ABSTRACT

Prostate cancer is currently the most common visceral malignancy in American men with over 234,000 new cases diagnosed annually. In addition, prostate cancer is second only to lung cancer as a cause of death. The large “baby boomer” population is now moving into the age group where prostate cancer rates dramatically increase and the number of men afflicted will “skyrocket”. The conquest of prostate cancer will require the integrated efforts in the areas prevention, early diagnosis, and therapeutics.

Accumulating epidemiologic and laboratory evidence support the hypothesis that prostate cancer risk is the result of combinatorial impacts of crucial environmental exposures, inherited susceptibility, and modifying influences of diet and lifestyle factors. The concept that a single dietary variable will prove to be a singular and exclusive cause is overly simplistic. However, progress can only be made with a focused line of research that will contribute key pieces of the puzzle. Among the many nutritional components under investigation, the role of energy balance is emerging as a potential risk factor for prostate cancer. This insight is critically relevant considering the rapid explosion of obesity in America.
The search for mechanistic evidence supporting specific causal relationships between dietary variables, such as energy balance, and prostate cancer has been exceptionally complex and difficult by epidemiology alone. Thus, rodent models have provided tools for evaluating crucial relationships and hypotheses emerging from human observation. The first component of this dissertation employs a carefully controlled rodent model of prostate cancer tumorigenesis with rats fed diets consumed ad libitum or restricted in total energy/intake by 40%. We focused our attention upon the hypothesis that energy intake modulates the IGF-I endocrine and autocrine/paracrine axis to alter tumor growth via regulation of tumor expression of the critical angiogenic promoter called vascular endothelial cell growth factor (VEGF). As background for this study, prostate tumor angiogenesis has emerged as one of the critical biological properties of a “successful” cancer, and the aberrant expression of pro-angiogenic growth factors is a common feature in clinical studies. Our studies demonstrate that dietary energy intake increases the biological activity of the IGF-I axis and is correlated with enhanced expression of VEGF in vivo and in vitro.

Ultimately, evidence derived from humans provides stronger support for causal relationships. We chose to pursue studies of diet and prostate tumor angiogenesis using a novel source of human prostate cancer tissue. The Health Professionals Follow-up Study was initiated in 1984 with the recruitment of over 50,000 men volunteering to be prospectively monitored for subsequent decades. Approximately 10 years ago, Dr. Clinton and collaborators obtained NIH support to create a prostate tissue bank for all men that undergo prostatectomy. By 2002, his team procured approximately 600 samples, and this tissue became the primary resource for studies of prostate cancer
angiogenesis and diet that are reported in the final sections of this thesis. We first defined our methods of quantitation of prostate angiogenesis by employing computerized digital image analysis of vascular architecture using biomarkers of blood vessels (Factor VIII and CD34). We then proceeded to complete a pilot study evaluating changes in vascular architecture between normal (nonmalignant) and cancer tissues in a series of 100 prostatectomy specimens. We clearly documented that vascular number, size, and shape (irregularity) change during the process of carcinogenesis. Based upon these encouraging results, we then examined approximately 500 cases of prostate cancer and assessed the relationships of vascular architecture to several biomarkers of aggressive prostate cancer, including Gleason grade and pathological stage, as well as critical biological outcomes such as risk of death. Our studies suggest that vascular architecture may be a strong predictive factor for lethal prostate cancer. Finally, we have conducted preliminary studies correlating prostate tumor vascular architecture with dietary exposures using the epidemiologic data (food frequency questionnaires) provided by men prior to diagnosis with prostate cancer. We observed a significant reduction in tumor vascularity with increased vitamin D exposure, supporting recent observations that this nutrient is associated with a lower risk of aggressive prostate cancer. Secondly, we observed a lower vascularity with increased body mass (obesity) in the cohort of men from the HPFS that chose prostatectomy for their primary therapy. This finding, upon first review, may be counterintuitive based upon our original hypothesis and rodent findings. However, we now propose that this finding is due to selection bias. Indeed, we propose that obese men with high risk factors at diagnosis, such as higher PSA values, higher Gleason score, and more advanced clinical stage, should be encouraged to pursue other therapeutic options
such as external beam irradiation and brachytherapy rather than prostatectomy. The surgical approach, which is more difficult in obese men, is being reserved for men with excellent prognostic feature. Thus, a selection bias may confound our sample by making it difficult to evaluate the role of obesity in tumor vascular architecture.

In conclusion, our studies provide novel data regarding the relationships between energy intake, IGF-I, and prostate tumor VEGF expression in a rodent model. Our studies with an epidemiologically linked set of human prostatectomy specimens shows that prostate tumor vascular architecture is different from normal prostate tissue and tumor vessels of greater number, smaller size, and irregular shape are associated with poor prognosis.
Dedicated to Krystian and Grażyna Powolny
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Prostate cancer is one of the most commonly diagnosed cancers in men in United States. Over 234,000 new cases will be diagnosed and over 27,000 men will die from this disease [1].

Screening for the prostate specific antigen (PSA) was initiated in the 1990’s and resulted in a sharp increase of prostate cancer incidence. Since 1992 rates of prostate cancer incidence were slowly declining [1]. Widespread use of PSA screening allows for more frequent detection of early stages of prostate cancer, a period when radical prostatectomy is considered curative [2]. Despite advances in detection and in treatment of this disease, prostate cancer death rates remain largely unchanged since the 1950’s. This is largely due to the heterogeneous biology of prostate cancer, which is characterized by a wide spectrum of clinical behavior ranging from indolent to a rapidly progressing disease. The challenge is to differentiate between the subpopulation of patients who may benefit from more aggressive treatment and those who may opt to avoid additional therapy and its side effects. New predictors of disease progression and patients’ survival are necessary because the use current predictors of prostate cancer
progression such as PSA, Gleason grade and tumor stage are often insufficient to accurately identify more aggressive disease.

Early detection and accurate diagnosis are essential to provide the most appropriate treatment course and novel biomarkers are necessary to more accurately predict prostate cancer progression and patient survival. This manuscript will first review the characteristics of prostate cancer and discuss the importance of tumor vascularization of prostate cancer progression and then describe our original research projects. The first project employs an animal model to examine the effects of energy balance on prostate cancer angiogenesis. The second research project uses image analyses of the vasculature as a tool for examining the relationship between prostate tumor angiogenesis, disease progression and survivorship. The third study assesses the correlation between selected dietary factors and vascularity of the tumor. Together these projects elucidate the importance of angiogenesis and tumor vascularity in prostate carcinogenesis and how this process may be affected by patients’ dietary habits providing ground for future investigations.
HYPOTHESIS

1. Dietary energy restriction can mediate its affects through the insulin-like growth factor system and affect tumor angiogenesis by altering expression of vascular endothelial growth factor.

