matched case-control study, statin use was associated with a decrease in prostate cancer mortality (OR=0.37, *p* value <0.0001).(39) Further, a study of both PSA and prostate cancer risk among statin users concluded that although PSA was lower among statin users, the relative risk decrease in prostate cancer risk was not accounted for by the decrease in PSA. (40) These scenarios highlight the notion that there may potentially be biologic effects of statin use on the progression of prostate cancer, other than to lower PSA and induce a detection bias.

The strengths of this study include a long follow up period (mean=94.9 months) and a unique cohort of men with inherited forms of prostate cancer/early onset prostate cancer. Statin medication use was high among survey respondents. The PCGP cohort has been previously reported to have higher education and income levels.(41) The high statin use reported in this study may indicate a healthy user bias. Participants may be more likely to be screened frequently, possibly leading to less differential in PSA screening practices between statin and non-statin users. Men enrolled in this study do so voluntarily and may be more likely to accurately report health updates, statin use and PSA testing history. The mean age at time of prostatectomy in this study was 56.5 years. This finding is consistent with early-onset of initial prostate cancer diagnosis previously reported in men with familial/hereditary prostate cancer (42, 43) and highlights that this group of men an ideal group to study chemoprevention given the greater number of years they spend after prostate cancer diagnosis as compared to sporadic prostate cancer cases. The mean follow-up period (post-surgery) was significantly longer in ever-statin users as compared to ever-statin users (101.5 months vs. 88.8 months, *p* value =0.009). The longer follow-up period observed among statin users may indicate that there is a differential loss to follow-up by exposure in our study population.

This study has some limitations. Statin medication dose information was not collected from subjects and we were not able to examine a dose effect other than what we could characterize by the potency of the statin. We did not collect

information on co-morbidities, cholesterol levels or overall health measures. Confounding could have resulted from not controlling for other factors associated with statin use and risk factors for recurrence. Studies have shown that cholesterol levels may be associated with cancer risk.(14) Diabetes (treated or untreated) has been associated with an increased risk of biochemical recurrence after prostatectomy.(44). Further, even if a subject was on statins, we did not measure cholesterol levels and there is also a complex interplay between cholesterol and prostate cancer malignancy. A review of prostate cancer and cholesterol found that hypercholesteromeia is likely a risk factor for prostate cancer progression.(45) Such potential unmeasured confounders could have altered our association between statin use and biochemical recurrence risk.

Although participants reported statin use and start and end dates over the past 10 years prior to survey, we did not have information as statin use at the time of surgery for subjects who had their RRP prior to 1999 (the first year subjects were asked about statin use) (n=154, 28.6%). We were limited in our ability to determine whether a potential biologic effect of statins varied as to the timing post-RRP of statin initiation among all respondents.

Our eligibility criteria specific response rate was low (41%). Give that the base for our study is men who responded to the 2009 HUS, there is likely a response bias. We were unable to collect statin medication use data on men who were deceased, or would not have been able to respond to the survey due to poor health. This limitation prevented us from measuring competing risks. Conclusions from this retrospective study based on self-report are limited by the nature of the

study design.

Future prospective studies of statin use may be well suited to this cohort. This group represents a motivated group and is likely to have accurate recollection of PSA tests and medication use. In addition to statin use and recent PSA testing information asked on the 2009 HUS, participants were also asked to recall the date and treatment they received related to their initial prostate cancer diagnosis in the same survey. Among survey respondents, the concordance between information reported on the 2009 HUS and information previously confirmed through medical record review was very high (>98%) indicating that information provided by these participants was accurately recalled and reported. 5-ARIs, which have been shown to lower prostate cancer risk, have a low prevalence of use among men with a family history of prostate cancer(25), presumably due to potential side-effects and potential for an increase in higher-grade prostate cancers. Elucidating the biologic mechanisms and effects of a potential chemopreventive agent, which may confer decreased prostate cancer risk but also has minimal side effects has the potential for substantial public health significance, particularly for men with an increased risk of prostate cancer. The high incidence, prevalence and treatment-related morbidities associated with prostate cancer make an ideal target for a chemopreventive agent in either the primary or secondary prevention setting. Future studies attempting to elucidate the effect of statin use on the risk of prostate cancer recurrence should focus on prospectively designed studies or clinical trials beginning at the time of initial

prostate cancer treatment with uniform screening protocols in men with a inherited forms of prostate cancer.

2.5 Conclusions

Statins (3-hydroxy –3– methyl-glutaryl-coenzyme reductase inhibitors) are a potential prostate cancer chemopreventive agent. In this study of men with inherited forms or early-onset prostate cancer, statin use was not associated with the risk of biochemical recurrence after prostatectomy. However, due to the limited number of men in this study, this association may warrant further investigation, particularly in men with high-grade cancers who at risk for recurrence.

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Figure 2.1 Potential Actions of Statins in BCR

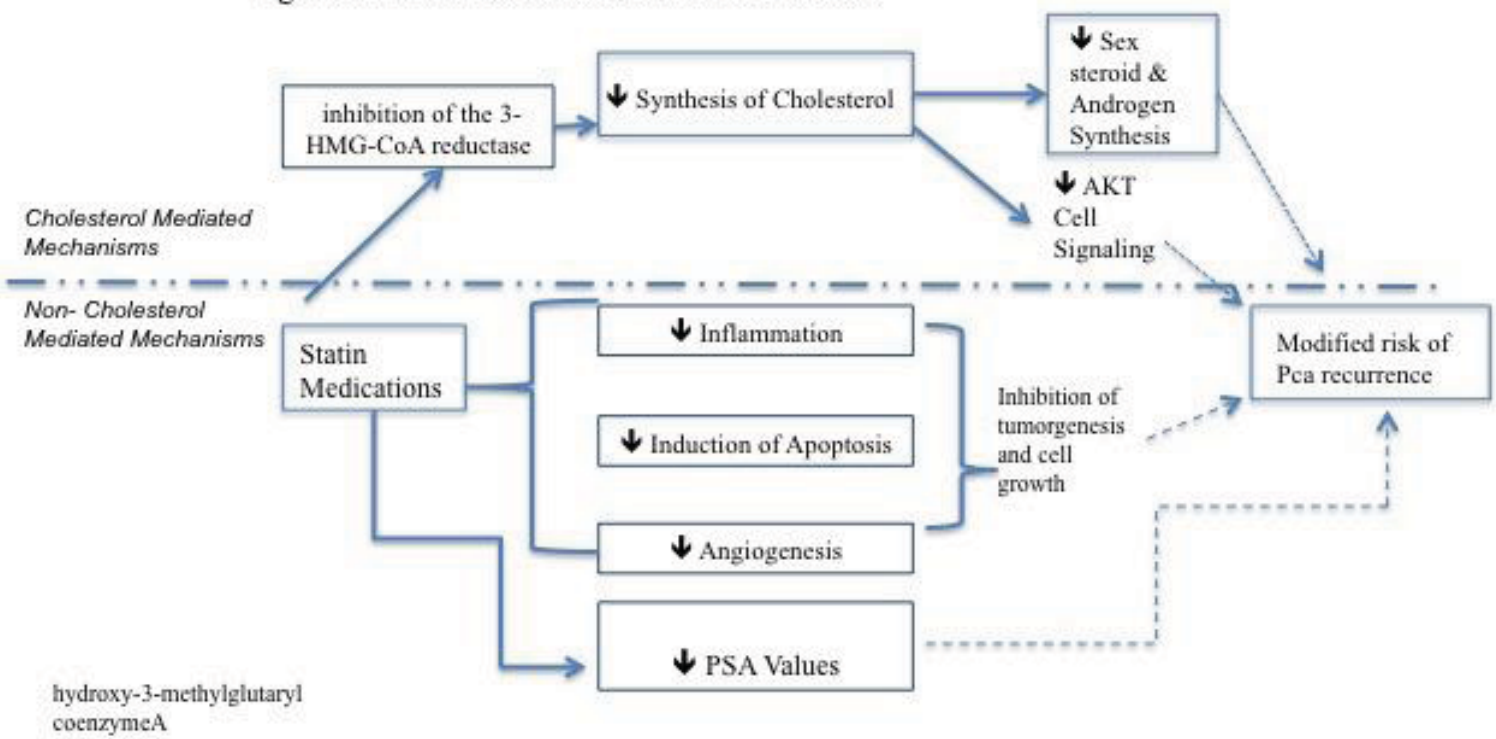

Figure 2.2: Schematic of associations between statin use and BCR

Dashed lines indicate associations which are hypothesized but have not yet been shown

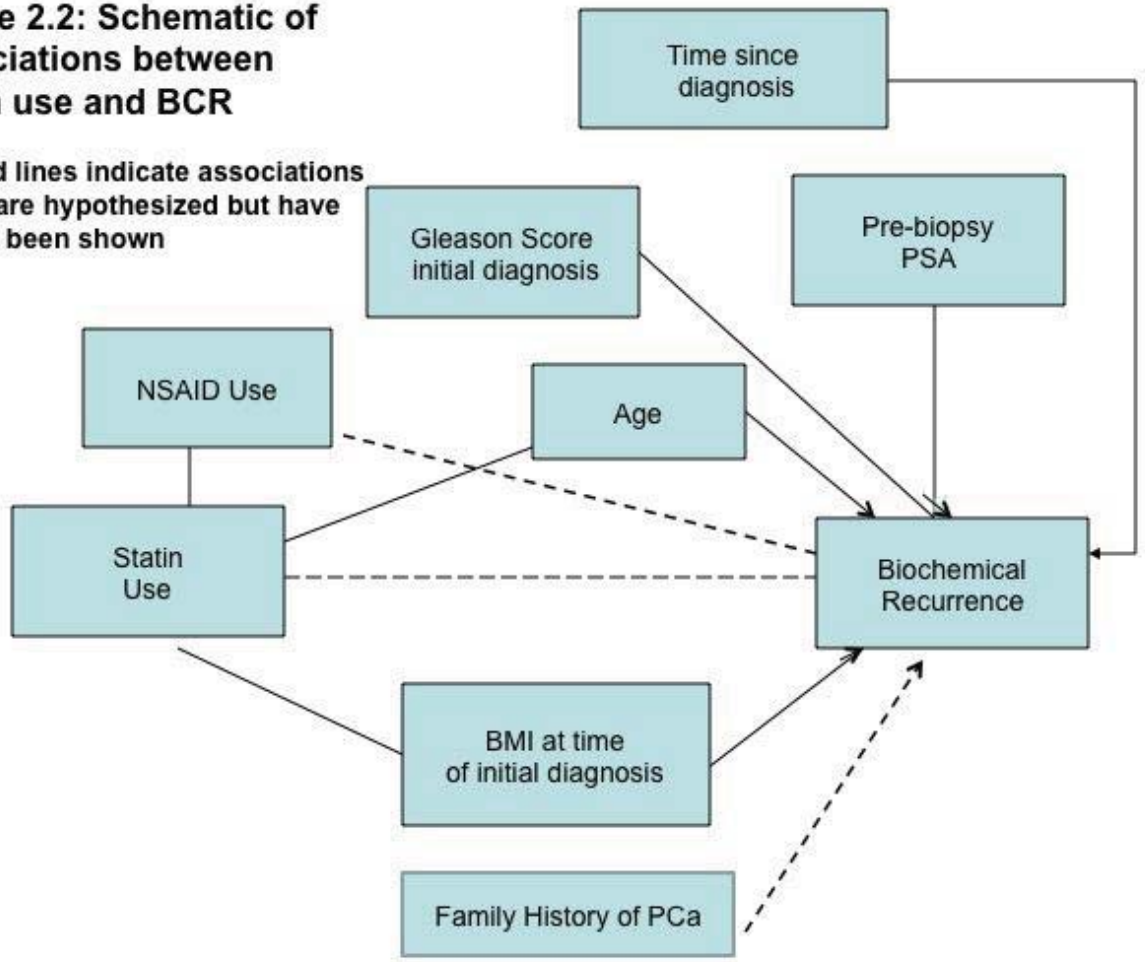

Table 2.1 Demographic, Clinical and Pathological Characteristics of Study Participants according to Statin Use

Characteristic	All participants (n=539)	Statin Users * (n=258, 47.9%)	Non-Statin User (n-281, 52.1%)	P value
Age at survey, Mean (SD)	64.5 (9.4)	66.5 (9.1)	62.6 (9.3)	<0.001
Age at surgery, Mean (SD)	56.5 (7.6)	58.0 (7.4)	55.2 (7.6)	<0.001
Follow-Up Time (months), Mean (SD)	94.9 (56.6)	101.5 (56.5)	88.8 (56.2)	0.009
BMI (kg/m^2) at time of initial PCa diagnosis, Mean (SD)	26.9 (3.4)	27.4 (3.5)	26.5 (3.3)	0.004
BMI categories, No. (%)				
< 25.0 kg/m^2	148 (27.5)	53 (20.5)	95 (33.8)	0.003 [a]
25.0- 29.9 kg/m^2	305 (56.6)	159 (61.6)	146 (52.0)	
≥ 30 kg/m^2	86 (16.0)	46 (17.8)	40 (14.2)	
Lipophilic Statin Users		196 (36.4)	n/a	----
Hyrdrophilic Statin Users		51 (9.5)	n/a	
Most Recent PSA Test Value	2.1 (19.1)	1.37 (13.4)	2.8 (23.0)	0.44
Current NSAID Use, No. (%)				
Yes	270 (50.1)	156 (60.5)	114 (40.6)	<0.001 [a]
No	269 (49.9)	102 (39.5)	167 (59.4)	
Race, No. (%)				
White	523 (97.0)	249 (96.5)	274 (97.5)	0.83 [b]

African-American	13 (2.4)	7 (2.5)	6 (2.1)	
Other	3 (0.56)	2 (1.0)	1 (0.4)	
Characteristic	**All participants (n=539)**	**Statin Users * (n=258, 47.9%)**	**Non-Statin User (n-281, 52.1%)**	**P value**
Gleason Grade**				
<= 6	240 (44.5)	116 (45.0)	124 (44.1)	0.87 [a]
7	180 (33.4)	83 (32.2)	97 (34.5)	
8-10	94 (17.4)	47 (18.2)	47 (16.8)	
Unknown	25 (4.7)	12 (4.6)	13 (4.6)	
Pre-Operative PSA, Mean (SD) [§]	7.5 (9.7)	7.9 (10.2)	7.1 (9.1)	0.38
Year of Surgery, Median		2000		
Prostatectomy Year categories, No. (%)				
< 2000	190 (35.2)	107 (41.5)	83 (29.5)	0.004 [a]
2000 - 2009	349 (64.8)	151 (58.5)	198 (70.5)	
Clinical Stage, No. (%)				
T2a	74 (13.7)	25 (9.7)	49 (17.4)	0.05[a]
T2b	294 (54.5)	145 (56.2)	149 (53.0)	
T3	113 (21.0)	54 (20.9)	59 (21.0)	
Unknown	58 (10.8)	34 (13.2)	24 (8.5)	
Current Statin Users, No. (%)				
Yes	226 (42.4)			
No	307 (57.6)			

Duration of Statin Use, months, Mean (SD)		86.5 (57.5)	n/a	---
Statin Use less than 5 years		100 (40.2)		
Statin use ≥ 5 years		149 (59.8)		
Characteristic	**All participants (n=539)**	**Statin Users * (n=258, 47.9%)**	**Non-Statin User (n-281, 52.1%)**	**P value**
Experienced a recurrence, No.(%)				
Yes	115 (21.3)	60 (23.3)	55 (19.6)	0.30 [a]
No	424 (78.7)	198 (76.7)	226 (80.4)	

* Ever users

** Pathological Gleason Grade except 20 observations where path Gleason was missing and Biopsy Gleason was substituted

§ Pre-diagnostic PSA missing for 58 subjects

[a] P value obtained using the Chi-square test, [b] p value obtained using Fisher's exact test, [c] p value obtained from Student's t test

Table 2.2 Crude and Adjusted Proportional Hazards Model for Statin Use and Risk of BCR

	Crude HR (95% C.I.)	Crude *p* value	Adjusted HR (95% C.I.)+	Adjusted *p* value
Current Statin Use (n=226)	0.96 (0.66-1.39)	0.82		
Ever User (n=258)	1.04 (0.72-1.49)	0.86	1.06 (0.68-1.64)	0.81
Age at time of surgery, yrs	0.99 (0.96-1.01)	0.23	0.97 (0.95-1.00)	0.07
BMI (kg/m^2), <25	ref			
BMI (kg/m^2), >=25 -30	1.25 (0.86-1.82)	0.25	1.31 (0.78-2.20)	0.31
BMI (kg/m^2), >30	1.20 (0.74-1.97)	0.46	1.35 (0.70-2.63)	0.37
NSAID Use	0.94 (0.65-1.35)	0.72	0.96 (0.62-1.48)	0.03
Pathological Gleason				
Gleason 6	ref		ref	
Gleason 7 (3+4)	1.07 (0.72-1.60)	0.74	1.62 (0.95-2.77)	0.08
Gleason 7 (4+3) & 8-10	3.78 (2.59 -5.55)	<0.01	3.63 (2.07-6.37)	<0.001
Pre-Diagnostic PSA* (ng/mL)	1.45 (1.14-1.84)	0.01	1.32 (1.02-1.70)	0.03
Clinical Stage				
T2a	ref		ref	
T2b	0.39 (0.26-0.59)	<0.01	0.58 (0.32-1.07)	0.08
T3	2.86 (1.94-4.22)	<0.001	1.26 (0.65-2.42)	0.50
Decade of Surgery				
<1999	ref		ref	
2000-2009	1.99 (1.26-3.13)	0.003	2.27 (1.36-3.80)	0.002

* Pre-Diagnostic PSA was log transformed

\+ Models adjusted for age at time of surgery, BMI, NSAID use, Gleason grade, pre-diagnostic PSA, clinical stage and decade of surgery

Table 2.3 Cox Proportional Hazards Models of the Association between duration of statin use and BCR

	No.	Unadjusted HR (95% C.I.)	Unadjusted p value	No.	Adjusted HR * (95% C.I.)	Adjusted p value
Duration of statin use (years)						
>=5	149	0.93 (0.53-1.63)	0.93	127	1.11 (0.58-2.09)	0.76
< 5	100	1.07 (0.61 -1.88)	0.81	91	0.91 (0.48-1.71)	0.95
Total Statin Use, months	249	1.001 (0.99-1.005)	0.81	218	1.001 (0.99-1.01)	0.76

*Multivariate models adjusted for decade of prostatectomy, Gleason 7=3+4 pattern, Gleason ≥7 =4+3, log transformed pre-diagnostic PSA

Table 2.4 Cox Proportional Hazards Models of association between a) Lipophilic statin use and BCR and b)Hydrophilic statin use and risk of BCR

	No.	HR (95% C.I.)	Unadjusted p value	No.	Adjusted HR * (95% C.I.)	Adjusted p value
Lipophilic Statin User	196	1.16 (0.80-1.68)	0.43	169	1.20 (0.80-1.81)	0.37
Hydrophilic Statin Use	81	0.75 (0.38-1.48)	0.41	51	0.74 (0.35 -1.54)	0.42

*Multivariate models adjusted for decade of prostatectomy, Gleason 7=3+4 pattern, Gleason ≥7 =4+3, log transformed pre-diagnostic PSA

Table 2.5 Cox Proportional Hazards Models of association between statin use and BCR comparing high Gleason grade cancers to low Gleason grade cancers

	No.	Adjusted HR * (95% C.I.)	Adjusted p value
Gleason 7=4+3, 8=10	87	1.01 (0.51-1.99)	0.98
Gleason 7=3+4	255	1.15 (0.72-1.83)	0.57
Gleason ≤ 6	197	Ref	

*Multivariate models adjusted for decade of prostatectomy, log transformed pre-diagnostic PSA, NSAID use and BM

Table 2.6 Gamma Frailty Models comparing ever-statin users to non-statin users accounting for relatedness among subjects

	No. (%)	**HR (95% C.I.)**	**p value**	Θ	Kendall's **T**
Model 1: Ever-Statin User*	539	1.03 (0.7-1.52)	0.87	0.361	0.15
Model 2: Ever-Statin User**	467	0.97 (0.62-1.52)	0.89	0.464	0.19

*Model 1: Crude Cox Proportional Hazards Model

** Model 2: Adjusted Cox Proportional Hazards Model adjusted for BMI, NSAID use, decade of surgery, Gleason734, Gleason743, Pre-diagnostic PSA (log transformed)

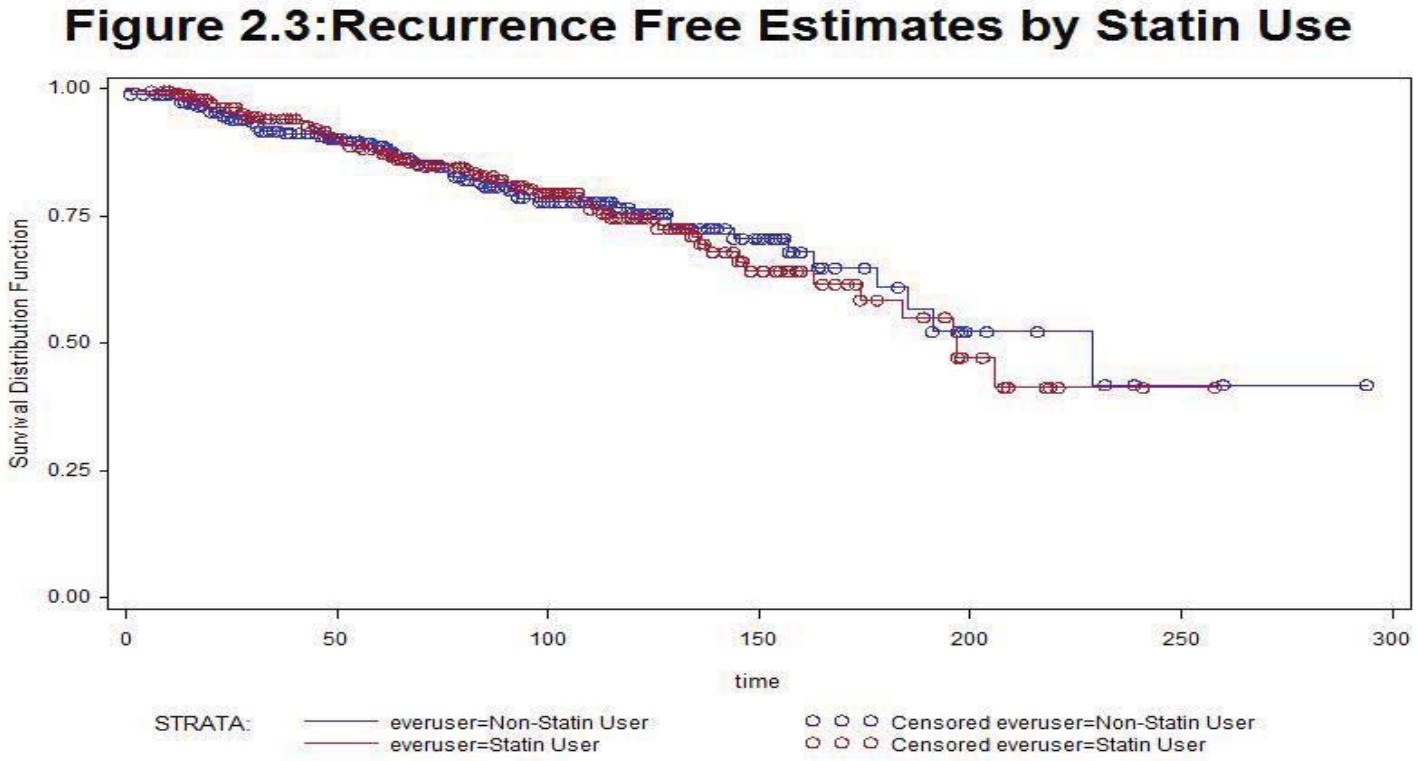

Figure 2.3 Association between ever-statin use and BCR-free survival is shown using Kaplan-Meier survival function. Ever statin users shown in red and Non-statin users shown in blue.

Figure 2.4:Recurrence Free Estimates by Statin Use Duration

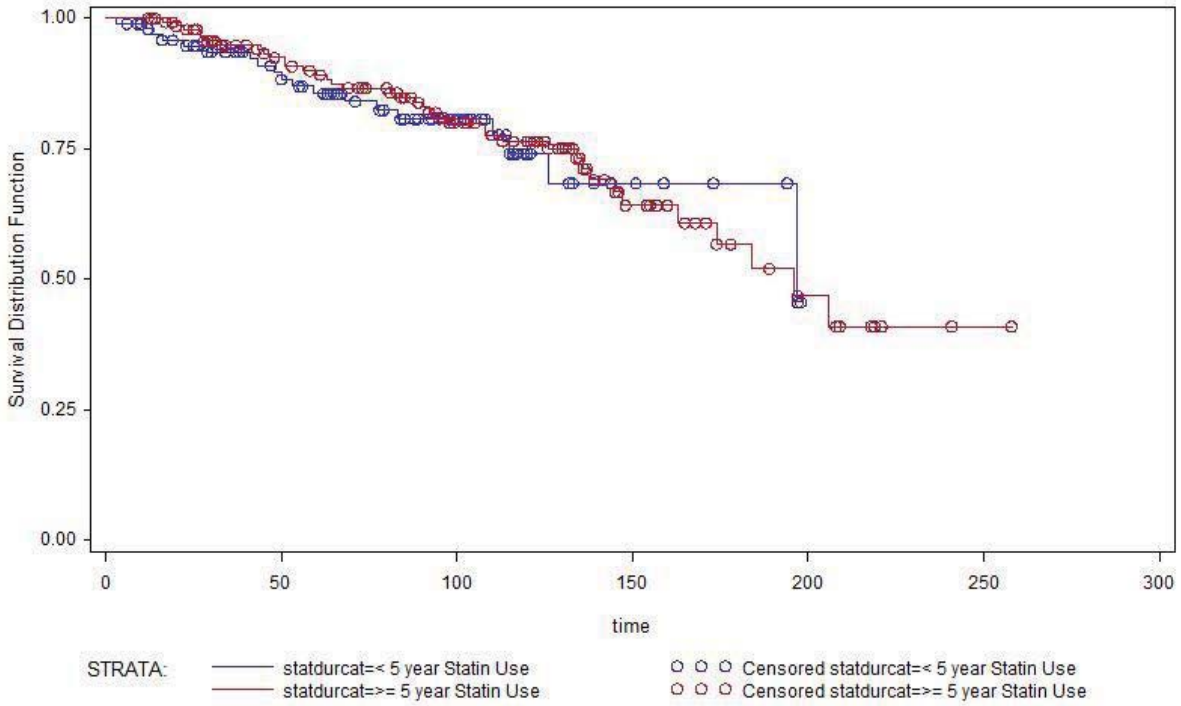

Figure 2.4 Association between duration of statin use (≥5 years (in red) vs. < 5 years (in blue) and BCR-free survival is shown using Kaplan-Meier survival function.

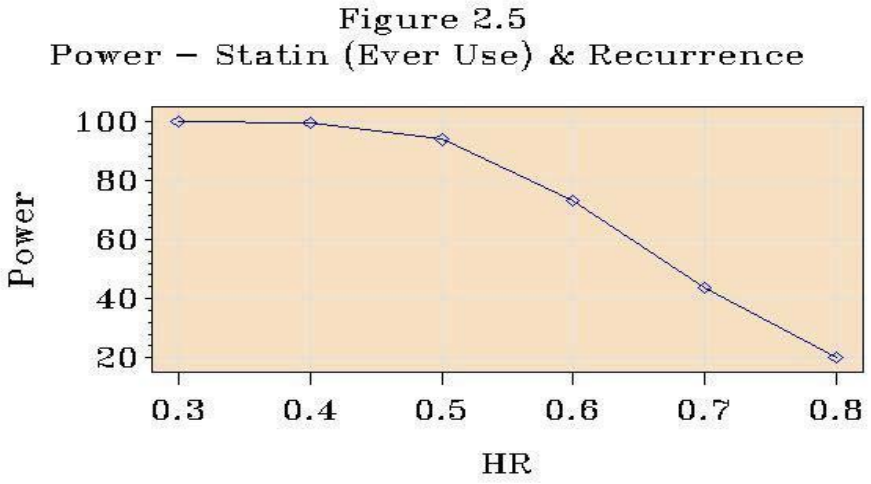

Figure 2.5 Power to detect an association between statin use and BCR in men with inherited forms of prostate cancer.

CHAPTER 3

Rare variants in *MAP3K14, RND2* and *ARHGAP27* and hereditary prostate cancer

3.1 Introduction

The established risk factors for prostate cancer are increasing age, positive family history and African-American race.(1) Familial clustering of prostate cancer has been well established, and results from segregation analyses strongly suggest that genetic risk factors explain a significant amount of the familial aggregation of prostate cancer.(2) Compelling evidence exists for a genetic component to prostate cancer susceptibility. A family history of prostate cancer in a first-degree relative confers a 50% increased risk of developing the disease. A family history of three first-degree relatives with prostate cancer gives rise to an increased risk of 11-fold in male relatives. (3) Further, concordance for prostate cancer is substantially higher among monozygotic twin pairs as compared to dizygotic twin pairs, (27.1% vs. 7.1%, respectively).(4) The definition of hereditary prostate cancer has been subject to debate, however the "Hopkins Criteria"(5) set forth defines hereditary prostate cancer as families meeting at least one of the following criteria: a) three or more first degree relatives with

prostate cancer, b) prostate cancer in three successive generations through the paternal or maternal lineage, and/or c) two first-degree relatives diagnosed with PC at an early age (≤ 55 years). Sporadic prostate cancer (which includes men with no family history) accounts for the majority of prostate cancer cases (75%-85%). Familial prostate cancer (which includes men with a family history which do not meet the strict criteria of HPC above) accounts for 10% to 20% of PC cases, while HPC accounts for 5 – 10% of prostate cancer cases. (5)

Linkage analysis has been used to attempt to identify genetic loci predisposing to familial/hereditary prostate cancer using families with multiple affected members, however there has been little success in identifying genes and variants involved in HPC.(6) Strong evidence for prostate cancer linkage has been consistently reported at chromosomal region 17q21-22. In 2003, the first evidence for the existence of a prostate cancer gene on chromosome 17q was reported based on findings from a genome- wide linkage scan (GWS) on 175 pedigrees from the University of Michigan Prostate Cancer Genetics Project (PCGP). (7) In this report, a nonparametric LOD score of 2.36 consistent with suggestive evidence for linkage was observed at approximately 60 cM and the nonparametric LOD score increased to 3.27 when restricting to pedigrees with four or more prostate cancer cases. Linkage evidence supporting chromosome 17q as a potential prostate cancer susceptibility locus was subsequently reported in two additional analyses, one study including 426 HPC families detected a LOD score of 3.16 (8) and another combined analysis of 1,233 prostate cancer families from the International Consortium for Prostate Cancer Genetics (ICPCG) also

found evidence of linkage at 17q21 and reported a LOD score of 1.99. (9) Both of the confirmatory studies included the 175 HPC families from UM and an additional 188 HPC families from John Hopkins Hospital (JHU). Further, A fine-mapping study conducted in a subset of 147 families (including families from UM-PCGP and JHU) with 4 or more affected members or an average age of diagnosis less than or equal to 65 years found a maximum LOD score of 5.49 at 78cM with a 1-LOD support interval of 10cM.(10) A genome-wide linkage scan of 69 HPC families from within Finland (no families in common with the previous linkage scans mentioned above) further confirmed linkage at 17q21-22 with a LOD score of 3.14 detected among these families.(11)

A strong inherited component in prostate cancer has been consistently reported. Inherited susceptibility is estimated to account for up to 40% of variation in prostate cancer risk; this percentage exceeds what has been found in solid tumor cancers such as breast and lung.(12) However, attempts to identify susceptibility genes and/or variants have been largely unsuccessful. Previous prostate cancer linkage studies have identified other chromosomal regions of linkage such as 17p11(13), 8p(14), and 1q23-25(15, 16). Further attempts to identify suceptibility genes in each of these linkage regions were focused on isolating known variants within strong candidate genes within each region. *HPC1* and *RNASEL*(1q24), *ELAC2* (17p11) and *MSR1* (8p22) have all been identified and studied as potential prostate cancer susceptibility genes, however, confirmatory studies have been conflicting and no high penetrant variants have been found. (17) Limitations of such approaches may be due to genetic and

phenotypic heterogeneity. Linkage analyses often provide conflicting results dependent on the subset of families used in each study potentially resulting from genetic and phenotypic heterogeneity among pedigrees studied. One of the major causes for difficulty in mapping prostate cancer genes is reduced statistical power due to multiple susceptibility genes, incomplete penetrance, and high rates of sporadic prostate cancer in the general population. Recent genome-wide association studies (GWAS) in prostate cancer have identified around 30 common mutations in the genome associated with prostate cancer. (18) However, these mutations have low magnitudes of association and their clinical utility may be limited. The utility of GWAS identified SNPs in prostate cancer risk prediction was assessed in two large population-based case-control studies of men in the US and Sweden. Among men with a family history of the prostate cancer, having over 14 GWAS-identified risk alleles was associated with an increased risk of prostate cancer..(19) However, the > 30 variants identified through GWAS appear to only account for about 25% familial clustering in prostate cancer. (20) The current understanding of inherited variation in prostate cancer risk, whether from linkage, genome-wide association studies or candidate gene association studies, does not appear to explain the significant portion of familial clustering in prostate cancer. Although having a large number of commonly occurring variants with small magnitudes of association is associated with an increased risk of prostate cancer, common variation does not appear to explain the hereditary component of prostate cancer. These findings point to the existence of rare, highly penetrant allele(s) that may be underlying the strong aggregation of prostate cancer in HPC

families.

The observation of strong LOD scores obtained from a subset of HPC families with relatively large proportion of early age-of-onset cases strongly suggests the existence of a chromosome 17q21-22 prostate cancer susceptibility gene(s). A potential susceptibility gene in the 17q21 region is the breast cancer susceptibility gene *BRCA1* (located within 5cM of the LOD peak (D17S1868). *BRCA1* has been investigated as a potential prostate cancer susceptibility gene, as male carriers in breast cancer/ovarian cancer families tend to have an increased risk of prostate cancer, however most studies of have found little evidence of an association between prostate cancer (early onset/FPC) and deletrious *BRCA1* mutations. Further, among 65 unrelated individuals from the 175 HPC UM-PCGP families sequenced for full gene mutation analysis *BRCA1* gene, no deleterious mutations accounting for the linkage evidence were discovered. (21)

Among the genes located within this 10 cM linkage peak are *RND2, MAP3K14* and *ARHGAP27*. *MAP3K14* is located at 17q21 and is known as mitogen-activated protein kinase kinase kinase 14, which is a serine/threonine protein-kinase. *MAP3K14* encodes NIK (NF-kB inducing kinase) which induces production of active NF-kB dimers in the non-canonical pathway.(22) The NF-kB -inducing signaling cascade is common to receptors of the tumor-necrosis/nerve-growth factor (TNF/NGF) family and to the interleukin-1 type-I receptor and is believed to contribute to tumor cell invasion and metastases.(23) *RND2* is located on 17q21 and neighbors the *BRCA1* gene. *RND2* encodes a member of the Rho GTPase family and has been implicated in the regulation of

neuronal morphology and endosomal trafficking.(24) *ARHGAP27* is located on 17q21 and ARHGAP family genes are associated with cancer, because their genetic alterations lead to carcinogenesis through the dysregulation of Rho/Rac/ Cdc42-like GTPases.(25)

Next-generation sequencing technologies have provided new opportunities to interrogate large genomic intervals that are implicated in human disease in a rapid and comprehensive manner. This study utilizes next-generation sequencing approaches to sequence the complete coding region (exome) in the genomic interval of interest, 17q21-22. Exome sequencing has the potential to locate causative mutations in key genes in complex diseases by interrogating the 1% of the genome, which results in protein coding changes, which previously has not been possible due to limitations in traditional methods. Targeted sequencing also provides an opportunity to identify novel variants within coding regions. Given the consistent evidence of prostate-cancer linkage to the 17q21-22 region in multiplex HPC families, a targeted sequencing strategy was undertaken to analyze 2009 exons (coding regions) of 202 genes located within the 16cM 2-LOD support interval at chromosome 17q21-22. The goal of this study is to investigate the variation in genes with biologic plausibility, *MAP3K14*, *RND2*, and *ARHGAP27* through next-generation sequencing techniques in order to discover potential mutations associated with HPC.