2. Image analyses of tumor vascular architecture using CD34 and Factor VIII stained tissues may be an objective and reliable tool for evaluating prostate cancer biology. Vascular architecture and vessel number is predictive of the presence of more aggressive disease and increased risk of death from prostate cancer in men from the Health Professionals Cohort.

3. Vascular architecture and vessel number is affected by dietary habits of the men diagnosed with prostate cancer which in turn affects the aggressiveness and progression of disease.
OBJECTIVES AND SPECIFIC AIMS

1. Use an animal model to evaluate whether dietary energy restriction can alter the hormonal status of the body by influencing the insulin-like growth factor system and thus affecting tumor angiogenesis by reducing expression of angiogenesis promoters.
   a. To determine the effects of diet restriction on the body and organ weights of the animals.
   b. To determine effects of the diet restriction on the levels of hormones in the IGF-I system, such as IGF-I, IGFBP3, and growth hormone.
   c. To examine the effects of diet restriction on the hepatic expression of IGF-I and IGFBP3.
   d. To evaluate effects of the diet restriction on local expression of the IGF-I and its receptor in the tumor and normal prostate tissue.
   e. To assess the effects of diet restriction on expression of a major angiogenic promoter vascular endothelial growth factor in tumor, normal prostate tissue and plasma.

2. To evaluate whether image analyses of CD34 and Factor VIII immunostained prostate cancer tissue provide an objective and quantitative tool for assessing vascular architecture within the tumor.
a. To establish a complete data base of vascular parameters including vessel number, area, diameter, perimeter, percent area and vessel shape that can be used to quantify effects of various lifestyle factors on prostate cancer.

b. To correlate parameters of vascular architecture with the Gleason grade and tumor stage to evaluate relationship between angiogenesis and tumor biology.

c. To assess the relationship between vascular parameters and risk of death from prostate cancer, after adjusting for age at diagnosis and Gleason grade.

3. To evaluate effects of selected dietary factors on vascular architecture in the prostate cancer tissue of men in the Health Professionals Follow-up Study.

   a. Use previously validated analyses of CD34 and FVIII immunostained tumor tissues evaluate the vascular density, vessel size and shape

   b. To establish a correlation between vascularity biomarkers and selected dietary factors utilizing the Health Professionals Follow-up Study database containing information collected in the food frequency questionnaires.
2.1 Introduction

Prostate cancer is the most commonly diagnosed visceral cancer and third most common cause of cancer-related death in American men [1]. It is a disease with a wide spectrum of biological/clinical behaviors ranging from indolent disease to a very aggressive course. Heterogeneity in the biological behavior of prostate tumors may be a result of genetic variability or a result of exposure to different environmental factors, which influence genetic instability and types of mutation present [3, 4]. Genetic polymorphisms may contribute in part to the observed higher incidence of prostate cancer observed in African Americans [5, 6] and lower rates in Asian countries [7, 8]. However, inherited variability is not the only factor influencing the prevalence of this disease in different populations. Numerous epidemiological studies indicate that prostate cancer incidence is 10 – 20 times higher in United States and Northern and Western Europe than in Asian countries [8].
Epidemiological studies suggest environmental factors such as diet may be critical factors in prostate cancer risk. Migration studies examining populations of Japanese immigrants in United States demonstrate that within a few generations Japanese-Americans develop prostate cancer with an increasing frequency though still at a slightly lower rate than Caucasian and particularly African Americans [9-12]. A recent study by Chang demonstrated that the trends for prostate cancer incidence in China begin to resemble those observed in United States with increasing westernization of the Asian culture [13]. Similar trends have been observed in Japan since World War II, where a dramatic increase of milk, meat, eggs and saturated fatty acids consumption was followed by a linear 25 fold increase in prostate cancer death rates [14]. This global variability of changes in risk over time and with migration, points to the importance of environmental factors and their ability to impact prostate cancer development and progression.

Although many environmental factors should be considered, a key difference between Asian and Western lifestyles are the nutritional habits, which we propose are among the most critical variables that may impact prostate cancer development and progression [13].

The purpose of this review is to briefly describe the data supporting the influence of diet, with an emphasis on energy balance and obesity, as etiologic risk factors for prostate cancer. The second section of this literature review will address how dietary patterns may influence changes in hormonal status, which may impact the prostate gland or the prostate tumor environment to influence the carcinogenic cascade. Finally, the third part of this review will examine the potential role of diet induced changes in prostate vasculature and prostate tumor angiogenesis during carcinogenesis. A key aspect
of this review is to evaluate the utility of various biomarkers of angiogenesis as potential predictors of prostate cancer progression, as well as to define which markers could possibly serve as tools to assess dietary impacts upon tumor angiogenesis.

2.2 Review of prostate cancer development and progression

The biology of prostate carcinogenesis is not clearly understood and many hypotheses are under consideration regarding the development of this disease. Some hypotheses focus on the effects of carcinogens such as those in tobacco or cooked meats, while others emphasize viral or bacterial infections that may be associated with chronic inflammation. The following is a brief review of prostate cancer development and progression based upon a synthesis of the most prevalent ideas about prostate carcinogenesis at this time.

The process of prostate carcinogenesis from a normal glandular epithelium to cancer is complex and heterogeneous. Epidemiological studies linked sexually transmitted diseases and associated with them prostatitis with increased risk of prostate cancer [15, 16]. Furthermore, chronic or recurrent inflammation has been linked with prostate cancer development [17]. One of the proposed mechanisms explaining the effects of inflammation on prostate tissue suggests that presence of reactive oxygen species, nitric oxide and other reactive molecules released from activated macrophages results in massive tissue damage[17-19]. In the attempt to regenerate lost/injured tissue epithelial cells proliferate at a higher rate. This gives rise to a lesion called proliferative
inflammatory atrophy (PIA). PIA is characterized by increased proliferation in an attempt to regenerate injured tissue and infiltration of inflammatory cells which release reactive oxygen species contributing to the tissue damage [18, 19]. Moreover, this lesion demonstrates reduced expression of p27, a cyclin-dependent kinase inhibitor, and increased expression of Bcl-2, an anti-apoptotic protein [18]. These gene expression patterns resemble those observed in prostatic epithelial neoplasia (PIN). Interestingly, PIA appears usually in the peripheral zone where most carcinomas arise [19, 20] and recent studies suggest this may be one of precursors to PIN [20]. PIN is characterized by increased proliferation of the intraductal and intra-acinal epithelial cells associated with presence of multiple layers of cells and nuclear atypia (hyperchromasia, variability in nuclear shape and size). Glands containing PIN are sometimes larger than normal with disrupted layer of basal cells, that usually difficult to observe [21]. PIN lesions may be detected even as early as in the third decade of life, but the number, frequency and size of detected PIN increases with age [22][23]. Discovering PIN is not synonymous with prostate cancer, in fact often men die of other, unrelated causes and PIN is recognized during autopsy [22, 23]. The hypothesis that PIA and PIN are precursors to prostate cancer is supported by the fact that both benign lesions originate in the peripheral zone, like prostate carcinoma [19, 20]. Observations of premalignant and malignant lesions in prostate demonstrate that PIA frequently merges directly with PIN and PIN is topographically associated with prostate carcinoma. Finally, presence of transitional morphology between PIA and PIN and genetic changes typical for prostate cancer further support the notion of PIA and PIN being the precursors of prostatic carcinoma [18-20, 24, 25].
Both PIN and PIA are associated with mutations or epigenetic modifications in a variety of genes regulating cell proliferation such as p27 and retinoblastoma (RB [26, 27]). The reduces the ability of the cells to respond to stressors glutathione S-transferase (GSTP), thus promoting further genetic instability [24, 26]. One of the key mutations essential for PIN to progress toward prostate cancer is inactivation of NKX3.1 gene which encodes prostate specific homeobox and is mutated in over 90% of prostate cancers [24, 26, 27]. Over time and with repeated exposure to environmental stressors additional mutations in genes such as PTEN arise, which are hypothesized to contribute to the development of localized prostate cancer [24, 26, 27]. Interestingly, a recent study by Henrique et al [25] demonstrated that genes involved in prostate cancer progression, such as GSTP and APC, undergo inactivating epigenetic modifications as normal prostate tissue progresses towards cancer. Based on 30 prostatectomy tissue samples, it was observed that normal prostate GSTP had 10% lower percentage of methylated alleles vs. 28.1 % in high grade PIN and 80 % in prostate cancer (P <0.0001) [25]. Current research indicates that epigenetic modifications are visible in the entire genome. For example, hypermethylation affects genes regulating cell cycle (such as CDKN2A), hormonal responses (i.e. AR, RARβ), DNA repair (GSTP1), signal transduction and inflammatory response [28]. Aside from global hypomethylation typical for most tumors, oncogenes such as ras, c-jun and c-MYC are hypomethylated in prostate cancer [28]. Other epigenetic alterations include histone modifications, specifically histones in the vitamin D receptor and RARβ are hypoacetylated and those in the GSTP region are methylated [28]. This suggests that prostate cancer progression is not only associated with accumulation of mutations in key regulatory genes, but also with epigenetic
modifications. Since those alterations are reversible they are of particular interest as potential targets for cancer treatment.

Other steps essential for progression toward prostate cancer include developing independence from growth factors which provide survival signals inhibiting apoptosis, gaining limitless proliferative potential and resistance to anti-growth signaling [29]. However, as is the case for any solid tumor, growth and metastasis of prostate cancer are limited by the blood supply. Therefore, gaining ability to induce formation of new blood vessels (angiogenesis) is one of the essential steps in carcinogenesis [29, 30]. New blood vessels provide the tumor with adequate supply of nutrients and remove metabolites, and also facilitate local tissue invasion and facilitate distal metastasis [30]. Frequently, deregulation of the angiogenic process and formation of new blood vessels in the developing tumor is associated with mutations in p53, which is known to regulate cell cycle progression and is associated with stimulation of angiogenesis [26, 31].
Figure 2.1: Stages in molecular development of prostate cancer [24, 27]. RNASEL – interferon induced latent endoribonuclease; MSR1 – macrophage scavenger receptor1; GSTP – glutathione S-transferase; AR – androgen receptor; PTEN- phosphatase and tensin homologue; NKX3.1– codes for prostate specific homeobox; RB – retinoblastoma.
Development of local invasion and distal metastasis is associated with alterations in the tumor microenvironment. Various tumors, including prostate cancer, lose or inactivate E-cadherin, which is a cell-cell adhesion molecule acting as a suppressor of invasion and metastasis [26]. Another group of molecules affected by tumors are the integrins, which bind to extracellular matrix molecules (ECM). Tumor cells usually express integrins favoring binding to degraded elements of ECM which promotes invasion and metastasis. Finally, tumors frequently have upregulated expression of matrix degrading proteases which aid in the local invasion of tumor cells through surrounding stroma and through walls of blood vessel [26, 29]. Prostate cancer commonly invades seminal vesicles and surrounding organs and usually forms distal metastasis in bones, lungs and lymph nodes [32, 33].

Growth of normal prostate epithelium is dependent on and regulated by androgens. Interestingly, this dependence on androgens remains true for the prostatic tumor and even metastatic prostate cancer relies on androgen to promote its growth. Clinicians have been able to take advantage of this relationship and used treatment with anti-androgens and androgen suppressors to attenuate prostate cancer growth and progression [24]. In order for the prostate tumor to gain independence of the androgen, androgen receptor signaling is subject to various mutations which increase its sensitivity, allow for non-specific ligand binding, or amplify the number of receptors in the cellular membrane. Androgen independence is achieved through activation of ligand-independent signaling pathways in some cases [24, 26, 27]. Figure 2.1 depicts the stages
in prostate cancer development and most common genetic alterations associated with them.

Each one of the stages in prostate cancer development described above typically occurs over years and the entire process requires decades. Thus, long term exposure to environmental stressors and/or lifestyle risk factors may have a major influence on disease progression [8]. The most common behavioral risk factors include dietary components [34], energy balance or obesity [35], physical inactivity [35, 36] occupational exposure to carcinogens [37, 38], smoking [39, 40], chronic inflammation of the prostate [17], and sexually transmitted diseases [15, 16].

2.3 Prostate cancer and dietary factors

There is a dramatic difference between prostate cancer incidence observed in Asian countries and in populations of the Western world (United States or Northwestern Europe, Figure 2.2) [8]. Migrant studies on Japanese Americans demonstrate vividly that once immigrants take on the Western lifestyle their risk of prostate cancer increases gradually (usually over generations) to levels comparable to that of Caucasian males in US [11, 12]. Numerous studies, though not all [41], point to the western diet, energy balance, and obesity as potential modifiable risk factors for prostate cancer prevention [42-44]. Several components of the western lifestyle and diet have been implicated. These include high consumption of fat, especially animal fats, refined sugars, red meat, and dairy products in parallel with a lower intake of fruits and vegetables, fewer complex
carbohydrates and fiber [8, 45]. A comprehensive review of all of these factors is beyond the scope of this thesis and a number of review articles have generalized the current literature [8, 46]. I will focus attention on several of the dietary components that are particularly relevant to the findings in this thesis i.e., energy balance and vitamin D.