3.2. Materials and methods

3.2.1 Study subjects

The data for this study came from two study populations. The University of Michigan (UM) PCGP is a large, ongoing family-based study designed to map and identify genes predisposing to inherited forms of prostate cancer. Enrollment into PCGP is restricted to (1) families with two or more living members with prostate cancer in a first- or second-degree relationship or (2) men diagnosed with prostate cancer at ≤55 years of age with or without a family history of the disease. All participants are asked to provide a blood sample, extended family history information, and access to medical records. The majority of the PCGP families were recruited directly from the University of Michigan Comprehensive Cancer Center. Other sources included direct patient or physician referrals. Diagnosis of prostate cancer was confirmed by review of pathology report and medical record review. The Institutional Review Board at the University of Michigan Medical School approved all aspects of the protocol, and all participants gave written informed consent, including permission to release their medical records.

Data from JHU came from HPC families each with at least three first-degree relatives with prostate cancer. Diagnosis of prostate cancer was confirmed through medical record review. All of the families included have been previously included in linkage studies performed in HPC families.(7, 8, 10) A study schema is presented in **Figure 3.1**.

Ninety-four unrelated individuals from the UM-PCGP and JHU HPC families (54 from the PCGP and 40 from JHU) were selected for this study ("probands"). Individuals selected belonged to families that have demonstrated linkage to the 17q21-22 region (i.e. informative for non-parametric linage

analysis) in prior studies and belonged to families with three or more confirmed cases of prostate cancer within their family. From these families, the individual with the youngest age at diagnosis was selected for targeted sequencing. Genomic DNA was extracted from whole blood using 5-PRIME ArchivePure DNA purification kit (Fisher Scientific).

3.2.2. Targeted sequencing

A primer library was designed for amplification of ~2800 amplicons representing 2009 exons from our target region. We then used the RainDance RDT 1000 system (RainDance Technologies, Inc., Lexington MA) to amplify 3 ug of sheared genomic DNA from each sample using our primer library. Purified amplicons were used as template for sequencing using the Life Technologies SOLiDTM system, version 4.0 fragment library methodology (Life Technologies Corporation, Carlsbad, CA). Sequence data processing was performed using Life Technologies Bioscope to align the sequences to the genomic reference (Build 36, hg18). Variant detection was performed using SamTools 1.31 and SolSNP 1.1. Variants were analyzed for potential pathogenicity using Polyphen (http://sift.jcvi.org/) and SIFT (http://genetics.bwh.harvard.edu/pph/) for coding variants. All nonsynynomous variants detected in the *MAP3K14*, *RND2*, and *ARHGAP27* genes were selected for confirmation.

3.2.3 Confirmation by Sanger Sequencing

Five nonsynonymous variants were detected within the genes of interest. Direct PCR and Sanger sequencing were used to validate mutations found in the

genes of interest among probands. In addition, family members of individuals with confirmed mutations (both those affected and unaffected with prostate cancer) were subject to Sanger sequencing if DNA was available (n=75). Sequencing of variants in *RND2, MAP3K14,* and *ARHAP27* was performed using DNA extracted from whole blood with the primers presented in **Table 3.1.** For the H548Q variant, PCR reaction mixtures (50µL) contained 4 µl of 20ng/ µl genomic DNA, 10µl of 5X AccuPrime GC Rich PCR Buffer A, 31 µL of water, 2 µL of 5µM forward and reverse primers each, and 1.0 µL of AccuPrime GC-Rich DNA Polymerase (All reagents are Invitrogen). The PCR reaction mixtures for all other detected variants (50µL) contained 2 µl of 20ng/µl genomic DNA, 5µl of 10X PCR buffer, 30.25 µL of water, 1.5µL of 50mM MgCl2, 1µL of 10mM dNTP (New England Biolabs) and 5µL of 5µM forward and reverse primers, and 0.25 µL of Platinum Taq DNA polymerase (Invitrogen). Cycling conditions for all variants were as follows: amplification was performed through 30 thermal cycles (denaturation at 95°C for 1 minute, annealing under temperature gradient of 59.6 °C for 1 minute and extension at 72 °C for 1 minute). An initial annealing step at 95°C for 5 min was performed before thermal cycling and an extension step at 72 °C for 10 min was performed after the thermal cycling. PCR products were sequenced using standard Sanger sequencing, capillary electrophoresis technology and BigDye® Terminator chemistry (Applied Biosystems, Carlsbad CA). All nonsynonymous variants within the candidate genes were selected for confirmatory sequencing.

3.2.4 Mutation frequency comparison

Allele frequencies were compared to those reported in the Exome Variant Server (EVS) within the Exome Sequencing Project (ESP) funded by National Heart Blood and Lung Institute (NHLBI). (http://evs.gs.washington.edu/EVS). (26) The current EVS data release (**ESP5400**) is taken from 5379 samples drawn from multiple ESP cohorts and represents the first data freeze of the ESP exome variant data. Sequences were aligned to NCBI build 37 human genome reference. For purposes of our analyses, we considered only European-American (EA) samples from EVS. ESP does not report data with respect to cancer diagnoses or other diseases on individuals included in their study therefore, we used the ESP data only to compare the frequency of mutation occurrence in this sample of populations collected by NHLBI with the frequencies found in this study.

3.2.5 Statistical analysis

Subject characteristics were presented as median and interquartile ranges for continuous variables and as number and percentages for categorical variables. Fisher's exact tests were performed to compare allele frequencies of detected variants among the 94 probands from our study with publicly available data jn ESP (over 3000 EA samples EVS data). Statistical models were performed using the R statistical package (http://cran.r.project.org). Adjusted *P* value thresholds were calculated for each gene using a Bonferroni adjustment to adjust for all nonsynonymous variants in each gene among EA samples cataloged in EVS in order to correct for multiple hypothesis testing.

Allele sharing among affected individuals within each pedigree was

calculated using a pairwise approach using the non-parametric linkage (NPL) pairs statistic, which provides a multipoint approach to calculating the identity by descent (IBD) probabilities within each pair of individuals in a pedigree. The computation of the NPL pairs statistic relies on the total number of alleles shared IBD between pairs of affected individuals in a pedigree. The IBD sharing tests can be used to determine if a pedigree selected on the basis of the presence of the gene shows stronger evidence of linkage as measured by increased allele sharing IBD among affected family members. I used the King and Cox NPL pairs statistic (27) to test for allele sharing among affected individuals using the MERLIN (Multipoint Engine for Rapid Likelihood Inference) software program.(28) I used the exponential model to estimate linkage given the small number of pedigrees. Different pedigree structures have different sets of possible IBD-sharing configurations. The null hypothesis is all IBD states are equally likely and the alternative hypothesis is that an increase (or decrease) in probability of each state occurs. To combine signals from different pedigree structures, a scoring function is required to assign a numerical value to each IBD-sharing configuration (i.e. S pairs) Given S, the number of alleles shared IBD (0,1,2) between a pair of individuals, and given for a sibling pair, for example, $E(S_{pairs}) = \frac{1}{4}(nIBD_0)^2 + \frac{1}{2}(nIBD_1)^2 + 3/4(nIBD2)^2$ then:

$$Z_{pairs} = \frac{S_{pairs} - E(S_{pairs})}{[Var(S_{pairs})]^{1/2}}$$

NPL pairs statistic can be computed as follows:

$$Z = \Sigma\ (1/\sqrt{m})(Z_{pairs})$$

where m = number of pedigrees, $Z_{(pairs)i}$= normalized score for pedigree i

In addition, family-based association testing (FBAT) was performed using the PLINK software package (version 1.07) (http://pngu.mgh.harvard.edu/purcell/plink) to test for association between each gene and prostate cancer.(29) The DFAM procedure within PLINK was used to perform family-based tests of association using discordant sib-pair data in the absence of parental genotype and phenotype data. Family-based association tests are a class of generalized score statistics that use within- and between-family marker-inheritance patterns to test for association (32, 33). The null hypothesis in family-based association testing is that the marker or gene has no association and no linkage with the trait or that the marker or gene and trait are not associated in the presence of linkage and the alternative hypothesis is that the marker or gene has an association with the trait in the presence of linkage. This approach to family-based tests of association builds on the original transmission disequilibrium test (TDT) method but is extended to allow testing data from nuclear families, sibships, pedigrees, or any combination and provides unbiased tests even in the absence of parental genotypes. (30) The association was analyzed using data from both affected and unaffected family members to maximize power. Family-based association tests provide a marker-specific Z value and a 2-sided *p* value based on the normal approximation.

An aggregate NPL score was calculated for each marker using the NPL

scores from previous linkage studies for each family harboring the variant for that particular marker.

3.3 Results

Targeted sequencing was performed in the 17q21-22 candidate region among 94 unrelated probands belonging to HPC families from UM-PCGP and from JHU. All families selected for targeted sequencing previously demonstrated linkage to 17q21-22. There were 202 genes and 2009 exons interrogated within this region and the average depth of coverage across all loci was 49.5X. An average of 705 variants per sample were detected across the target region. Approximately 694 variants on average were previously described in dbSNP and on average about 12 novel variants were detected per individual. The sample characteristics are presented in **Table 3.2** There were 54 probands from UM families and 40 probands were from JHU families. The average age of diagnosis was 52 years among PCGP probands and 62 years among JHU probands. **[Table 3.2]**. Pre-diagnostic PSA was 5.8 ng/mL among UM probands and 12.6 ng/mL among JHU probands. Of the 94 probands sequenced in this study, 9 (9.6%) were non-Caucasian. The mean confirmed number of men affected with prostate cancer in each proband's family was 4.67 and 4.9 among PCGP participants and among JHU participants, respectively.

There are 202 genes within this chromosomal region. The exons interrogated included 17 exons within *ARHGAP27*, 15 exons within *MAP3K14*

and 5 exons within *RND2*. The location of these genes with respect to the 2-LOD support interval at 17q21-22 is presented in **Figure 3.2**. Within the these three candidate genes, targeted sequencing detected no nonsynonymous variants within *RND2*, two nonsynonymous variants within *MAP3K14* and three nonsynonymous variants within *ARHGAP27* and all five variants are missense mutations. No truncating mutations were detected among these candidate genes. *ARHGAP27* F211C(A-->C) was detected in 1 proband , G118V (C--> A) was detected in 2 probands, H854Q (G --> C)was detected in 8 probands. The *MAP3K14* variant E215K (C-- >T) was detected in 1 proband and S140N (C->T) was detected in 3 probands. All variants were predicted to be damaging by either Sorting Intolerant from Tolerant (SIFT)(31)[1] and/or PolyPhen (Prediction of functional effect of human SNPs)(32) functional predictions, with the exception of G118V, which was predicted to be tolerated. The minor allele frequencies are presented in **Table 3.3.** All probands harboring these 5 mutations were Caucasian. S140N (rs11574819), F211C (rs143997699), G118V (rs112715622) and H548Q (rs3479364) have been described in the database of DNA sequence variants of the National Center for Biotechnology Information (dbSNP), *MAP3K14* E215K is a novel missense mutation not previously described in ESP or in dbSNP.

The allele frequencies of the variants detected in our study were compared to the frequencies reported among European-Americans samples in ESP. Four of the five variants have been described in ESP: *MAP3K14* S140N and *ARHGAP27*

[1] SIFT is a sequence homology-based tool that **s**orts **i**ntolerant **f**rom **t**olerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect.

G118V, F211C, H548Q. *MAP3K14* E215K was not reported in ESP. **Table 3.4** provides results of comparing the carrier frequency in our study (among Caucasians only n=85) with the carrier frequencies among European-Americans reported in ESP for the four variants. The carrier frequencies in men in our study did not differ significantly from those observed in ESP for all four variants. *MAP3K14* S140N and *ARHGAP27* F211C and G118V occurred with more frequency in our study as compared to data from ESP, however none of these results were statistically significant. An adjusted *P* value threshold is presented to account for account for multiple nonsynynomous SNPs within each gene cataloged in ESP which were not detected by our targeted sequencing approach. The adjustment for multiple testing was made using a conservative Bonferroni adjustment: adjusted *P* value =0.05/(# of nonsynynomous variants in each gene in ESP - # of nonsynynomous variants in each gene detected in this study) However none of the comparisons reached statistical significance at either an unadjusted *P* value threshold (p value <0.05) or the *P* value adjusted for multiple testing.

Carrier status for all five variants among probands detected through targeted sequencing (n=15) was successfully confirmed using Sanger Sequencing in all instances. Sequencing of all additional family members with DNA (n=75), both affected and unaffected with prostate cancer, was performed to identify mutation carrier status among family members.

The family-based association test between *MAP3K14* and *ARHGAP27* variants and prostate cancer is presented in **Table 3.5**. Family-based association test results demonstrate that the variants detected within *ARHGAP27* were preferentially transmitted to affected men (Z score=2.87, *p* value=0.004). No associations were observed between the presence of the minor allele of variants within *MAP3K14* and prostate cancer. The lack of findings with respect to *MAP3K14* variants may be driven by the low number of pedigrees available for analysis.

IBD sharing presented is presented in **Table 3.6.** The NPL_{pairs} statistics measures allele sharing among affected family members. For *ARHGAP27* F211C, G118V and H548Q are the NPL pairs statistic Z score ranges from 1.16-1.18, corresponding to a *P* value of 0.12. There is marginal evidence of allele sharing with *ARHGAP27*. Likewise, the Z score for *MAP3K14* E215K and S140N correspond to a *P* value of 0.13 An aggregate NPL score is presented for each variant in **Table 3.7**. The aggregate NPL score is the combined peak LOD scores for each family carrying the variant allele of each marker obtained from previous linkage studies. The highest aggregate NPL scores were observed for *MA3K14* S140N, 2.35 and for *ARHGAP27* H548Q, 2.39. The evidence for linkage at these 5 variants was above 1 for all variants except *MAP3K14* E215K.

With respect to functional relevance, the mutations, H548Q and F211C, occurred in or close to highly conserved regions of the genome **[Figure 3.3]**

providing evidence that these variants may potentially alter the function of the protein and contribute to prostate carcinogenesis. The location of H548Q with respect to a conserved region (354-540) is consistent with the association observed using family-based association testing. F211C occurs in a conserved Pleckstrin homology-like (PH) domain.

Sequencing of family members of probands with mutations revealed some evidence of cosegregation of mutant alleles and disease. **Figure 3.4** shows the pedigree for F211C variant shows an affected sibling pair. Cosegregation of the F211C mutant allele with disease status was observed in the affected sibling pair. **Figure 3.5** shows one of the pedigrees from a family where the proband was a S140N mutation carrier and the mutant allele cosegregated with disease affection in all four affected siblings with available DNA. Further, **Figure 3.6** shows three pedigrees for carriers of the H548Q variant. Within these three pedigrees, the mutant H548Q allele cosegregated with disease in all 10 affected family members.

3.4 Discussion

Next-generation targeted sequencing of the exons of the 17q21-22 chromosomal region among 94 HPC family probands revealed five nonsynonymous missense mutations within *MAP3K14* and *ARHGAP27.* These genes were selected *a priori* based on their location relative to a previously reported linkage peak among HPC families with early age at onset and multiple

affected members and based on their potential biologic plausibility in cancer biology. These five variants are rare, one of which has not been reported in ESP or in dbSNP. The *ARHGAP27* H548Q mutation was observed in 8 probands, the G118V mutation was observed in 1 proband and the F211C mutation was observed in 2 probands. The *MAP3K14* E215K mutation and S140N mutation were observed in 1 and 3 probands, respectively. Given the potential biologic relevance of these genes in prostate cancer, we confirmed the carrier status among probands and investigated mutation status among other family members with available DNA. Although these variants did not occur more frequently in our study as compared to frequencies reported in publicly available data reported in ESP, there was some evidence of cosegregation in A*RHGAP27* F211C, H548Q and *MAP3K14* S140N. Family-based association tests demonstrated that the *ARHGAP27* gene is associated with HPC.

Prostate cancer risk variants detected in genome-wide association studies do not explain a significant portion of familial clustering and may indicate that the variants underlying genetic susceptibility to prostate cancer are rare, highly penetrant alleles. Our approach of performing targeted sequencing of coding regions (exons) in a candidate chromosomal region implicated in HPC is a promising strategy to identify unknown variants with potential functional significance. Further, the enrichment of the study population to focus on families with high number of affected men and early age at disease onset address issues limiting other studies of inherited forms of prostate cancer by minimizing genetic

and phenotypic heterogeneity. Exomic sequencing in complex disease is increasingly being used to detect rare and potentially yet unknown causal variants. Although targeted sequencing is often associated with Mendelian inheritance traits, targeted exomic sequencing offers an opportunity to uncover causal variants not yet associated with complex disease. Recently, data from targeted sequencing in this same study population revealed a rare missense mutation within the *HoxB13* gene associated with a significantly increased risk of HPC and the mutation was more common in men with early-onset prostate cancer.(33) After accounting for the linkage signal attributable to families carrying the *HoxB13* mutation there remains unexplained evidence of linkage at 17q21-22 (data not shown). This highlights that the variants described in this study within *ARHGAP27* and *MAP3K14* may play a role in hereditary prostate cancer and that there may be multiple prostate cancer susceptibility genes even in the same linkage region. Exomic sequencing in 96 familial pancreatic cancer cases led to the identification of a rare truncating mutations in the gene, *PALB2*, associated with hereditary pancreatic cancer (34) illustrating that complete sequencing of protein-coding regions of the genome can lead to the identification of a genes involved in complex, hereditary diseases.