Prostate cancer is influenced by energy balance and obesity

Energy balance is influenced by energy intake (diet) and energy expenditure. Energy expenditure is comprised of basal or resting metabolic rate, physical activity (voluntary and non-voluntary exercise) and the thermic effect of food (that is the energy cost of absorbing consumed calories) [47]. Resting metabolic rate which contributes to most of the daily energy expenditure is related to one’s ability to gain or loose weight [47, 48]. Polymorphisms in genes coding for uncoupling proteins-2 (UPC-2) and -3 (UPC-3) were implicated in regulation of metabolic rate even though in vivo studies were not able establish a clear physiological explanation for their effects [48]. Interestingly, some studies suggest that UPC3 is involved in lipid oxidation and research demonstrated that lower fat oxidation has been related to weight regain [49]. Activity of the sympathetic nervous system (SNS) also affects energy expenditure. In fact low activity of SNS, assessed by levels of urinary excretion rate of norepinephrine, was correlated with development of obesity [50]. Physical activity levels have dramatically decreased over the past century due to technological advances [51] which resulted in a shift towards more positive energy balance and increased obesity [52-54]. Simultaneously, technology also allowed some people more time to engage in recreational physical activity [47, 48] which may prevent the positive energy balance. Finally, some research suggests that
non-voluntary physical activity, such as fidgeting, affects energy expenditure in a significant manner and may contribute to expenditure of additional 300 kcal a day [55]. Figure 2.3 represents components of energy balance.

Currently much effort is focused on elucidating the impact of the diet on energy balance, especially in the light of recent studies pointing to the western diet as one of the culprits of the increasing obesity in United States, Northwestern Europe [56, 57] and even in Australia [58]. Body Mass Index (BMI) is a commonly used measure of obesity. BMI is an indicator of optimal weight for health and based on the information from the National Institute of Health, NHLBI Clinical Guidelines on Overweight and Obesity established in June 1988. It is calculated based on person’s weight in kilograms and height in meters (Equation: $\text{BMI} = \frac{\text{kg}}{m^2}$). A person is considered underweight if their BMI is less than 18.9 and range of 18.5 to 24.9 indicates person has appropriate weight. A person is considered obese if their body mass index (BMI) is over 30 and overweight if their BMI is over 25. Current research indicates that in United States over 60% of adults are overweight and almost 35% of them are obese [59, 60]. Even more alarming are the rates of obesity in children and adolescents, which skyrocketed over the past few decades and currently over 30% of children and adolescents are considered obese or overweight [61]. Mounting evidence suggests that obesity is linked to an array of illnesses including diabetes, cardiovascular disease and various types of cancer [62, 63].
Figure 2.2: Age-adjusted incidence rates of prostate cancer in selected countries and populations of North America, Western Europe and Asia (per 100,000 person-years [8].
The relationship of obesity and prostate cancer risk has been examined in several studies. Study conducted in men from the Cancer Prevention Study I and II (CPS I and CPS II) cohorts demonstrated that obese men had higher relative risk of prostate cancer death than the non-obese ones (RR = 1.27, 95% CI = 1.04 - 1.56 CPS I and RR = 1.21, 95% CI = 1.07 - 1.37 in CPS II) [64]. Similar results were observed in a study conducted by Calle et al., who used CPS II cohort population and observed that men with BMI > 35 had increased relative risk of prostate cancer death (RR = 1.34, 95% CI = 0.98 - 1.83) [44]. A positive association was observed between obesity and higher grade and more advanced stage of the disease in two studies examining about 3,000 men who underwent a radical retropubic prostatectomy. BMI > 30 was an independent predictor of higher Gleason score (P < 0.001) and was associated with presence of positive margins (P = 0.007) in the study by Amling et al. [65]. Furthermore, obesity was an independent predictor of shorter time to biochemical recurrence after prostatectomy in multivariate analyses, with hazard ratio 1.20 for obese vs. non-obese men (P = 0.028) [65]. These results were supported by findings in the study by Freedland et al [66] who examined the influence of obesity in a cohort of 1,250 men who underwent radical prostatectomy at 5 medical centers. Results of this study indicate that moderately to severely obese men had higher rates of biochemical failure than men with normal weight (P = 0.009). As in the previous study, BMI > 35 was a significant predictor of time to PSA recurrence, with hazard ratio (HR) of 4.09 (95% CI = 1.67 - 10.01) compared to the normal weight men in multivariate analyses. In the moderately to severely obese group 50% of men experienced recurrence after 40 months of follow-up [66]. The above studies suggest that obesity significantly increases the risk of being diagnosed with
Figure 2.3: Components of energy balance based on manuscript by Ravussin 1999 [47]. CHO – carbohydrates, RMR – resting metabolic rate, TEF - thermic effect of food.
advanced disease, recurrence after radical prostatectomy and death from prostate cancer. These results support detrimental effects of obesity on prostate cancer development and also its progression.

However, impact of obesity on prostate cancer progression and development is far from straightforward. There are quite a few studies which point out that obesity lowers the risk of prostate cancer [67-70]. For example, in a study nested within the HPFS cohort obese men under 60 had lower risk of prostate cancer (RR = 0.52, 95% CI = 0.33 - 0.83, P<0.001) than those with BMI between 23 and 24.9 [68]. The same study reported that BMI was inversely associated with prostate cancer incidence in men with family history of this disease (RR = 0.74, 95% CI = 45 – 1.19, P = 0.01) [68]. One of the mechanisms explaining the protective role of obesity suggests that adipose tissue contains aromatase which converts testosterone to estradiol [71]. Peripheral conversion of androgens results in lower levels of circulating testosterone and thus reduces stimulation of prostate tissue. This hypothesis is supported by a population-based (cases were found in the Greater Bay Area Cancer Registry) case-control study which examined relationship between the age of 30 and risk of prostate cancer (OR = 0.53, 95% CI = 0.28 – 1.00)[70]. Results of the study demonstrate that obesity was inversely associated with advanced prostate cancer especially for men 20-29 (OR = 0.40, 95% CI = 0.20 – 0.88) [70]. In a study by Daniell obese men were found to have lower frequency of advanced tumors (P < 0.05) [72] and author contributes the protective effects obesity to increased estradiol and reduced endogenous testosterone. Furthermore, obese man demonstrated significantly lower tumor specific mortality rates than their normal weight counterparts (10% in obese
vs. 27% for non-obese men) [72]. Finally, there is also research on effects of obesity on prostate cancer recurrence after brachytherapy, which indicates that after 8 year follow-up obesity had no effects on biochemical progression [73]. Results of this research points out the complexity of changes induced by obesity. Clearly, further investigations are necessary to clarify the relationship between obesity and prostate cancer and elucidate the mechanism behind its effects.

Obesity is a result of a positive energy balance, which, as mentioned earlier, may be caused by either excessive food intake or limited energy output (for example through physical activity), or combination of both of those factors. The correlation between energy intake and prostate cancer risk was examined in a population-based case-control study which recruited 605 Caucasian and African-American men, aged 40-64, who were diagnosed with prostate cancer and 592 age-matched controls [74]. Diet over the 3-5 years before diagnosis (or interview) was evaluated using self-administered food-frequency questionnaires. Diet analyses demonstrated that increased total energy consumption was associated with increased risk of localized prostate cancer (OR = 2.15, 95% CI = 1.35 - 3.43) and regional/distant disease (OR = 1.96, 95% CI = 1.08 – 3.56) [74]. When energy intake was examined in men in the HPFS cohort the results revealed that although there was no correlation between energy intake and total risk of cancer, increased energy intake was associated with higher risk of metastatic disease and fatal prostate cancer (RR = 1.36) [75]. A study utilizing Prostate Cancer Prevention Trial (PCPT) cohort suggests that it may be the excess energy from high fat diet is contributing
to the increased risk of prostate cancer [76]. Overall, these studies support the link between positive energy balance, obesity, and increased prostate cancer risk.

Prostate cancer commonly develops over years or even decades, which makes it virtually impossible to conduct dietary intervention studies in human population. That is why animal studies provide additional evidence for the importance of energy intake in development of various tumors. Tumor formation was delayed in response to 40% energy restriction the in the p53 deficient mouse model which develops spontaneous tumors in various organs [77]. Apc\textsuperscript{min} mice, a model of intestinal tumorigenesis, placed on 40% calorie restriction exhibited more than 50% reduction in frequency of intestinal polyps [78].

There are also several studies examining the effects of energy restriction on prostate cancer models. The first report on the effects of diet on prostate cancer development come from Pollard et al., who observed that aging conventional and germfree Lobund–Wistar rats had reduced incidence of metastatic prostate cancer and extended survival in response to 30% dietary restriction [79]. Research done in a carcinogen induced (N-methyl-N-nitrosourea) rat model of prostate carcinogenesis revealed that 20% diet restriction prolonged cancer free survival and reduced the incidence of prostatic tumors [80]. Another study was done utilized a transplantable model of androgen-dependent prostate cancer (R3327H) in rats, which went on 20% or 40% energy restriction. Diet restriction decreased tumor size and altered the architecture of the tumor tissue and its vasculature [81]. Interestingly, energy restriction led not only
to lower incidence of tumors but also to decrease in levels of insulin-like growth factor 1 (IGF-I; [77, 78]) and leptin [77, 78]. Calorie restriction was demonstrated to have effects even on the progression of the tumors. When transgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice were initiated on a 40% calorie restriction diet after lesions were detected, dietary restriction lowered the mean grade of the observed lesions and reduced the weight of the prostate and seminal vesicles [82]. These data indicate that dietary changes are able to influence both tumor development and progression, potentially through altering hormonal balance.

Prostate cancer risk in relation to dairy products and vitamin D consumption

Another component of western diet associated with increased risk of prostate cancer is milk and other dairy products. Dairy products, especially milk, are consumed in much higher amounts in Northern Europe and United States than in Asian countries such as Japan [83, 84]. Epidemiological studies examining the consumption of dairy and associated with it intake of calcium demonstrated that moderate consumption of milk and calcium are not associated with increased risk of prostate cancer [85-88]. However, increased consumption of milk products and calcium was positively associated with increased overall risk of disease [89-91] and metastatic disease [74, 92]. For example, high calcium intake was associated with increased risk of advanced disease (RR = 2.97, intake ≥ 2000 mg/d vs. 500 mg/d) and metastatic prostate cancer (RR = 4.57, [85]) in HPFS study. Similarly, in a Swedish cohort, calcium intake increased the risk of prostate cancer (RR = 1.99 for intake ≥ 1183 mg/d vs. < 825 mg/d) and metastatic tumors (RR = 2.64) even after adjusting for other established risk factors [92]. The levels of dietary
calcium which these studies found to increase prostate cancer risk are within the levels of intake recommended to protect bone health and prevent osteoporosis [93]. Some authors suggest that increased consumption of dairy results in higher calcium intake which in turn inhibits vitamin D pathway (Figure 2.4) [85, 89, 90, 92, 94]. This hypothesis is supported by the observation that men who consumed large amounts of calcium from skim milk had lower plasma levels of 1,25 (OH)₂D₃, a vitamin D metabolite (85 pmol/L down to 71pmol/L) than those men who consumed less skim milk (600g/d Ca vs. 150g/d; P = 0.005 [89]. Several groups have attempted to establish a relationship between vitamin D metabolites and biomarkers and prostate cancer risk in humans.
**Figure 2.4:** Hypothesized mechanism of prostate cancer prevention through regulation of vitamin D blood levels and its cellular signaling
Ahonen et al. [95] found a 70% increased prostate cancer risk in men with 25 hydroxyvitamin D (25-OH D) levels below the median, especially in men < 52 years old who entered the study with low serum 25-OH D (adjusted odds ratio = 3.5). Corder et al. [96] found that mean 1,25(OH)$_2$D levels were significantly lower in men with prostate cancer and that prostate cancer risk was decreased in men with higher 1,25(OH)$_2$D levels, especially if serum 25-OH D levels were low. Finally, Tuohimaa et al. [97] recently showed that low vitamin D status measured within 10 years of diagnosis with prostate cancer, increased risk of prostate cancer in Finish and Norwegian men by 3.1 to 4.2 fold. In contrast, several studies in men from sun-rich regions where vitamin D insufficiency is uncommon did not detect a relationship between serum vitamin D metabolites and prostate cancer [98-100]. Although not uniform, majority of these studies suggest that high serum 25-OH D or high 1,25(OH)$_2$D are associated with a lower risk of prostate cancer. These results also indicate it is necessary to establish a healthy level of sun exposure and dietary intake of vitamin D and calcium that would be optimal for prevention of prostate cancer as well as for bone and skin health. Vitamin D is hormonally active and alters cell biology by activating nuclear vitamin D receptor (nVDR)-mediated gene transcription [101]. Importantly, nVDR is expressed in central and peripheral zones of the human prostate [102]. In vitro studies showed that in primary or transformed prostate cells 1,25(OH)$_2$D and its analogues inhibit proliferation, promote differentiation and apoptosis, and limit migration and invasiveness of the cells [103-111]. These data suggest that vitamin D signaling through nVDR controls epithelial cell proliferation and differentiation and thus, may affect prostate cancer growth and development.
Effects of fat intake on prostate cancer

One of the characteristics of the western diet is very high consumption of animal fats, especially those from red meat [34, 112]. In one of the early studies, Giovannucci et al. used the Health Professionals Follow-up Study (HPFS) cohort to assess the relationship between the risk of advanced prostate cancer and consumption of fat [34]. Their results indicated that high total fat consumption was directly related to the risk of advanced prostate cancer (RR = 1.79). Increased intake of saturated, monounsaturated fatty acids and alpha-linolenic acids were associated with increased risk of advanced disease. Similar results were observed in a population-based case-control study (703 men), where high consumption of total fat, as well as saturated and monounsaturated fatty acids, was associated with increased risk of localized/regional [74].