None of the variants identified in this study have been previously associated with prostate cancer or in disease processes. Although *MAP3K14* has not been implicated in prostate cancer, cell proliferation and differentiation depend on mitogen-activated protein kinase (MAPK) cascades, which are initiated

upstream by the MAPK kinase kinase kinase (MAP3K) families.(35) The larger class of mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that play a role in the response to extracellular stimuli (osmotic stress, heat shock and proinflammatory cytokines) and regulate various cellular activities, such as gene expression, differentiation, proliferation, and cell survival and apoptosis.(36) The *ARHGAP27* variant H548Q occurs just outside a highly conserved region of the gene and F211C is within a highly conserved region. Although the potential mechanism of these variants in prostate cancer is unknown, highly conserved DNA sequences are thought to have functional value and may indicate areas of potential biologic significance. The conserved region near H548Q involves the GTPase-activator protein (GAP) domain for Rho-like GTPases. Small GTPases cluster into distinct families, and all act as molecular switches, active in their GTP-bound form but inactive when GDP-bound. The Rho family of GTPases activates effectors involved in a wide variety of developmental processes, including regulation of cytoskeleton formation, cell proliferation and the JNK signaling pathway.(37) *ARHGAP27* consists of an SH3, WW, PH and RhoGAP domain and isoform 1 had 17 exons. (25)

Further, focusing linkage analyses on men with younger age of onset is affirmed by a segregation analyses that showed that the most likely model of inheritance in men with an earlier age at diagnosis is a rare dominant susceptibility gene, while a rare recessive susceptibility gene for men who are older at time diagnosis.(38) Restricting pedigrees based on age at onset reduces

biases created by genetic heterogeneity and allows the isolation of variants, which may differ between early and late age at onset disease.

The strengths of this study include a novel approach to identifying putative prostate cancer genes in a highly enriched study population. Further, the combination of two study cohorts increases the power to discover causal variants. This study also has several limitations. Due to the late onset of prostate cancer and study design, parental genotypes were missing in a large proportion of the pedigrees. Another limitation of this study is the low frequency of markers in this study population may violate the assumption of the normal distribution used to calculate the *P* value in the family-based association test. Results from this study may not be generalizable to all men at risk for prostate cancer or even to men with a family history of prostate cancer. The men included in this study come from prostate cancer families with a high number of affected men and experience disease onset at an early age and variants detected in this study should be validated in larger sets of both hereditary and sporadic prostate cancer cases before findings can be extended to larger populations of men at risk for prostate cancer.

Certain assumptions are contained within this study and caution should be exercised in interpreting the result. Tests of proportionality between allele frequencies in our study as compared to ESP, assume that individuals sequenced in ESP are prostate-cancer free. Further, given the rarity of these variants, even in

a study population enriched for HPC and early age at disease onset caution should be exercised in interpreting the results from association tests. A possibility exists that the low number of individuals informative for linkage may have created biased results. Further, in order to increased power in association testing, I included both affected and unaffected family members and assumed that members currently classified as "unaffected" are truly "unaffected". Due to the relatively late onset of prostate cancer, a potential exists for disease misclassification due to the age-dependent penetrance of prostate cancer. Men may also be classified as unaffected based on differential screening practices. I did not have data on screening frequency to address this point.

Linkage studies for prostate cancer and other complex diseases may lead to conflicting results, given the small number of pedigrees in studies due to late age of onset and difficulty in collecting multi-generational samples, a substantial degree of genetic heterogeneity in prostate cancer, with potentially multiple modes of inheritance (dominant, recessive and X-linked) and locus heterogeneity. Further, disease heterogeneity may exist even among HPC due the high prevalence of sporadic prostate cancer in the general population and potential environmental risk factors, which may also affect risk in sporadic prostate cancer. The targeted sequencing undertaken in this study addresses many of these issues by increasing the number of affected men in each family and concentrating on early onset disease, which may decrease the genetic and phenotypic heterogeneity.

3.5 Conclusions

Targeted sequencing offers a novel approach to uncovering causal variants in complex disease. A targeted sequencing approach interrogating the coding regions of genes of interest has revealed mutations in diseases, particularly diseases with a genetic component or familial clustering. Five mutations were detected in *MAP3K14* and *ARHGAP27*, genes that were selected *a priori* based on biologic plausibility and location within a previously reported linkage peak in HPC. *ARHGAP27* demonstrates an association with prostate cancer in family-based association testing and some of evidence of cosegregation in pedigree analysis. Given the limited power in this study, these findings should be confirmed in larger studies in order to determine the mutation frequency in these genes and their association with prostate cancer in men with sporadic and hereditary prostate cancer.

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Figure 3.1 Study Schema – Targeted Sequencing of 17q21-22 in HPC Families

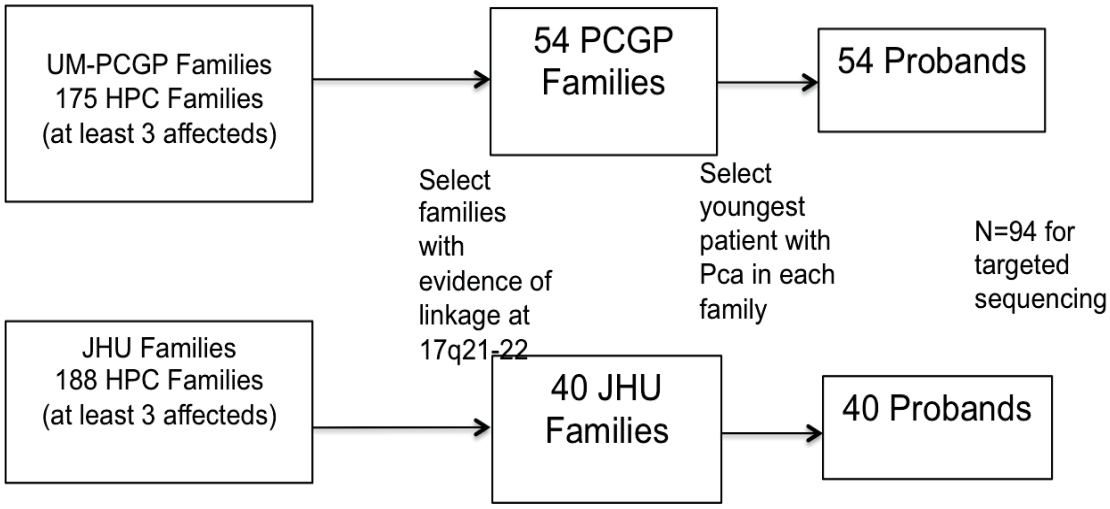


Figure 3.2 Candidate gene genetic map locations relavie to 2-LOD interval at Chromosome 17q21-22.

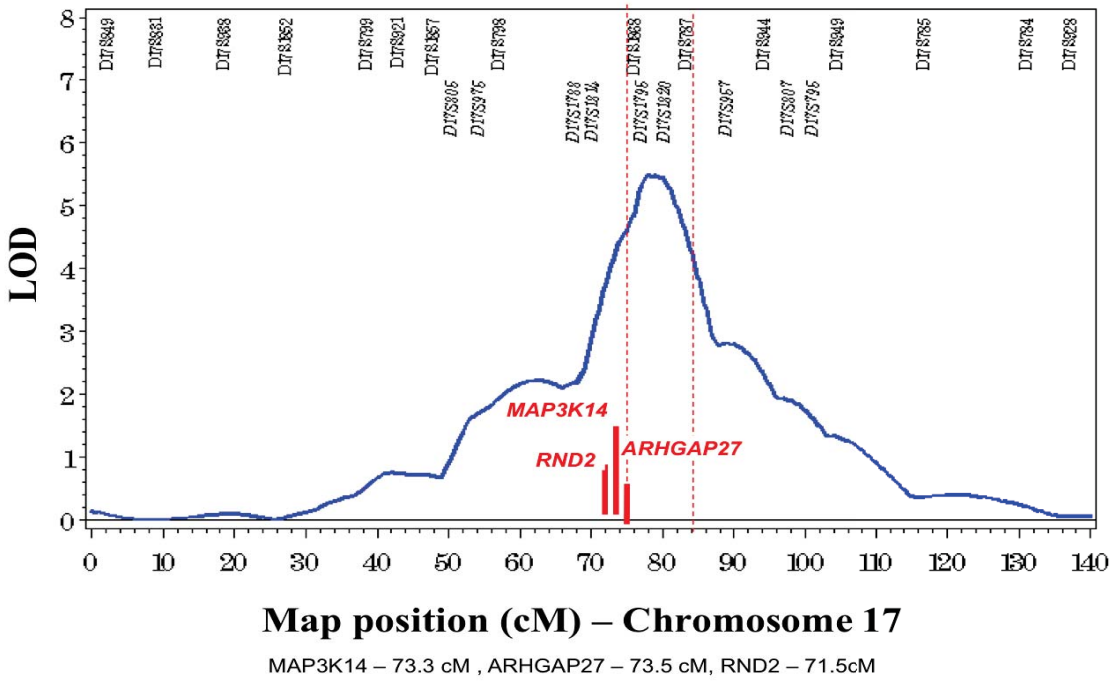


Figure 3.2: Non-parametric multipoint linkage analysis for prostate cancer on chromosome 17 reported in Lange et al. *Hum Gen* 2007:121(1). The solid curve represents the 2-LOD (logarithm of the odds) support interval for 147 families with BOTH four or more affected men and an average age of prostate cancer diagnosis ≤ 65 years. The dashed lines represent represents the 1 LOD support interval. The location of *MAP3K14*, *RND2*, *ARHGAP27* are represented by the red lines.

Figure 3.3 Gene Structure and Variant Location. Within *ARHGAP27*, F211C is located within the Pleckstrin homology-like domain and H548Q is located just outside of the RhoGAP Domain.

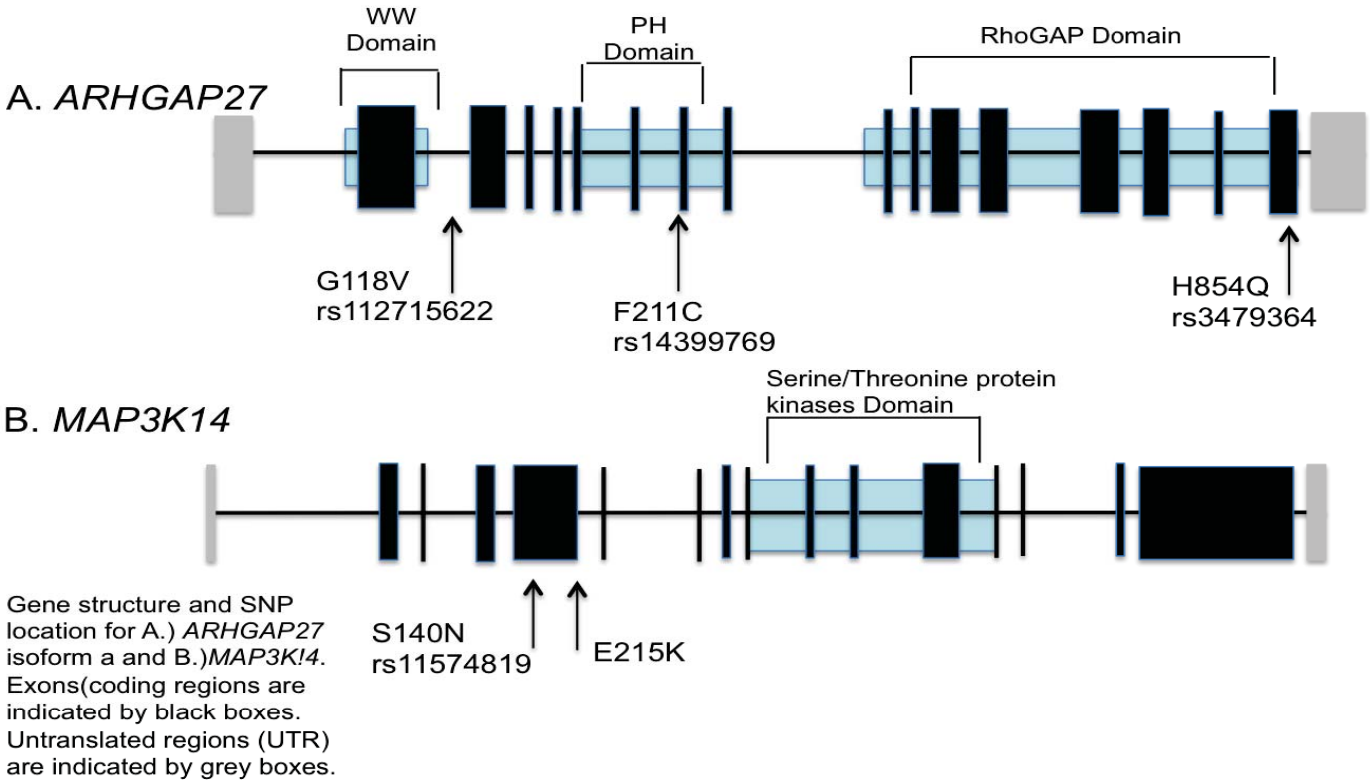


Table 3.1: Sequencing Primers for *RND2*, *MAP3K14*, *ARHGAP27*

Gene	**Codon**	**Primer**	**Sequence**	**AT***	**Size**
MAP3K14	E215K	Forward	TCTGGACCAGTCCATCTTCC	59.6	643 bp
		Reverse	AGGGATGAGGCAGTCTGCTA		
MAP3K14	S140N	Forward	TCTGGACCAGTCCATCTTCC	59.6	643bp
		Reverse	AGGGATGAGGCAGTCTGCTA		
ARHGAP27	F211C	Forward	GTGGGTGGGCAGCAGTGGACAT	59.6	851bp
		Reverse	GGGAAAGGGACCTGGGGAGAAAGA		
ARHGAP27	G118V	Forward	CAGGTAACAACCTGGGGAAA	59.6	324bp
		Reverse	GCTGTGGCAGGAGACTTC		
ARHGAP27	H548Q	Forward	GATGCTCTTCCAGCCCTCT	59.6	563bp
		Reverse	AACCGAATTAACCCCCAAAC		

*Annealing Temperature, ° Celsius

Table 3.2 Demographic and Clinical Characteristics of 94 Probands

	UM Families (n=54)	JHU Families (n=40)
Race		
Caucasian	51 (94.4)	34 (85)
African American	2 (3.7)	5 (12.5)
Asian American	1 (1.9)	1 (2.5)
Hispanic or Latino	n/a	0 (0)
Ashkenazi Jewish	1 (1.9)	5 (0.125)
# Confirmed Affected Men	4.67 (2-12)	4.9 (3-9)
Average Dx Age of Confirmed Affected Men	61.2 (48-69.3)	n/a
NGWS NPL at Peak (78 cM)	1.23 (-0.50-2.49)	1.4 (0.8-3.0)
CIDR NPL at Peak (76 cM)	1.24 (-0.58-3.45)	n/a
	N(%) or Median[Interquartile Range]	
Age at Diagnosis	52 (47.25-55)	62 (55-66)
Pre-Diagnosis PSA (ng/ml)	5.8 (4.245-12.0)	12.6 (5.9-36.3)
Prostatectomy + Gleason	40 (74.1)	25 (62.5)
<7	30 (55.6)	12 (41.4)
7	21 (38.9)	10 (34.5)
>7	3 (5.6)	7 (24.1)
Stage*^		
Local	39 (73.6)	11 (34.4)
Locally Advanced	10 (18.9)	17 (53.1)
Metastatic	4 (7.5)	4 (12.5)
Clinically Aggressive PCa**	18 (33.3)	25 (62.5)

*Stage missing for 1 UM proband and 8 JHU probands

^Localized = T1 or T2, N0 and M0 or Pre-Dx PSA <20ng/ml
Locally Advanced = T3 or T4, N0 and M0 or Pre-Dx PSA >20ng/ml but <100ng/ml
Metastatic = N1 or M1 or Pre-Dx PSA >100ng/ml

**Clinically Aggressive = Gleason >7 or Stage T3/T4 or Pre-Dx PSA>15 or Gleason=7 and Pre-Dx PSA>10 or Gleason=7 and Surgical Margins Positive

Table 3.3 *MAP3K14, RND2, ARHGAP27* variation identified through targeted sequencing in 94 probands

Gene	Chromsome 17 location*	Ref Allele/ Variant Allele	dbSNP ID	Variant Type	Substitution	Sift/ PolyPhen Prediction	# Alleles, MAF** in our study
MAP3K14	43,364,306	C/T	n/a	Nonsynonymous Missense	E215K (Glutanic Acid->Lysine)	Damaging	1, 0.53%
MAP3K14	43,364,638	C/T	rs11574819	Nonsynonymous Missense	S140N (Serine->Alanine)	Damaging	3, 1.60%
ARHGAP27	43,480,168	A/C	rs143997699	Nonsynonymous Missense	F211C (Phenylalamine->Cysteine)	Damaging	1, 0.53%
ARHGAP27	43,481,848	C/A	rs112715622	Nonsynonymous Missense	G118V (Glyceine-> Valine)	Tolerated	2, 1.06%
ARHGAP27	43,472,825	G/C	rs34793644	Nonsynonymous Missense	H548Q (Histidine-> Glutamine)	Damaging	8, 4.26%

*Locations from hg19 reference sequence
** MAF, Minor Allele Frequency

Table 3.4 Summary of comparison of minor allele frequencies (MAF) between 94 probands in our study and Exome Sequencing Project (ESP) data

Gene	Variant	No. of subjects in ESP §	Allele Count in ESP	Allele Count in our study	MAF in ESP,	MAF in our study **	p value*	P value adj. threshold¶
MAP3K14	E215K	n/a	n/a	1	n/a	0.59	n/a	0.002
MAP3K14	S140N	3371	78	3	1.16	1.76	0.45	0.002
ARHGAP27	F211C	3504	12	1	0.17	0.59	0.27	0.003
ARHGAP27	G118V	3510	32	2	0.46	1.06	0.19	0.003
ARHGAP27	H548Q	3407	441	8	6.47	4.70	0.43	0.003

* p value generated from Fisher Exact Test
** Among Caucasian families in our study (n=85)
§ Control subjects in the Exome Sequencing Project – European Americans (EA)
¶ p value threshold after Bonferroni adjustment for multiple nonsynynomous SNPs in each gene were not detected by targeted sequencing within the overlapping region in ESP

Table 3.5 Results from Family-Based Association Test

Gene	**Number of Probands with mutation**	**Allele Frequency**	**Z Score¶**	**P value**
MAP3K14	4	4.26%	0	1
ARHGAP27	11	11.7%	2.87	**0.004**