Later studies in the HPFS cohort further examined the correlation between specific types of fatty acids (n-3 and 6-n fatty acids) and risk of prostate cancer. The results of the investigations indicate that alpha-linolenic acid was positively associated with risk of total and advanced prostate cancer [113]. Furthermore, eicosapentaenoic and decosahexaenoic acids were both correlated with reduced risk of prostate cancer. None of the n-6 examined in that study was associated with disease risk [113]. Outcomes of this and other studies indicate that the source of fat has an influence on the associated risk. It was apparent that relationship between high consumption of fatty acids and prostate cancer was primarily due to the animal fat, especially that from red meat [34, 45]. Higher consumption of fish fatty acids actually lowers the risk of prostate cancer.
[114], while there was no association between prostate cancer and fat derived from dairy products [34, 115].

Results of the epidemiological research quoted above suggest that fatty acids are associated with progression of prostate cancer. Impact of low and high fat (42% calories from fat) isocaloric diet on prostate cancer progression was evaluated in an immunodeficient mice implanted with a slow androgen-dependent prostate cancer cell line (Los Angeles Prostate Cancer 4, LAPC-4). Low fat diet (12% calories from fat) slowed tumor growth, improved survival of the mice, and slowed progression of prostate cancers to androgen independence [116]. However, dramatic results of this study were not supported by other researchers who examined effects of high fat diet in Noble rat sex hormone induced prostate cancer model [117], in a carcinogen induced Wistar-derived rats [118]. Further research is necessary to clarify the effects of high fat diets and potential mechanisms used to modulate prostate carcinogenesis.

**Prostate cancer risk and meat consumption**

Consumption of red meat is high in western culture. When intake of red meat was evaluated as a separate variable, it had the strongest positive associations with advanced prostate cancer among all food groups [34]. Increased consumption of red meat was associated with higher risk of metastatic prostate cancer in another study on the HPFS population [115]. This association can be contributed in part to the content of saturated and monounsaturated fatty acids in red meat. However, the rest of the risk can be assigned to other substances present in red meat or formed during its preparation.
(grilling, broiling and doneness of meat), such as heterocyclic and polycyclic amines such as 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), benzo[a]pyrene (B[a]P) and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) [112]. Animal studies revealed that consumption of PhIP induces prostate tumors [119]. Consumption of very well done meat was associated with 1.4-fold higher risk of prostate cancer and 1.7-fold higher risk of incident disease (diagnosed during the trial follow-up) in a study using the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) cohort. Moreover, consumption of high amounts of PhIP was associated with statistically significant increased risk of prostate cancer [112]. Recent studies demonstrate that carcinogens such as PhIP and B[a]P may promote prostate carcinogenesis by forming DNA-PhIP adducts or inducing DNA single strand breaks [120, 121]. Interestingly, activity of GSTP1 is able to inhibit the formation of PhIP-DNA adducts in cell culture. Future studies should establish whether dietary interventions promoting GSTP1 activity are able to effectively attenuate carcinogen activity.

Vegetables and fruits influence prostate cancer risk

Western diet is characterized by a high consumption of animal products and relatively low intake of fruits and vegetables. Mounting evidence from epidemiologic, animal, and in vitro studies indicate that nutrients found in fruits and vegetables have preventative effects on development of prostate cancer. A study examining the diet of Japanese men, who have 10 times lower incidence of prostate cancer than men in USA, demonstrated that soy products such as tofu and natto reduced risk of the disease [122].
Similarly, lower incidence of prostate cancer is also observed in men of Southeast China. This may be in part contributed to their diet rich in colorful fruits and vegetables (tomatoes, pumpkin, spinach, watermelon, citrus fruits) containing large amounts of carotenoids [123]. Surprisingly, unlike for other malignancies, data gathered in Western countries does not observe a strong relationship between prostate cancer risk and consumption of fruits and vegetables [124, 125]. Beneficial effects of fruits and vegetables were not observed even in the largest a prospective cohort study conducted in Europe (European Prospective Investigation into Cancer and Nutrition, EPIC), where overall intake of fruits and vegetables was not associated with risk of disease [126]. These results do not negate potential effects of specific fruits and vegetables (cruciferous, soy, or carotenoid rich) on prostate cancer. Probably the largest study employing the HPFS cohort demonstrated reduced prostate cancer incidence and lower risk of advanced disease in men who consumed high amounts of lycopene and tomatoes [127]. A multiethnic case control study conducted in men living in United States, Canada and British Columbia also showed that higher consumption of yellow and orange vegetables (containing high concentrations of carotenoids) and cruciferous vegetables correlates with reduced risk of advanced prostate cancer [128]. Another study from Netherlands conducted in a cohort of over 58,000 men (the Netherlands Cohort Study, NLCS) detected significant correlation between consumption of retinol, alpha-carotene and beta-carotene and reduced risk of prostate cancer [94]. Interestingly, it is still not clear whether beneficial effects of the fruits and vegetables are contributed solely to the influence of specific compounds they contain, for example lycopene, or is it a combination of those substances with other components of fruits and vegetables.
Furthermore, it is important to clarify the effects of food preparation on bioavailability of the beneficial compounds. Future intervention trials may provide answers to those questions and further evidence for benefits of specific fruit and vegetable components on prostate carcinogenesis.

2.4 Energy balance alters the IGF-I axis

Energy balance and associated with it obesity affect levels of variety of hormones including insulin, growth hormone, thyroid hormones, testosterone, estradiol and numerous factors in the IGF-I family of protein, all of which influence prostate cancer development [43, 129-134]. Our intention is not to describe complex relationships between diet and various hormones. In stead, we would like to give a brief review of the effects of energy balance on the IGF-I system. IGF-I family of proteins consists of two growth factors IGF-I and IGF-II, their cell surface receptors (IGF-IR and IGF-IIR), six IGF binding proteins (IGFBP 1 through 6), IGFBP related proteins (IGFBP-rPs), insulin-related receptor (IRR) and a group of IGFBP proteases (Figure 2.5) [135, 136]. Due to similarities in structure, IGF-I is able to bind to IGF-IR, IGF-IIR and has low affinity for insulin receptor [135]. IGF-I is produced in almost all tissues, but the majority of circulating IGF-I is made in liver [137]. Over 90% of IGF-I circulating in plasma is bound to one of the IGFBPs and out of that 75 % is bound to IGFBP-3. IGF-I signaling is able to induce hypoglycemia [138] and signals through IGF-IR and activates Akt and MAPK signaling, thus promoting cell proliferation and providing survival signals which inhibit apoptosis [139]. The role IGFBPs is to regulate availability of IGF-I, extend its
half life, and control its transport between tissues and blood vessels. IGFBPs control the levels of bioactive (free) IGF-I, affect its ability to interact with receptors and prevent over stimulation of proliferation and inhibition of apoptosis [135, 137].

Numerous studies, conducted both in United states and in Europe suggest that total energy intake is positively correlated with IGF-I levels [130-132]. Levels of IGFBP3 were reduced by higher intake of energy from saturated fat in healthy adults [132, 133]. In addition to the impact of total energy specific components of the diet influence the levels of IGF-I in different manner. Participants of a population-based study in Greece who consumed red meats, fats and oils had significantly higher levels of IGF-I [133]. In Nurses Health Study (NHS), women who consumed higher amounts of protein from milk exhibited higher blood levels of IGF-I [132]. Furthermore, as observed in NHS and in a multiethnic study in UK, consumption of saturated fats was also significantly associated with increased IGF-I [130, 132]. Interestingly, two studies revealed that intake of energy from carbohydrates was inversely associated with IGF-I levels [130, 133]. Overall, these studies indicate that excessive energy intake from a typical western diet is contributing to increasing IGF-I levels.
Figure 2.5: Overview of the insulin-like growth factor 1 system. Overview includes major components of its signaling pathways and cellular phenotypes regulated by IGF1R signaling. Figure created based upon information contained in review articles by LeRoith D et al, Howarth GS, Moschos SJ et al, and Bahr C et al[135, 140-142].
Another example of the impact of energy balance on hormonal levels comes from a prospective exercise study. This study examined the effects of over (excess of 15% energy) or underfeeding (33% energy restriction) in 19 healthy men undergoing high intensity aerobic training [134]. Measurements of total and free IGF-I levels revealed that men in the diet restriction group had lower levels of that hormone while the overfed men had no change. Furthermore, IGF binding protein-1 was also increased in the group who underwent diet restriction [134]. This would indicate that negative energy balance had major regulatory effect on IGF-I levels.

2.5 IGF system affects prostate cancer risk

Due to its pro-proliferative and anti-apoptotic characteristics, IGF-I is thought to promote growth of variety of neoplasms, including prostate cancer [139]. There are several studies examining the correlation between levels of IGF proteins and risk of developing prostate cancer. The first prospective study to suggest association between IGF-I and prostate cancer was a case-control study in the Physicians Health Study (PHS, [143]). It demonstrated that men in the highest quartile of the IGF-I levels had relative risk of 4.3 compared to than those in the lowest quartile. IGF-I remained a significant predictor of malignancy after including weight, body mass index, lycopene and levels of other hormones. Furthermore, multivariate analyses of the IGF-I and IGFBP3 independent of each other, revealed an inverse association between IGFBP3 levels and prostate cancer risk. This study demonstrated usefulness of IGF-I as a predictor of prostate cancer risk which provides additional information at the time of diagnosis. Most
of the studies available, though not all [144], support the positive association between IGF-I and risk of prostate cancer [129, 145-147]. Additionally, levels of IGF-I were positively associated with higher risk of advanced disease (RR = 5.1, 95% CI = 2.0 - 13.2), but did not show a link with Gleason score. Moreover, when extreme quartiles of IGFBP3 blood levels were compared, men with the highest IGFBP3 levels had relative risk of advanced disease of 0.2 (95% CI = 0.1 – 0.6) compared to men in the lowest quartile. Combination of low IGFBP3 with high IGF-I levels resulted in relative risk for advanced disease equal 9.5 (95% CI = 1.9 – 48.2) [129].

Unlike IGF-I, IGFBP3 levels were not consistently correlated with risk of prostate cancer. As mentioned above, some studies indicate that low IGFBP 3 concentrations are associated with increased risk of disease [129, 143]. However, a prospective study in Sweden and another one nested in the HPFS cohort demonstrated a positive relationship between IGFBP3 and prostate cancer [145, 147]. These contradictory results suggest that circulating IGFBP3 levels may not be directly related to the development of prostate cancer, even though its levels in tissues may be.

Studies in animal and in vitro cell culture models indicate that IGF-I has properties suggesting that it is one of the key factors in development and progression of prostate cancer. To examine effects of persistent overexpression of IGF-I in prostatic epithelium, a transgenic mouse model expressing human IGF-I under bovine keratin 5 promoter (BK5) was used. BK5 promoter targets expression of IGF-I to the epithelium of various tissues including prostate. Utilizing this model DiGiovanni et al. observed a
stepwise progression towards adenocarcinoma, starting in the early hyperplastic lesions to frank neoplasia. About 50% of the mice developed adenocarcinoma of the prostate by 6 months of age. These results support the idea of direct link between IGF-I signaling and prostate cancer development [148].

Interestingly, there is a multiple studies report on the interrelationship between androgen receptor and IGF-IR. Research utilizing LNCaP cells as a model of androgen sensitive prostate cancer cell line revealed that stimulation of androgen receptor (AR) results in six-fold increase in expression of the IGF-IR. This effect is independent of the genomic activities of AR and mediated through Src extracellular signaling pathway. Up-regulation of the IGF-IR is likely to result in increased sensitivity to IGF-I, increased proliferation and survival of the cancer cells [149]. Research focused on the β-catenin pathway indicates that IGF-I induces AR activation in the absence or with low levels of ligand. The effects of IGF-I are mediated through up-regulation of cellular and nuclear β-catenin in cancer cells. Interestingly, IGF-I-induced activation of AR resulted in enhanced expression of Prostate Specific Antigen (PSA), which is one of the genes targeted by AR [150]. Research quoted above indicates that IGF-I may play a role in progression from androgen –dependent to androgen independent prostate cancer. This hypothesis is supported by another in vitro study, which demonstrated that IGF-I anti-apoptotic signaling and enhanced expression of IGF-IR assists in the progression towards androgen independence [151]. Finally, a recent study from Marelli et al. [152] indicates that IGF-I is also able to assist in metastasis in androgen-independent manner by altering expression of αvβ3 integrin through PI3K/Akt signaling pathway. Altered expression of
this integrin results in changed cell morphology and increased cell migration of the prostate cancer cells which is one of the steps in cancer progression towards metastatic disease.

In an in vitro model of human pancreatic cancer, IGF-I was demonstrated to promote expression of VEGF. Furthermore, \textit{in vivo} inhibition of IGF-IR signaling in a mice orthotopic model of pancreatic cancer resulted in reduction in VEGF expression and decreased angiogenesis. Results of this study suggest that IGF-I signaling promotes proliferation, attenuates apoptosis and promotes angiogenesis which essential for tumor invasion and metastasis [153].

\textbf{2.6 Development of a hypothesis: Energy balance, changes in IGF-I, and altered tumor angiogenesis.}

Angiogenesis is a process of formation of new blood vessels, which normally occurs in the body during embryogenesis and in adulthood during reproduction and wound healing [154-156]. It is tightly orchestrated by a balance of pro- and anti-angiogenic factors of which many have been identified (\textbf{Table 2.1} and \textbf{Table 2.2}). For example vascular endothelial growth factor (VEGF), one of the most potent promoters of angiogenesis, and thrombospondin 1 (TSP-1), an inhibitor of neovascularization [30, 157]. Interestingly, ability to form new blood vessels is one of the key steps necessary for progression of solid tumor development [29]. Without angiogenesis, the growth of solid tumors cannot exceed 1mm in diameter, because the rate at which tumor cells undergo
apoptosis matches the rate of the tumor cell proliferation due to nutrient and oxygen deprivation [29, 30, 158]. The 1mm size restriction is dictated by the ability of the nutrients and oxygen to diffuse within the tumor tissue without additional blood supply. It is assumed that tumors gain the ability to secrete pro-angiogenic factors or induce their secretion in the surrounding normal/host stromal tissues [30, 159], thus changing their microenvironment towards one favoring neovascularization. This process, called the angiogenic switch [158, 159], stimulates sufficient vascularity to provide cancer with substrates and oxygenation, as well as an adequate removal of toxic metabolites. Angiogenesis is also thought to be essential for local invasion and formation of distal metastasis [159, 160], thus affecting progression of the disease to its lethal phenotype.