¶Combined Affecteds and Unaffecteds

Table 3.6 IBD Sharing Probabilities Test Results

Gene	**Z Score**	***P* value**
MAP3K14	1.14	0.13
ARHGAP27	1.18	0.12

Table 3.7 Marker specific aggregate NPL scores for all families with mutation

Gene	Variant	No. of Families	NPL statistic (LOD Score)
MAP3K14	E215C	1	0.81
MAP3K14	S140N	3	2.35
ARHGAP27	F211C	1	1.40
ARHGAP27	G118V	2	1.51
ARHGAP27	H548Q	8	2.39

* Based on peak NPL Scores for each family detected in previous linkage studies

Figure 3.4: F211C variant – UM Family 1

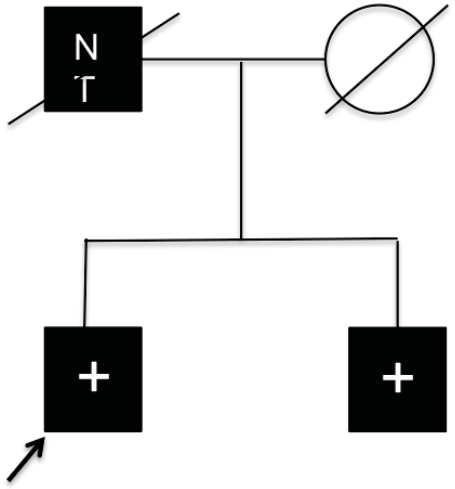
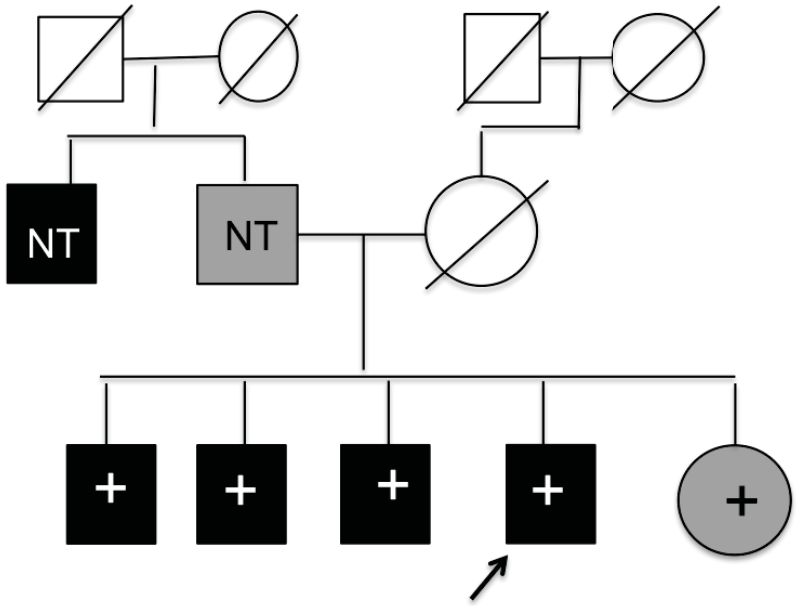
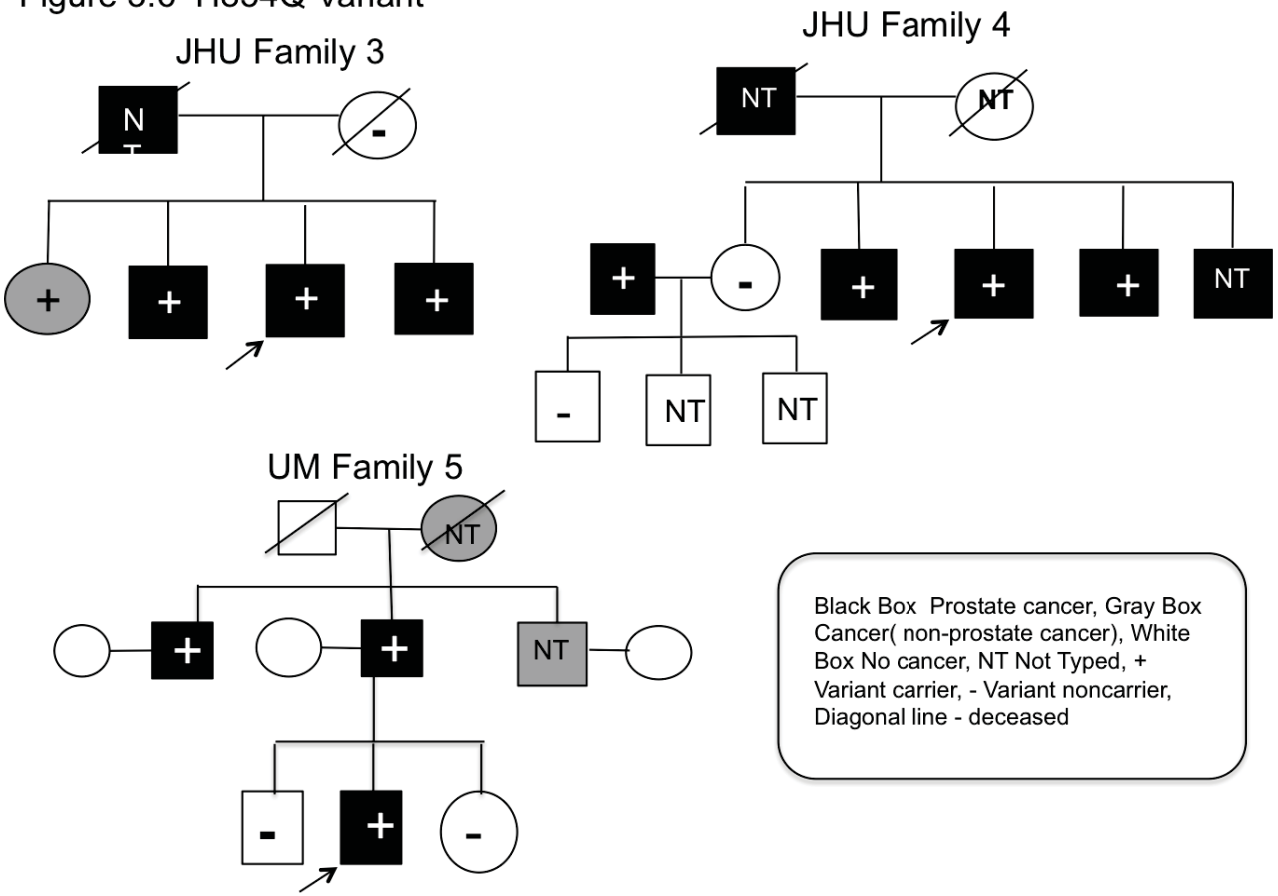


Figure 3.5: S140N – JHU Family 2



Black Box Prostate cancer, Gray Box Cancer(non-prostate cancer), White Box No cancer, NT Not Typed, + Variant carrier, - Variant noncarrier, Diagonal line - deceased

Figure 3.6 H854Q Variant



Chapter 4

The role of germline variation in genes involved in androgen synthesis and metabolism and response to androgen deprivation therapy in men with metastatic prostate cancer

4.1 Introduction

Prostate cancer-related mortality is secondary to metastatic disease with a long time course of morbidity. Despite improved diagnostic techniques (namely PSA testing) and consequently increased early detection of prostate cancer,(1) a subset of men will still progress to develop metastatic disease. The first-line of therapy for metastatic prostate cancer is androgen deprivation therapy (ADT). Death from prostate cancer results subsequent to the transition from a hormone sensitive prostate cancer (HSPC) state, which is responsive to hormonal treatments, to a state of castrate-resistant prostate cancer (CRPC), which eventually becomes unresponsive to standard doecetaxel-based chemotherapy. (2) CRPC is characterized by poor prognosis and once the CRPC or androgen independent prostate cancer (AIPC) stage occurs, the median survival is 9- 30 months. Median survival in men with CRPC and metastatic disease is 9-13 months.(3) The risk of death from metastatic prostate cancer is highly variable, and clinicians have long sought methods to predict survival outcomes accurately in individual patients. In one retrospective study, high serum alkaline

phosphatase, high Gleason score, and intense bone metastasis (>6) were found to be the only predictors of progression and impaired survival in men with metastatic disease.(4)

ADT usually involves agents that suppress gonadotropins through a pituitary mechanism. Gonadotropin-releasing hormone agonists and antagonists both suppress gonadal release of testosterone, although their activity profiles vary. ADT down-regulates androgen receptor (AR) transcriptional activity in the tumor but the response in metastatic disease is transient and tumors often progress to a CRPC state. Although serum testosterone concentrations decline dramatically with ADT, CRPC growth remains largely dependent on AR activity. (5) Despite an initial response rate to ADT in approximately 80% of those receiving treatment, predictable and irreversible resistance to androgen deprivation will occur in the vast majority of patients. (6)

PSA is a biomarker for diagnosis, risk prediction and monitoring disease activity in men with all stages of prostate cancer. The PSA nadir is the lowest PSA reading achieved after any treatment for prostate cancer. Failure to reach PSA nadir of PSA ≤ 4 ng/mL after the initiation of ADT is compared to progression of prostate cancer to androgen independence while on ADT and indicates less likelihood of success on ADT. (7) In men with metastatic prostate cancer undergoing ADT, a short PSA doubling time after the PSA nadir was also found to be associated with shorter PSA nadir duration and poorer median cancer specific survival.(8) Further, a faster time to reach a PSA nadir after the initiation

of ADT was associated with shorter survival duration in men with metastatic hormone-sensitive prostate cancer, indicating that a rapid initial response to ADT indicates more aggressive disease; however research has yielded no reliable predictors of whether PSA nadir will be reached or maintained or of which men will have improved survival prior to treatment initiation.(9) Data from 573 participants in a randomized controlled trial (SWOG-9916) assessing survival under different treatment modalities found that median survival was 18 and 11 months for those who reached PSA nadir and those who did not reach PSA nadir, respectively.(10) These results highlight the use of PSA as a valid intermediate end point in estimating the response to ADT and potential for survival.

The ability to better assess prognosis at an individual level is crucial for maximizing clinical benefit while minimizing exposure to unnecessary toxicities associated with ADT. These toxicities include loss of libido, erectile dysfunction, hot flashes, fatigue due to mild anemia, reduction in bone density, loss of muscle mass, and a possible increased risk of both diabetes mellitus and cardiovascular disease. (11)

The exact biologic mechanisms and underlying processes involved in CRPC have not been fully elucidated. The androgen pathway is commonly accepted to have a critical role in the survival of prostate cells. Androgens impact the development, maturation and maintenance of the prostate, by effects on the differentiation and proliferation of the luminal epithelium.(12) Testosterone and

its metabolite dihydrotestosterone (DHT) are essential for prostate gland growth**.** **Figure 4.1** highlights many genes involved in the synthesis of androgens/estrogens from cholesterol. During this complex process, testosterone may be further metabolized by 5α-reductase to DHT, which has higher affinity for binding to the AR. Although the mechanisms be which a prostate cancer cell survives ADT are not well understood, there are multiple AR dependent and AR independent pathways hypothesized.

AR status and a functional androgen signaling axis are particularly important for patients who are either diagnosed with, or subsequently develop, metastatic prostate cancer, as they determine the success of androgen ablation.(13) There is evidence of variation in androgen receptor gene expression patterns as prostate cancer tumors progress to androgen independence. (14, 15) CRPC is often marked by up-regulation of androgen synthesis enzymes, which lead to biologically relevant levels of androgens in prostate cancer tumors, even in cases where androgen levels in the blood are undetectable.(16) Androgen synthesis by the adrenal glands persists even after testicular production is diminished. Further, progression of CRPC could also be the result of ligand-independent receptor activation and activation of cell signaling pathways.(17) In CRPC metastatic tissue, expression levels of genes involved in mediating androgen synthesis are increased.(18)

Genes coding for enzymes or hormones that are involved in the synthesis and metabolism of testosterone and other androgens are compelling biological

candidates for study as targets for therapy or as potential modifiers of response to therapy in metastatic prostate cancer.(19) Somatic genetic changes and specifically AR reactivation have been shown to be important in the development of castrate-resistant prostate cancer.(20) However, understanding somatic genetic changes that occur during and after ADT in the tumor are not helpful in determining *a priori* how individuals will respond to ADT. More recent studies have suggested that germline genetic variation may contribute to response to hormonal therapy. Three studies have identified single nucleotide polymorphisms (SNPs) associated with response duration to ADT in the *CYP19A1*, *HSD3B1*, *HSD17B4*, *SLCO1B3* and *EGF* genes.(21-23) None of the SNPs identified in *CYP19A1*, *HSD3B1*, *HSD17B4* or *EGF* genes are in coding regions, therefore the molecular mechanism that accounts for the association between these SNPs and the response to ADT remains unknown. *SLCO1B3* encodes for proteins involved in testosterone transport. Two coding SNPs in the *SLCO1B3* gene, which are in complete linkage disequilibrium, were found to be associated with survival in men with advanced prostate cancer.(23) *In vitro* studies have demonstrated that cells which are homozygous for both the 334G and 699A *SLCO1B3* alleles have impaired uptake of radiolabeled testosterone, suggesting a possible mechanism to explain the observation that men who are homozygous for both polymorphisms have an improved survival in the setting of castrate-resistant disease. Taken together, these preliminary studies focusing on limited candidate genes in relatively small populations suggest that germline variation in candidate genes

involved in hormone synthesis and metabolism may influence the response to androgen deprivation and the overall survival in men with CRPC.

Prostate cancer disparities in both incidence and mortality among African-American men as compared to Caucasian men have been well documented. The higher prostate cancer mortality among African-Americans has been hypothesized to occur because African-American men may be presenting at a more advanced stage of disease due to disparities in access to health-care and other socioeconomic factors.(24) However, the androgen and hormone related mechanisms involved in CRPC necessitate study among African-Americans as there exist notable differences between African-American and Caucasian men in endogenous hormone profiles in repeat lengths of the AR,(25) as well as consistent findings of higher levels of total testosterone and free testosterone in African-American men as compared to Caucasian men.(26, 27) In a recent analysis of data from two SWOG studies, African-American men with advanced prostate cancer who were treated uniformly on phase III clinical trials had a higher mortality rate (HR for death = 1.19, 95% CI = 1.05 to 1.35), after adjustment for other covariates (including income and education).(28) In another study, even after controlling for the effects of age, preoperative PSA level, pathological grade, and stage, the racial disparity in progression-free survival persisted among men diagnosed with clinically localized prostate cancer being treated with prostatectomy.(29) The disparity has also been observed in men with CRPC; a single-institution study found that African-American race was the only independent predictor of time to PSA progression.(30) To date, there have been

no definitive data as to the cause(s) of the disparity in outcome, however the findings of disparities that persist even in the context of randomized trials and in men who have progressed to CRPC indicate that biologic or genetic factors could be relevant. Further, ethnic and race differences are being recognized as factors accounting for variation in drug responsiveness among individuals and increasing interest in identifying genetic variants contributing to ethnic variation in sensitivity to cancer therapies.(31) Previous research on germline mutations associated with response to ADT has only considered Caucasian subjects and inferences found in those studies may not necessarily be extended to African-American men.

Objective:

The objective of this study is to comprehensively study the role of inherited genetic variation in genes involved in androgen synthesis and metabolism in the response to ADT. I propose to use DNA samples from both Caucasian and African-American men with newly diagnosed metastatic prostate cancer enrolled in a randomized controlled clinical trial. In addition to potentially identifying novel therapeutic targets for treatment of CRPC, findings from this study may elucidate the biological mechanisms involved in CRPC and in drug resistance. Further, identifying genetic variants associated with poor/improved performance on ADT may inform better clinical outcome and survival prediction among patients

4.2 Materials and methods

4.2.1 Study subjects

Study subjects are participants in the SWOG-9346 randomized, Phase III intergroup (SWOG, Cancer and Leukemia Group B, Eastern Cooperative Oncology Group, European Organization for Research and Treatment of Cancer and National Cancer Institute of Canada) trial. The objective of SWOG-9346 is to assess whether survival with intermittent ADT is not inferior to survival with continuous ADT in patients who have newly-diagnosed metastatic hormone-sensitive prostate cancer (HSPC). Key eligibility requirements for enrollment in the study included metastatic (M1 staging-distant metastases) prostate cancer and a minimum pretreatment PSA value of 5 ng/mL. The initial treatment regimen consisted of a 7-month induction course with ADT consisting of goserelin and bicalutamide. Patients whose PSA levels decreased to a PSA nadir of 4 ng/mL or less, with stable or declining trend, at months 6 and 7 of induction treatment were then randomly assigned to intermittent or continuous ADT. Patients whose PSA levels did not decrease to PSA nadir of 4 ng/mL or less at the end of the induction phase of the trial were removed from protocol but were observed for progression and survival. PSA levels were measured among participants at months 1, 4, 6, and 7 of the induction period; this was followed by monthly assessments after random assignment. For those patients not randomly assigned, PSA was assessed every 6 months after the end of induction and as clinically indicated. The SWOG-9346 trial began in 1993 and a total of 2,948 eligible participants accrued to SWOG-9346. Beginning in 2002, the SWOG-9346 protocol was amended to

institute the collection of whole blood specimens from newly enrolled participants recruited at study sites within the United States. Our study population consists of all men with whole blood available for DNA extraction and analysis (n=210). Whole blood samples are available from both men who achieved a PSA nadir of ≤4 ng/mL and men who did not. A study schema describing the SWOG-9346 trial and the subset of participants included in our study is shown in **Figure 4.2**. The outcome used in our analysis is defined as whether or not a subject achieved a PSA of ≤ 4 ng/mL (i.e. favorable response to ADT) after induction therapy. Evidence from SWOG-9346 and other SWOG trials have demonstrated that the attainment of post-induction therapy PSA nadir is a strong correlate of survival. Data from SWOG-9346 has already demonstrated that of 1,395 patients followed, the median survival times were between 44 and 75 months for patients who achieved PSA nadir of 4 ng/mL after induction therapy and 13 months for patients who did not reach PSA nadir of 4 ng/mL (*p* value <0.0001).(32)

4.2.2 Biospecimens

Whole blood was collected from participants at various timepoints during SWOG-9346 enrollment. Biospecimens were stored at the SWOG Cooperative Group repository. I extracted genomic DNA from whole blood samples using the Puregene DNA Isolation kit (Gentra Systems, Inc., Minneapolis, MN). DNA samples were quantified using the spectrophotometer (ND-1000, NanoDrop Technologies, Inc., Wilmington, DE). The concentration for all high quality samples was normalized to 100 ng/µl. A total of 210 samples were included in

this study. For quality control purposes, approximately 5% (n=11) randomly selected duplicates were included for genotyping and analysis. 99.6% concordance between duplicates was observed in the genotyping data among SNPs that succeeded in genotyping (call rates >90%). Also, genotype data from 3 CEPH individuals (Coriell Cell Repository, Camden, NJ) that were genotyped by the HapMap project were used to confirm reliability and reproducibility of the genotyping. Observed concordance among CEPH control DNAs was 99.9%.

4.2.3 Clinical Characteristics

Data was collected at baseline on SWOG-9346 participants including age at time of study enrollment (years), total Gleason grade measured at initial prostate cancer diagnosis (<7, 7 or >7), self-reported race, baseline PSA (ng/mL), disease severity (categorized as extensive or minimal, where extensive disease was defined as appendicular skeletal involvement, visceral metastasis, or both) (33), Eastern Cooperative Oncology Group (ECOG) Performance Status was also assessed (categorized as 0=Fully active, able to carry on all pre-disease performance, 1=Restricted in physically strenuous activity but able to carry out work of a light nature, 2=Ambulatory and capable of all self-care but unable to work, 3=Capable of limited self-care, confined to a bed or chair more than 50% of waking hours, 4=Completely disabled, and 5=Deceased).(34) No subjects included in our study had ECOG performance statuses of 4 or 5. The source of Gleason grade could be biopsy or prostatectomy pathology.

4.2.4 Candidate Gene and SNP Selection

Candidate genes were selected from an extensive literature search identifying genes that are involved in hormone synthesis and metabolism. An additional literature search was performed to identify genes in biological pathways implicated in the progression to and development of advanced prostate cancer, metastatic prostate cancer and/or castrate-resistant disease. A set of 38 candidate genes selected for inclusion in this study is presented in **Table 4.1.**

SNPs within all candidate gene regions (from 10 kb upstream and 10 kb downstream of all candidate genes) were identified from genotyped SNPs available in the HapMap Project [Rel 28/ Phase II &III, on National Center for Biotechnology Information Build 36 assembly] http://www.hapmap.org. Polymorphisms were selected from each gene provided that the minor allele frequency (MAF) was greater than 0.05 in at least one of three populations of interest: CEPH (Utah residents with ancestry from northern and western Europe) (CEU), Yoruba in Ibadan, Nigeria (YRI) and individuals with African ancestry from the Southwest United States (ASW). Using the TAGGER algorithm program, implemented in Haploview 4.2 (35), I used a pairwise-tagging approach to select tag SNPs capturing the unmeasured variants within a locus using an r^2 of ≥ 0.8. In addition, all nonsynynomous SNPs within the candidate genes of interest that have been described in either dbSNP or in the literature were included if the MAF was at least 0.02 in either a Caucasian or African-American population. This strategy increases the likelihood that associated SNPs are directly implicated in the response to ADT. Lastly, a literature search was

conducted to capture all SNPs associated with prostate cancer mortality, aggressive/recurrent prostate cancer, metastatic prostate cancer or response to ADT. These SNPs were included in our selection without regard to MAF. The genotyping platform for this study was the Illumina GoldenGate assay, which uses a discriminatory DNA polymerase and ligase to interrogate up to multiple loci simultaneously. The GoldenGate Assay system is based on Illumina's BeadArray technology using the Sentrix Array Matrix platform. A total of 1,536 SNPs were successfully converted to a custom Illumina GoldenGate genotyping assay; SNPs were selected to maximize likelihood of successful genotypig calls (Design score ≥ 0.7). All polymorphisms that failed assay design and were "singleton" SNPs (i.e., not tagging for any other polymorphisms) were not pursued further and the tagging strategy was replicated for any polymorphisms tagging other SNPs that failed assay design. Each SNP was tested for Hardy-Weinberg equilibrium (HWE) in control subjects (P <0.0001). A total of 153 SNPs (10%) were removed from analysis due to missing genotypes for more than 10% of study subjects. A chi-squared test was performed to determine if "missingness" differed by case-control status. For each SNP tested, the P value obtained was over the 0.05 significance threshold, indicating that degree of missing genotypes was not associated with case-control status.

4.2.5 Population Stratification

Population stratification occurs when there are both differences in disease risk and systematic differences in allele frequencies in genetic markers between

different populations and can lead to confounding in genetic association studies. An ancestry estimate for each subject, derived from multi-locus genotype data, can be used as a covariate to correct for population stratification in regression models.(36) Ancestry coefficients offer a more robust correction for ancestry over self-reported ancestry. Ancestry informative markers (AIMs) are genetic loci with large frequency differences between the major ethnic groups. Among the SNPs genotyped in this study, 106 SNPs considered AIMs were included to better describe the genetic variation between Caucasian and African-Americans subjects. Using genotype data from the AIMs, ADMIXTURE software was utilized to calculate an ancestry estimate for each subject and these estimates, used as model covariates, can correct for population stratification. ADMIXTURE is a software tool for maximum likelihood model based estimation of individual ancestries using multilocus SNP genotype datasets. (37)

4.2.6. Statistical Analysis

Data were descriptively summarized using medians and standard deviation for continuous variables and number and frequencies for categorical variables. Participants who failed to reach a PSA nadir of 4 ng/mL after induction therapy were considered cases. Unconditional logistic regression models were used to estimate odds ratios (OR) and 95% confidence intervals (CI) to test the association between each SNP and failure to reach PSA nadir (poor response to ADT). SNP associations were modeled assuming a multiplicative genotype-phenotype association. All models were adjusted for proportion of Caucasian ancestry estimates. Additional multivariate models were adjusted for clinical

covariates in addition to Caucasian ancestry estimates. Clinical covariates include Gleason grade (=7 versus <7, >7 versus <7), ECOG performance (0,1,2-3), age at enrollment (continuous), and baseline PSA value, (ng/mL; continuous). Caucasian and African-American subjects were analyzed jointly. Genotypes for 8 subjects with genotype call rates <75% were removed from analysis. In order to correct for multiple testing, one thousand permutation tests were generated by randomly shuffling case-control labels. The permutation testing adjusted *P* value is defined as the proportion of resampled data sets where the minimum pseudo *P* value is lower than or equal to the original *p* value. Since this data set is the first to consider response to ADT solely in men with metastatic disease and in African-American men, we consider these hypothesis-generating experiments and the strict control on potential type I errors may not be appropriate. There may be more than one SNP within a gene that is independently associated with response to ADT. For genes with multiple associated SNPs, I performed conditional analyses to assess whether after conditioning on the SNP in each gene with the strongest evidence of association (i.e. SNP with the lowest *P* value), any additional SNPs in the gene remained associated with response to ADT. In conditional analyses, using logistic regression models, the most significant SNP was included as covariate. In order to further explore the SNPs found to be associated with response to ADT, haplotypes were constructed and investigated within genes where one or more SNPs were found to be associated with response to ADT in single SNP analyses. Any haplotype where either a specific configuration or the global haplotype reached statistical significance are

presented. Due to the limited number of African-American subjects in this study, haplotypes were constructed using data from Caucasian subjects populations only. All statistical analyses were performed in PLINK (version 1.07). (38) All statistical tests were two-sided and significance was set at $p < 0.05$.

Pairwise linkage disequilibrium (LD), r^2, and D' between SNPs was calculated Haploview statistical software(35). LD visualization and analyses were conducted in Caucasians only. LD plots were generated in Haploview. Within each table the pairwise correlation coefficient (r^2) is presented. Standard color-coding was used, white ($r^2=0$), shades of gray ($0 < r^2 < 1$). Black squares without numbers indicate complete LD ($r^2=1.00$).

4.3 Results

Subject and clinical characteristics are presented in **Table 4.2.** The median age at time of study enrollment among participants is 68.2 years. In this sudy, 57 (27.1%) of subjects were classified as "cases" i.e. did not achieve PSA nadir of 4 ng/mL or lower after 7 months of induction therapy and 153 (72.9%) of subjects were classified as "controls". 45% of subjects had Gleason grade 8-10 cancer and the median (SD) baseline pre-treatment PSA value among participants was 46.5 ng/mL (5073). All subjects were enrolled in SWOG-9346 between the years 2002-2008. Of 210 subjects successfully genotyped, 24 (11.4%) were African-American and 186 (88.6%) were Caucasian, based on self-reported race.

A total of 1,383 SNPs were genotyped in 210 samples. Using 106 genotyped AIMs, an ancestry estimate was calculated for each subject assuming a structure with 2 clusters (ancestral populations). **Figure 4.3** shows the distribution a) of European ancestry proportion estimates among self-reported Caucasian subjects in this study and b) of African ancestry proportion estimates among self-reported African-American subjects in this study. The distribution of the percent European ancestry, calculated from AIMs, observed among Caucasian subjects was highly correlated with self-reported race. Likewise, the distribution of African ancestry observed among African-American subjects was highly correlated with self-reported race.

Logistic regression analyses were performed in the context of multiplicative (log additive) models. 8 subject samples were excluded from these analyses due to missing genotypes of 25% or more. 15 SNPs in or near *SLCO1B1*, *SRD5A2*, *CYP1B1*, *SRD5A1*, *ESR2 CYP3A43*, and *CYP19A1* were found to be have a nominal *p* value of <0.05 and were associated with response to ADT. The variants, their locations and functions are described in **Table 4.3**. Model 1 tests the association between single SNPs and response to ADT, after adjustment for ancestry estimates. SNPs with nominal *P* values that attained statistical significance in Model 1 are presented in **Table 4.4.** An inverse association was observed among carriers of a variant allele of rs2306283 (*SLCO1B1*) and response to ADT (OR=0.50, 95% CI=0.28-0.90, nominal *p* value =0.019). Carrying a variant allele of rs2306283 was associated with a 55% decreased odds of poor response to ADT. The risk among carriers of a variant allele of rs6543631

in *SRD5A2* (OR=0.57, 95% CI=0.34-0.95, nominal *p* value=0.03), rs9972359 in *CYP19A1* (OR=0.60, 95% CI= 0.37-0.97, nominal *p* value = 0.04) and r2900476 in *SLCO1B1* (OR= 0.35, 95% CI=0.13-0.996, nominal *p* value=0.049) was inversely associated with response to ADT. Also, within *SLCO1B1*, carriers of a variant allele of rs1463565 (OR=1.71, 95% C.I. 1.08-2.70, nominal *p* value=0.021) or rs17388851 (OR=1.73, 95% CI=1.08-2.75, nominal *p* value=0.023) were associated with increased odds of poor response to ADT. Within or near, SRD5A2, a carrier of the variant allele of rs12470143 (OR=1.75, 95% CI= 1.02-3.00, nominal *p* value=0.042) or rs2365778 (OR=1.63, 95% CI=1.02-2.62, nominal *p* value=0.042) was associated with increased odds of poor response to ADT. The largest magnitude of effect was observed for rs16964189, an intronic SNP in *CYP19A1*. Carriers of the variant allele of rs1694189 had 3-fold increased odds of poor response to ADT (OR =3.18, 95% CI=1.01-10.04, nominal *p* value=0.049). Variants in CYP3A43 (rs651430; OR=1.58, 95% CI=1.01-2.49, nominal *p* value=0.046) and CYP1B1 (rs10175368) were associated with an increased odds of failure to reach PSA nadir of 4 ng/mL or less. However, none of the reported associations in Model 1 reached an adjusted *p* value of 0.05 or less after multiple hypothesis testing corrections using permutation testing. Among the SNPs associated with response to ADT, rs2306283 is the only nonsynonymous SNP detected and is a missense variant and predicted to be "tolerated" by SIFT/PolyPhen. Rs17388851, rs2306283 and rs2900476 are located in the same LD block of *SLCO1B1* and are not in high LD with other SNPs spanning the 20kb region of *SLCO1B1* **[Figure 4.4].** rs12470143

is in high LD (r2 =0.91) with rs2300700, an intronic SNP in *SRD5A2*. Rs2365778 is in high LD (r2=0.96) with another intronic SNP in *SRD5A2,* rs6732223. Otherwise, the SNPs associated with response to ADT in *SRD5A2* are not in high LD with other SNPs within this gene region. **[Figure 4.5]** Within *CYP191A1*, rs9972359 and rs1694189 are neighboring alleles, however they are in low LD with one another.

The associations between single SNPs and outcome are presented in Model 2 adjusted for clinical covariates and Caucasian ancestry estimates. Due to the large number of missing values for disease severity (n=71), inclusion of this covariate did not improve model fit and was not included in the final multivariate model presented in **Table 4.4**. The effect estimates in models with and without adjustment for clinical covariates are similar for the 15 SNPs associated with response to ADT, although only 9 SNPs remained statistically significant in Model 2 as compared to Model 1. Those variants that were inversely associated with response to ADT (rs2306283, rs6543631, rss9972359 and rs2900476) remained so after adjustment for clinical covariates.

For any gene where more than one SNP within that gene was associated with ADT in single-SNP analyses, a conditional analysis was performed. In logistic regression, after controlling the effect of the most statistically significant SNP (i.e. SNP with the lowest nominal *P* value) in each gene detected in the single SNP-outcome models, the effects of all the other SNPs in that same gene

were no longer associated with response to ADT. **[Table 4.5].** Therefore, the secondary SNPs did not have an effect on the response to ADT independent of the effect of the conditional SNP. Once we condition on rs2306283, no other SNPs within *SLCO1B1* remain associated with the response to ADT. Conditioning on the most significant SNP in each of the genes, including *SRD5A2*, *CYP19A1*, *SRD5A1*, and *ESR2,* eliminated the association between secondary SNPs in that gene and response to ADT.

In Caucasian-only haplotype analyses, I estimated the effects of 3 SNPs in *SLCO1B1*, rs1651071, rs1691053 and rs2306283 and response to ADT. In single SNP analyses presented above, only rs2306283 was significantly associated with response to ADT. The A-A-G haplotype was associated with response to ADT and A-A-G haplotype conferred a 34% decreased odds of poor response to ADT as compared to the A-A-A haplotype. **[Table 4.6]** Haplotype association tests were not adjusted for multiple testing. I also estimated the effects of 3 SNPs within *SRD5A2*, rs1651071, rs1691053 and rs13166363 and response to ADT. Only rs13166363 was associated with response to ADT in single SNP analyses. In haplotype analysis, the A-G-G haplotype was associated with a 69% increased odds (*p* value =0.035) of poor response to ADT as compared to the A-A-A haplotype. **[Table 4.7]**

4.4 Discussion

In this study of 210 Caucasian and African-American men with metastatic prostate cancer, 15 SNPs in or near eight genes involved in androgen synthesis/metabolism were found to be associated with response to ADT. However, these associations did not achieve a *p* value of 0.05 or lower after adjustment for multiple testing using permutation tests. Rs2306283 located at position 130 in *SLCO1B1* is a nonsynonymous missense variant resulting in an amino acid substitution (Asn –Asp) and carriers of the variant allele had a decreased risk of poor response to ADT. After conditional analyses investigating multiple SNPs within genes associated with response to ADT, only 8 SNPs remained significantly independently associated with the ability to achieve PSA nadir of 4 ng/mL after induction therapy with goserelin and bicalutamide. The variants described here have not been previously described in response to ADT or in metastatic prostate cancer/CRPC. This study is unique in its inclusion of solely men with metastatic disease and is the first study to examine inherited variation in response to ADT in African-American men.

In comparing results from basic models adjusted only for ancestry with models adjusted for ancestry and clinical covariates, the effect estimates for single SNPs are similar in both models. These findings highly the utility of these SNPS to potentially predict outcome in patients receiving ADT over and above what is predicted by clinical and prognostic covariates such as Gleason grade, pretreatment PSA and age. These clinical covariates are potentially not true

confounders of the effect between inherited variation and response to ADT (i.e. are associated with both the outcome and the predictor but not an intermediate), they may likely act as intermediates in the causal pathway between inherited variation and response to ADT. Given the limited power in this study due to small sample size, it is important to note that all the variants associated with response to ADT have high minor allele frequencies (MAF ≥20%), indicating that there may be other variants where the minor allele frequencies are lower and are associated with response to ADT, however we may have been underpowered to detect such associations.

Although not all SNPs found to be associated with response to ADT were functional or in coding regions, rs2365778 (located 7kb downstream of *SRD5A2*) was found to be in near perfect LD with rs6732223. Rs6732223 has been associated with schizophrenia spectrum disorders and with significantly increased 5alpha-reductase activity among individuals with schizophrenia, (39) although the molecular mechanism of the SNP is unknown, it may be through expression of SRD5A2.

A notable and novel finding in this study is a nonsynonymous missense variant in *SLCO1B1*, rs2306283, associated with response to ADT. Rs2306283 results in a replacement of normal asparagine at codon 130 with aspartic acid. The role of rs2306283 has been studied in therapeutic outcomes and has found to be associated with statin medication intolerance(40) and has also been implicated in

response to cancer treatment and associated with increased toxicity associated with irinotecan, a drug used in the treatment of metastatic colorectal cancer patients.(41) *SLCO1B1* gene encodes for proteins involved in transport functions. Specifically, SLCO genes encode for organic anion-transporting polypeptides (OATPs), which are involved in hepatocyte influx mechanisms and are overexpressed in certain cancers.(42) Rs2306283 occurs in a highly conserved protein domain within the *SLCO1B1* OATP coding region. OATPs have been implicated in both the molecular etiology of prostate cancer as well as with clinical outcomes in prostate cancer. A *SLCO1B3* variant has been associated with impaired testosterone transport and improved survival in patients with prostatic cancer.(23) Another polymorphism in *SLCO1B3*, increases testosterone transport and is associated with shorter time to androgen independence in men treated with ADT(15), however we did not observe an significant associations between this variant and outcome in our study. *SLCO1B1* and *SLCO1B3* encode for the OATP1B1 and OATP1B3 proteins, respectively, which along with other OATPs are surfacing as potential transporters of cancer treatments and influx a number of pharmaceuticals.(43) OATP1B1 and OATP1B3 has been shown to influx several cancer treatments: OATP1B1 influxes ketoconazole, paclitaxel, and SN-38, while OATP1B3 including docetaxel, a drug used in the treatment of CRPC .(43) In addition, OATP1B3 may also play a role in disease etiology in that testosterone is a substrate for OATP1B3 and OATP1B3 protein was found to be overexpressed in prostate tumors, but not in normal prostate tissue.(44) Further study is warranted to determine if genetic variation and expression of OAT proteins may

have implications for prediction of treatment outcomes.

Steroid 5α-reductase catalyzes the conversion of testosterone to DHT, the most potent androgen. The type II isoform is encoded by the *SRD5A2* gene (located at chromosome 2q23) and plays a major role in prostate development and disease predisposition. Two missense variants in *SRD5A2*, A49T and V89L, have been associated with increased prostate cancer risk.(45, 46) In vitro studies suggest that A49T substitution is associated with a higher reaction rate resulting in higher intraprostatic DHT levels.(47) In addition, *SRD5A2* has been associated with pharamacogenetic variation for the steroid 5α-reductase inhibitor, finasteride. Finasteride has a reduced affinity for the A49T mutant enzyme. (48) The involvement of the *SRD5A2* gene in prostate carcinogenesis and in pharmacogenetic variation make it a compelling candidate for future studies of personalized therapy in men with CRPC.

All the variants found to be associated with response to ADT in this study were in or near genes involved in androgen synthesis and metabolism. *CYP19A1*, *CYP3A43* and *CYP1B1* have functions in steroid hormone synthesis, cholesterol synthesis and vitamin D metabolism. *SRD5A1* catalyzes the conversion of testosterone into DHT and is the first isoform of the steroid 5-alpha-reductase. Polymorphisms in *ESR2,* estrogen receptor 2, have been associated with prostate cancer risk but studies of *ESR2* in aggressive and metastatic prostate cancer have not found any associations between *ESR2* polymorphisms and risk of advanced

disease or with response to therapy.(49, 50)

An issue in our study and in most genetic association studies is that of multiple hypothesis testing. Several methods have been described to correct for multiple hypothesis testing: the conservative Bonferroni adjustment of a single P value, permutation testing, cross-validation techniques, or replicating results in an independent sample. Most of these methods operate on the principle of lowering the α-level necessary for rejecting the null hypotheses. It is expected that variants associated with complex, common diseases will have small effect sizes. Therefore, overly conservative adjustments of the α-level may miss a large portion of true associations. To address the issue of multiple testing and to reduce the number of false-positives, we implemented a Bonferroni adjustment and also permutation testing. However, we opted to report the unadjusted association given the small sample size in this study, particularly the small number of African-American subjects (n=24). We consider this study to be exploratory and although several SNPs were found to be associated with response to ADT, the associations did not remain statistically significant once we adjusted for multiple hypothesis testing under permutation testing or when using the more conservative Bonferroni adjustment. Our future research plans include attempting to replicate these findings in similar study cohorts uniquely of men with metastatic disease and in multi-ethnic/racial populations.

This study has several strengths. This study represents the first study investigating the association between germline variation and response to ADT in only men with metastatic disease and benefits from a platform of a randomized controlled trial with uniform enrollment criteria, disease and clinical measurements and administration of therapy. Likewise, this study is the first to consider African-American subjects in exploring the association between inherited variation in genes involved in androgen synthesis and metabolism and response to ADT.

A limitation of this study is that we were underpowered to stratify by race and to detect associations that may have been specific to African-American subjects. Further, we may have not been able to adjust for all confounders. Although I present models corrected for clinical characteristics, it is likely that these characteristics may not be true confounders and may actually be in the causal pathway between inherited variation and response to ADT. A potential confounder, in addition to race, would likely be family history, which would be associated with both outcome and predictor (SNPs). I did not have information on family history and could not adjust for it in association testing models. Although the potential impact of such correction is unknown, inherited forms of prostate cancer are associated with early-onset disease but not necessarily with more clinically aggressive disease.(51)

In this study, misclassification bias due to genotyping errors would likely be non-differential and bias results toward the null. In order to minimize differential misclassification bias in genotyping, measures including blinding case-control status and randomization of case and control samples during genotyping were implemented.

4.5 Conclusions

In this study of men with metastatic prostate cancer, 15 SNPs were found to be associated with either improved or worsened response to ADT. These associations did not remain significant after multiple hypothesis testing corrections. However, these variants warrant further study in larger populations of men with metastatic disease and in studies including African-American men. The variants identified are in or near candidate genes involved in androgen synthesis and metabolism and are plausible biologic targets for further therapeutic targets and outcome prediction. Due to the poor prognosis and treatment related morbidities associated with ADT, men with CRPC can benefit from prognostic markers which can aid in tailoring therapeutic regimens prior to the initiation of therapy.

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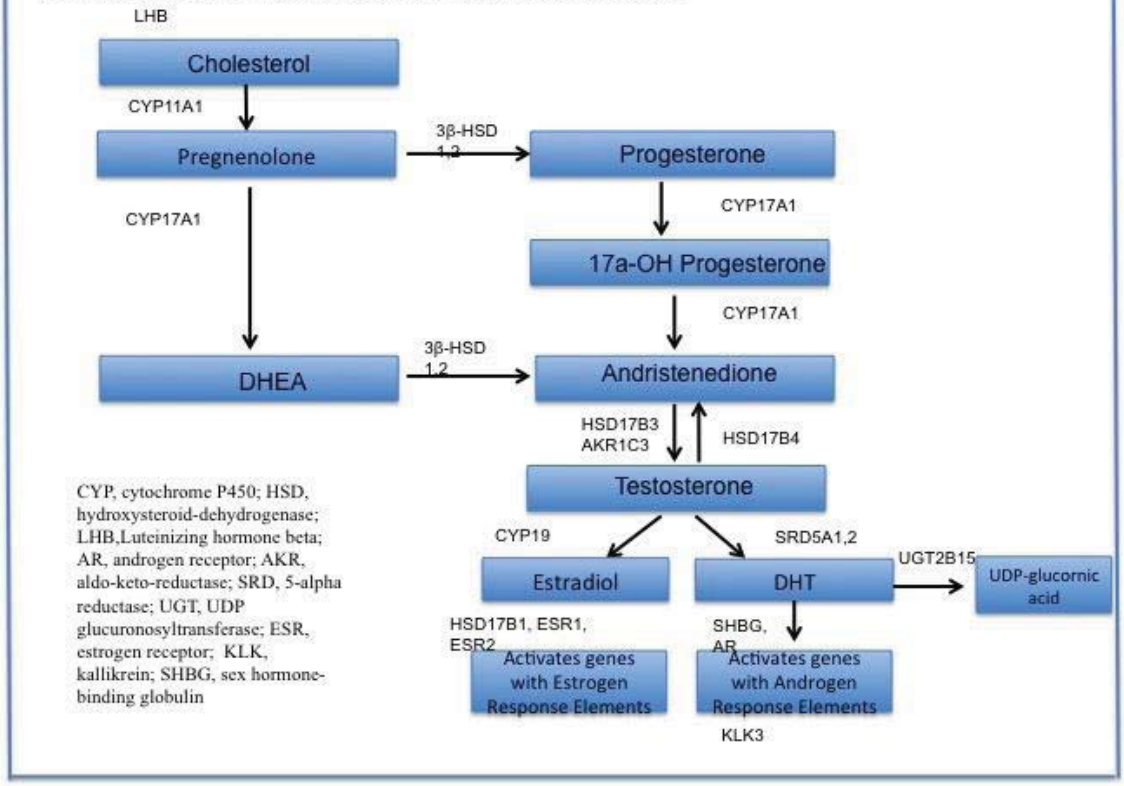
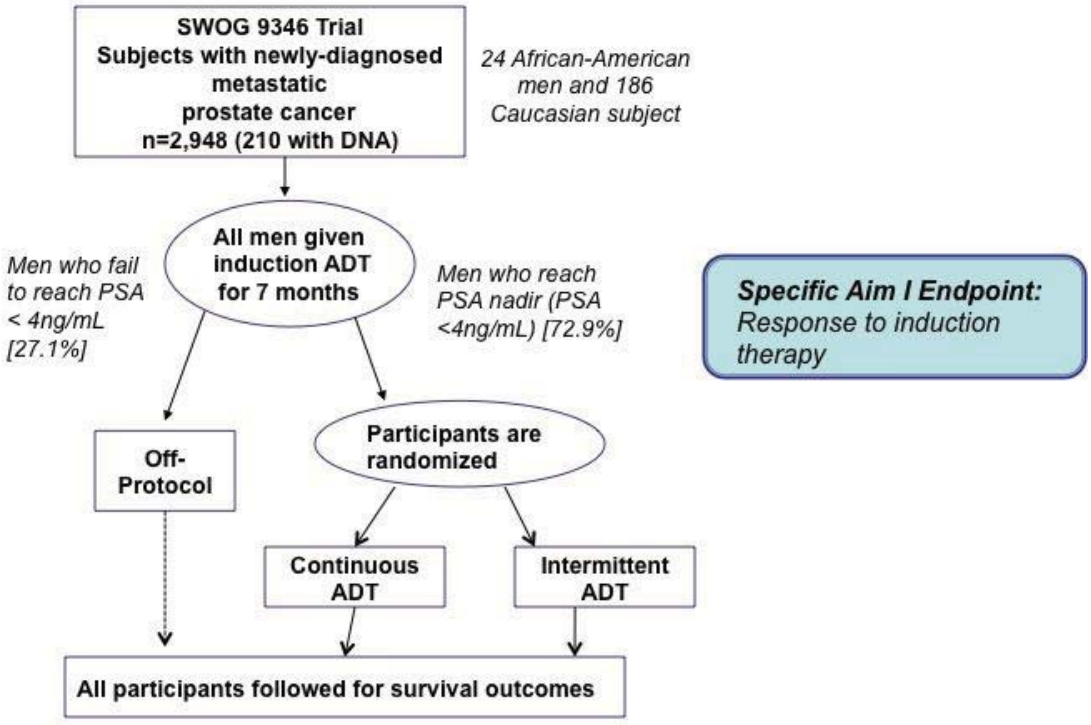
Figure 4.1:. A simplified schematic of the genes involved in the steroid biosynthesis pathway. The genes listed are necessary for the *de novo* synthesis of androgens/estrogens from cholesterol.





* Hussain et al, JCO, 2009

Figure 4.2: SWOG-9346 Schema of Participant Assignment

Table 4.1 Candidate Genes involved in hormone synthesis/metabolism included in our study

ABCB1	*CYP19A1*	*HSD17B4*	*SLCO2B1*
AKR1C1	*CYP21A2*	*HSD3B1*	*SLCO3A1*
AKR1C2	*CYP3A4*	*HSD3B2*	*SRD5A1*
AKR1C3	*CYP3A43*	*KLK3*	*SRD5A2*
AR	*CYP3A5*	*LHB*	*SREBF2*
COMT	*DHRS9*	*MAOA*	*SULT1A1*
CYP11A1	*EGF*	*SHBG*	*UGT1A1*
CYP11B1	*ESR1*	*SLCO1A2*	*UGT2B15*
CYP1B1	*ESR2*	*SLCO1B1*	
CYP17A1	*HSD17B3*	*SLCO1B3*	

Table 4.2 Clinical Characteristics of SWOG-9346 Study Subjects

Characteristic	Study Subjects
No of patients	210
Age, years	
Median	68.2 (10.3)
Range	46.5 - 93.0
Race	
Caucasian	186 (88.6)
African-American	24 (11.4)
Disease Severity*	
Extensive	78 (56.1)
Minimal	61 (43.9)
Gleason*	
<7	33 (17.3)
7	73 (38.2)
>7	85 (44.5)
Enrollment Year	
Median	2007
Range	2002-2008
Performance Status*	
0	136 (65.7)
1	53 (25.6)
2,3	18 (8.7)
Baseline PSA*, ng/mL	
Median (SD)	46.5 (5073)
Range	5.1 - 71500
PSA value, post-induction therapy	
>= 4 ng/mL	57 (27.1)
< 4 ng/mL	153 (72.9)

*Data on disease severity was missing for 71 subjects, Gleason score was missing for 19 subjects, Performance status was missing for 3 subjects and baseline PSA was missing for 9 subjects

Table 4.3 Description of SNPs associated with response to ADT in men with metastatic prostate cancer

Chr	SNP	Gene	Description	Ref/ Var Allele	Bp position	MAF in gen'l popn*	MAF in our study
12	rs2306283	*SLCO1B1*	Nonsynonymous, Missense variant	A/G	21329738	0.469	0.429
12	rs1463565	*SLCO1B1*	intron 7	C/G	21335381	0.534	0.422
12	rs17388851	*SLCO1B1*	intron 4	C/T	21323556	0.491	0.460
2	rs6543631	Intergenic	5' of *SRD5A2*	C/G	31742987	0.333	0.5
2	rs10175368	Intergenic	3' of *CYP1B1*	C/T	38307861	0.270	0.281
15	rs9972359	Intergenic	5' of *CYP19A1*	C/T	51491854	0.553	0.463
2	rs12470143	*SRD5A2*	intron 1	C/T	31763558	0.558	0.455
2	rs2365778	Intergenic	5' of *SRD5A2*	C/T	31738219	0.407	0.493
5	rs13166363	Intergenic	5' of *SRD5A1*	G/A	6677699	0.267	0.266
5	rs824811	*SRD5A1*	intron 4	T/C	6665781	0.229	0.212
14	rs2772163	Intergenic	5' of *ESR2*	C/T	64693385	0.288	0.308
7	rs651430	*CYP3A43*	intron 1	C/T	99429843	0.566	0.480
14	rs867443	*ESR2*	intron 9	A/G	64701042	0.308	0.283
15	rs16964189	Intergenic	5' of CYP19A1	C/T	51494237	0.195	0.141
12	rs2900476	*SLCO1B1*	intron 7	C/T	21336063	0.204	0.287

* MAF found in HapMap CEU (Utah residents with Northern and Western European ancestry) Phase III data

Table 4.4 Logistic Regression results for individual SNPs and response to ADT in men with metastatic prostate cancer

			MODEL 1*		MODEL 2**	
SNP	MAF in cases	MAF in controls	OR (95% C.I)	nominal P value	OR (95% C.I)	nominal P value
rs2306283	0.34	0.46	0.50 (0.28-0.89)	0.019	0.36 (0.16- 0.80)	0.013
rs1463565	0.53	0.38	1.71 (1.08- 2.70)	0.021	1.88 (1.06- 3.33)	0.031
rs17388851	0.56	0.43	1.73 (0.08- 2.78)	0.023	2.02 (1.09- 3.74)	0.026
rs6543631	0.42	0.53	0.57 (0.34- 0.95)	0.030	0.67 (0.36- 1.24)	0.205
rs10175368	0.35	0.26	2.01 (1.07- 3.77)	0.031	3.47 (1.65- 7.30)	0.001
rs9972359	0.38	0.49	0.60 (0.37- 0.97)	0.038	0.73 (0.42-1.26)	0.263
rs12470143	0.54	0.43	1.75 (1.02- 3.00)	0.042	1.50 (0.79- 2.85)	0.216
rs2365778	0.58	0.46	1.63 (1.02- 2.62)	0.042	1.47 (0.82- 2.61)	0.194
rs13166363	0.34	0.24	1.66 (1.02- 2.72)	0.043	1.87 (1.08- 3.24)	0.026
rs824811	0.25	0.20	1.91 (1.02 - 3.60)	0.044	2.53 (1.28- 5.01)	0.008
rs2772163	0.37	0.29	1.69 (1.01 - 2.82)	0.045	1.31 (0.68- 2.54)	0.418
rs651430	0.57	0.45	1.58 (1.01 - 2.49)	0.046	1.82 (1.04- 3.21)	0.037
rs867443	0.35	0.26	1.71 (1.01 - 2.91)	0.047	1.44 (0.74- 2.81)	0.280
rs16964189	0.24	0.16	3.18 (1.01 - 10.04)	0.049	4.11 (1.14- 14.88)	0.031
rs2900476	0.22	0.31	0.35 (0.13 - 0.99)	0.049	0.24 (0.06- 0.93)	0.039

*Model 1 is adjusted for ancestry

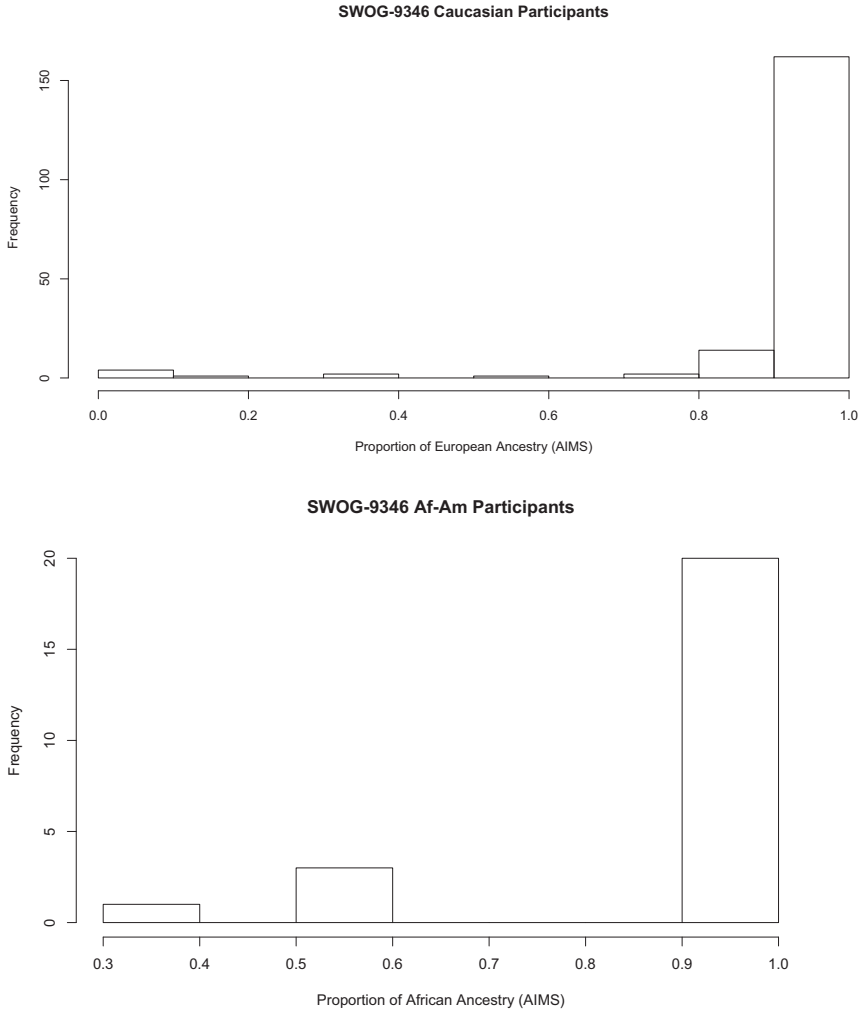
** Model 2 is adjusted for ancestry, age at enrollment, baseline PSA, Gleason >7, Gleason =7 (Ref Gleason <7), ECOG Performance Status (0-3)

Table 4.5 Analysis for genes with multiple SNPs associated with response to ADT

	OR (95% CI) ¶	*p* value
SLCO1B1		
rs2306283	Conditional SNP	
rs17388851*	1.631 (0.716 - 3.715)	0.244
rs1463565*	1.623 (0.8036 -3.277)	0.177
rs2900476*	0.5035 (0.1664 - 1.524)	0.225
SRD5A2		
rs6543631	Conditional SNP	
rs12470143**	0.4523 (0.5397 - 3.179)	0.551
rs2365778**	0.9472 (0.1774 - 5.058)	0.949
CYP19A1		
rs16964189	Conditional SNP	
rs9972359	0.65 (0.39-1.06)	0.08
SRD5A1		
rs13166363	Conditional SNP	
rs824811	1.40 (0.64 – 3.02)	0.398
ESR2		
rs2772163	Conditional SNP	
rs867443	2.19 (0.43-11.27)	0.348

¶ Gene-specific logistic regression models adjusted for ancestry & conditional SNP for each gene

Figure 4.3 Admixture estimation using 103Ancestral Informative Markers (AIMs). A) Proportion of European ancestry estimated using AIMs among SWOG-9346 self-reported Caucasian participants and B) Proportion of African ancestry estimated using AIMs among SWOG-9346 self-reported African-American participants



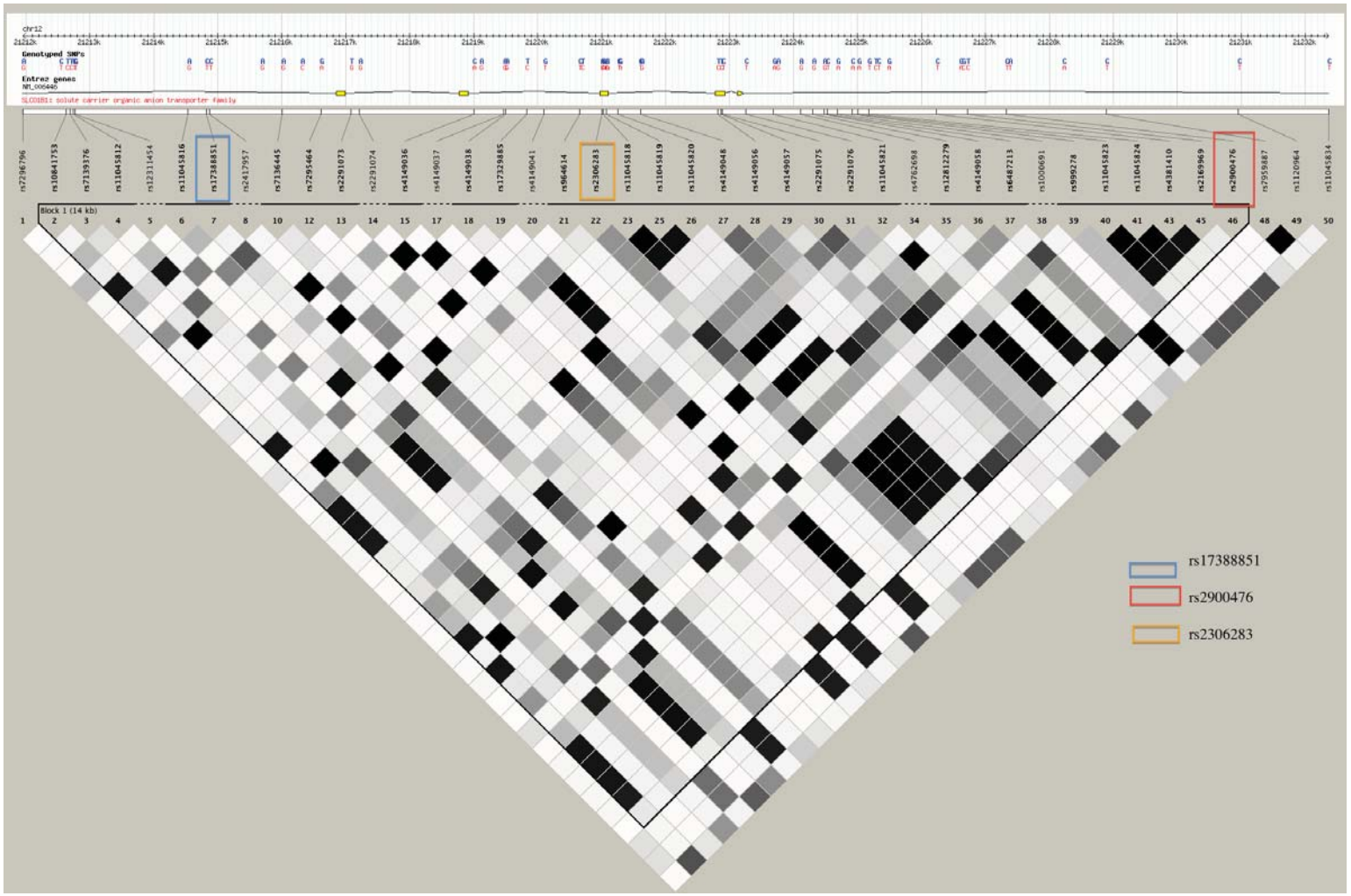


Figure 4.4 Linkage Disequilibrium (LD) patterns at *SLCO1B1.* A LD heatmap measured by R squared is shown for 3 SNPs in *SLCO1B1* along 25kB of chromosome12, measured using data from HapMap from CEPH Subjects. Black indicateshigh LD while white indicates low LD. Rs2900476 is in the red box, rs17388851 is in the blue box and rs2306283 is in the yellow box.

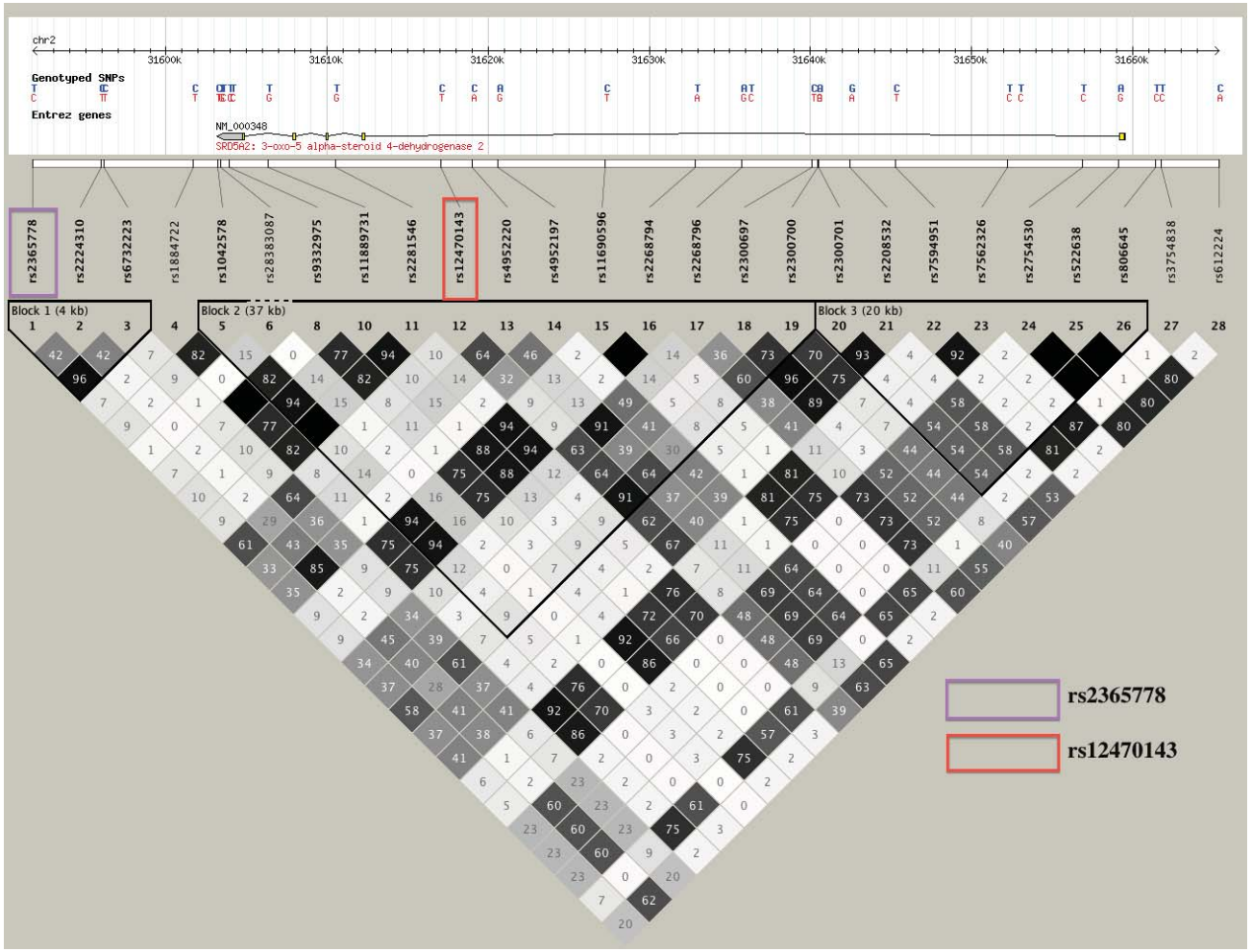


Figure 4.5 Linkage Disequilibrium (LD) patterns at *SRD5A2.* A LD heatmap measured by R squared is shown for SNPs in *SRD5A2* along 80kB of chromosome 2, measured using data from HapMap from CEPH Subjects. Rs2365778 is in the purple box and rs12470143 is in the red box. Black indicates high LD while white indicates low LD.

Table 4.6 Haplotype associations in *SLCO1B1* and response to ADT

Haplotypes	rs4149038	rs964614	rs2306283	Frequency	OR	*P* value
1	G	A	G	0.271	1.9	0.210
2	A	A	G	0.161	0.656	0.009
3	A	G	A	0.096	0.64	0.352
4	A	A	A	0.472	Ref	

Rare estimated haplotypes were not included

Table 4.7 Haplotype associations in *SRD5A2* and response to ADT

Haplotypes	rs1651071	rs1691053	rs13166363	Frequency	OR	*P* value
1	A	G	G	0.073	1.69	0.035
2	G	A	A	0.227	0.736	0.684
3	G	A	G	0.276	0.765	0.253
4	A	A	A	0.385	Ref	

Rare estimated haplotypes were not included

CHAPTER 5

Conclusions

In this dissertation, I have focused on using epidemiologic methods to improve understanding of forms of advanced prostate cancer. Within the context of epidemiologic studies, we have combined our understanding of molecular biology with advances in genetic sequencing and techniques in genetic epidemiology. This dissertation has contributed to our understanding of statins as secondary chemopreventative agents in prostate cancer and identified genes and variants potentially associated with hereditary prostate cancer. This dissertation has also considered African-American men, who are at increased risk for prostate cancer diagnosis and prostate cancer specific mortality, by studying the role of inherited variation in hormone synthesis/metabolism in response to ADT. We have identified variants not previously associated with response to ADT that may predict response to ADT in both Caucasian and African-American men with metastatic prostate cancer.

In my first study, I investigated to role of statins as potential secondary chemopreventative agents in prostate cancer in men with inherited forms of prostate cancer. Long-term (ever) statin use was not associated with a modified risk of recurrence in men treated with RRP (HR=1.04, 95%CI=0.72-1.49, *P* value=0.23). Although our findings do not support a protective association between statin use and risk of BCR,

future studies may be well suited to men with inherited forms of prostate cancer as they tend to be diagnosed earlier as compared to sporadic cases. There are currently no chemopreventative strategies recommended for men with a family history of prostate cancer to reduce incidence or recurrence and statins continue to be of interest as they are safe and widely used.

In my second study, I investigated variants in three candidate genes, *MAP3K14*, *RND2* and *ARHGAP27*, which are located in a chromosomal region of interest, 17q21-22. The chromosomal 17q21-22 region has been implicated through several linkage studies as potentially harboring one or more prostate cancer susceptibility genes. Using novel next-generation sequencing techniques to sequence all coding regions within 17q21-22, I identified five nonsynonynomous missense variants within *MAP3K14* (E215K and rs11574819) and *ARHGAP27* (rs143997699, rs112715622, and rs34793644). In family-based association tests, including mutation carrier status of family members, *ARHGAP27* is associated with HPC in the presence of linkage (Z score=2.40, *p* value=0.017). The variants associated with hereditary prostate cancer within *MAP3K14* and *ARHGAP27* in this study should be confirmed in other studies and studied in larger cohort of men with hereditary and sporadic cancer in order to determine their frequency and association with prostate cancer.

In the final chapter of my dissertation, I studied the role of inherited variation in response to ADT in men with metastatic prostate cancer. This is the first study to consider germline variation in African-American men who have an increased incidence of and mortality from prostate cancer as compared to Caucasian men. Identifying variants, particularly those with a biologic function, is of significant public health

importance. In this study, I identified 15 SNPs in genes involved in hormone synthesis and metabolism, including *SLCO1B1*, *SRD5A2*, *SRD5A1*, *CYP19A1*, *CYP1B1*, *CYP3A43*, and *ESR2* associated with response to ADT in African-American and Caucasian men. Although none of these variants remained significant after multiple hypothesis testing correction. The most interesting finding was the association between rs2306283 and response to ADT, where carriers of the variant allele are associated with a reduced risk of poor response to ADT (OR=0.50, 95%CI=0.34-0.95, *P* value=0.019). Rs2306283 is a nonsynonymous missense variant in S*LCO1B1*. Variants associated with response to ADT can be utilized to predict which men will respond to therapy and can inform personalized therapy regimens for men with advanced disease, thereby reducing exposure to toxicities associated with ADT. Pharmacogenomic studies in prostate cancer are rare and their importance is highlighted by the number of men being diagnosed with and treated for advanced prostate cancer.

Future Directions in Prostate Cancer:

Advancements in prostate cancer research will require a shift towards focusing on molecular and mechanistic analyses. Focusing on such studies will increase our understanding of those prostate cancers are clinically relevant and require treatment. In addition, these studies can inform future chemoprevention strategies and therapeutic targets. Primary and secondary prevention of prostate cancer remains an attractive goal because of the high prevalence of the disease and treatment-related morbidities associated with both initial and recurrent treatments. Recent studies have shown that supplements such as selenium nor vitamin E do not reduce the risk of prostate cancer, further 5-ARIs are not widely used. Chemopreventive agents with favorable safety profiles that provide

other disease–risk lowering benefits are ideal targets in prostate cancer. Statin medications continue to be of interest due to their widespread use and the body of consistent research from *in vitro* studies showing the potential of statins to inhibit prostate tumor initiation and progression. Epidemiologic studies have yielded inconsistent results, however better-designed prospective studies should concentrate on subgroups of men with high-risk profiles, such as men with a family history of the disease, If the use statins exhibited even a modest risk reduction effect in prostate cancer incidence or recurrence, this risk reduction could have significant public health impact given the large number of men being diagnosed with and dying from prostate cancer. Prostate cancer has a large component of genetic susceptibility that has yet to be explained and identifying variants accounting for prostate cancer heritability may inform improved screening practices for men with a family history of prostate cancer. Focusing on men with the most convincing evidence of inherited prostate cancer is highly relevant in reducing phenotypic heterogeneity that may be inhibiting the current studies from identifying highly penetrant mutations associated with familial and hereditary prostate cancer. Deep sequencing techniques represent novel strategies to identifying causal variants, which can be useful for screening of men with high-risk profiles and in understanding the etiology of various subtypes of prostate cancer. Further, replication of variants in additional populations such as men with sporadic disease and in men at high risk due to African-American race are an important subsequent step.

Among the most paramount priorities in prostate cancer is identifying either genetic or molecular signatures that can differentiate tumor subtypes and allow for expanded definitions of the various types of prostate cancer. The significance of variants

associated with HPC should be put in context by studying gene expression patterns related to those genes in prostate cancers. Given that the strongest risk factors for prostate cancer are age, race and family history, considering how gene expression varies with age and with degree of tumor differentiation will allow us to investigate the intersection of inherited mutations, increasing age and age-dependent and non-age-dependent somatic changes, such as DNA methylation. Understanding the complex genomic profile of men with prostate cancer will allow us to understand and distinguish which prostate cancers are clinically "important".

Lastly, future research in prostate cancer must focus on better, more targeted therapies in for the treatment of metastatic prostate cancer and CRPC. The development of novel treatments requires identification of molecular targets as well as designing personalized therapeutic regimens that can delay mortality and reduce treatment related toxicities and morbidities. Prostate cancer researchers are challenged with identifying critical pathways to target in CRPC treatment and understanding how to best utilize novel therapies with existing therapies and determining which stages should be targeted to delay or stop progression to the castrate-state. Targeted therapies should also be studied in such a way as to address the increased incidence and mortality among African-American men and to determine whether race differences impact variation of pharmacogenomics and drug response in prostate cancer.

Understanding the pathways involved in prostate cancer, whether it be the role of statins as a chemopreventative agent in prostate cancer, identifying the genes involved in familial/hereditary prostate cancer or elucidating the role of germline variation in response to ADT, requires study designs that will reduce phenotypic heterogeneity and

focus on subgroups of men which can aid in clarifying the biologic and molecular mechanisms involved in the most clinically relevant forms of prostate cancer.