Animal studies of dietary energy restriction and tumor growth suggest that diet may affect process of tumorigenesis in part by altering angiogenesis. The focus of this section will be on energy balance. An earlier study by our laboratory examined the effects of 20 and 40% restriction of energy intake on prostate cancer vascularity in rat and mouse models. Diet restriction resulted in a 62% decrease of vascularity as compared to control and 49% reduction in vascularity of tumors implanted in mice [81]. Furthermore, this diet restriction reduced the expression of VEGF in the tumors as detected by immunohistochemistry (P < 0.003) [81]. Finally, 20-40% diet restriction did not affect tumor cell proliferation, but it more than doubled the number of apoptotic cells (P < 0.001) [81]. This pattern of changes is similar to that observed in tumor treated with antiangiogenic agents, such as angiotatin [161]. In a rat carcinogen-induced model of mammary carcinogenesis, a 40% dietary restriction resulted in 31% to 39% reduction of
the vessel number, area, and density in florid intraductal proliferations, ductal carcinoma 
_in situ_ and in adenocarcinomas [162]. The changes in intratumoral vascularization, based 
upon their sample size and assessment methodology, induced by the dietary restriction 
were not statistically significant. Dietary energy restriction down-regulated tumor 
expression of VEGF receptor FLK-1 (P < 0.001) and VEGF, although this was not 
statistically significant [162]. Another study examining the effects of 40% dietary caloric 
restriction in animal model of different types of mouse and human brain tumors. Dietary 
caloric restriction decreased vascularity (from 118 ± 17 in _ad libitum_ group down to 
80±17 in diet restricted group, P < 0.05) and increased the apoptotic index (from 3.7±0.4 
in _ad libitum_ group, to 8.1±1.2 in 40% dietary restricted group, P < 0.01) observed in all 
tumors [163]. It also had differential effects on the proliferation and apoptosis of the 
tumor cells, and increased apoptosis but had no effect on the proliferation rates [163]. 
Altogether, the above studies indicate that dietary restriction may induce changes in the 
expression of factors promoting angiogenesis and affect vascularization of the tumors.
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<td>[169, 170, 173, 184, 185]</td>
<td>Transforming growth factor alpha (TGF-α) and beta (TGF-β)</td>
<td>[216-218]</td>
</tr>
<tr>
<td>Del-1</td>
<td>[187]</td>
<td>Tumor necrosis factor-alpha (TNF-α)</td>
<td>[222-224]</td>
</tr>
<tr>
<td>Entactin</td>
<td>[171, 173]</td>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>[229-232]</td>
</tr>
</tbody>
</table>

**Table 2.1:** List of factors promoting angiogenesis.
<table>
<thead>
<tr>
<th>Inhibitors of angiogenesis</th>
<th>Reference</th>
<th>Inhibitors of angiogenesis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-methoxy-estradiol</td>
<td>[234]</td>
<td>Interleukin-1,-4, -12</td>
<td>[235-242]</td>
</tr>
<tr>
<td>1,25Dihydroxyvitamin D3</td>
<td>[243, 244]</td>
<td>Ligands of PPARγ</td>
<td>[245, 246]</td>
</tr>
<tr>
<td>ADAMIS-1</td>
<td>[247-249]</td>
<td>Maspin</td>
<td>[250, 251]</td>
</tr>
<tr>
<td>Angiostatin</td>
<td>[252, 253]</td>
<td>Metalloproteinase inhibitor (TIMP)</td>
<td>[254-256]</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>[181, 257]</td>
<td>METH-1</td>
<td>[247, 249]</td>
</tr>
<tr>
<td>Antiangiogenic antithrombin III</td>
<td>[258, 259]</td>
<td>Pigment-epithelium-derived factor (PEDF)</td>
<td>[260-262]</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>[263, 264]</td>
<td>Pex</td>
<td>[265, 266]</td>
</tr>
<tr>
<td>Canstatin</td>
<td>[267, 268]</td>
<td>Placental ribonuclease inhibitor</td>
<td>[269]</td>
</tr>
<tr>
<td>Cartilage-derived inhibitor (CD1)</td>
<td>[270, 271]</td>
<td>Plasminogen fragment</td>
<td>[272-274]</td>
</tr>
<tr>
<td>Decorin</td>
<td>[216, 275]</td>
<td>Kringle 5</td>
<td></td>
</tr>
<tr>
<td>Endostatin</td>
<td>[278, 279]</td>
<td>Platelet factor 4</td>
<td>[177, 276, 277]</td>
</tr>
<tr>
<td>Fibronectin fragment</td>
<td>[207, 284]</td>
<td>Retinoids</td>
<td>[280-283]</td>
</tr>
<tr>
<td>Gro-β</td>
<td>[286]</td>
<td>Soluble VEGF receptor</td>
<td>[285]</td>
</tr>
<tr>
<td>Heparinases</td>
<td>[219, 288]</td>
<td>Tetrahydrocortisol-S</td>
<td>[220, 287]</td>
</tr>
<tr>
<td>Heparin chorionic</td>
<td>[294, 295]</td>
<td>Thrombospondin-1 and -2</td>
<td>[289-293]</td>
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<tr>
<td>gonadotropin (hGC)</td>
<td></td>
<td>Vascular endothelial growth inhibitor (VEGI)</td>
<td>[296, 297]</td>
</tr>
<tr>
<td>Inferterons α, β, γ</td>
<td>[281, 298-304]</td>
<td>Vasculostatin</td>
<td>[305]</td>
</tr>
<tr>
<td>Interferon-inducible protein (IP-10)</td>
<td>[306, 307]</td>
<td>Vasostatin</td>
<td>[263, 308]</td>
</tr>
</tbody>
</table>

**Table 2.2:** List of factors inhibiting angiogenesis.
2.7 Angiogenesis and prostate cancer

Development of prostate cancer is a multistep process that progresses over decades until tumors acquiring a variety of characteristics, such as limitless proliferation, evasion of apoptosis and ability to secrete their own growth factors (Figure 2.6). Although all of the steps in prostate carcinogenesis are essential for development of cancer, we are going to focus on the importance of angiogenesis in this disease. As mentioned above, solid tumors depend on the ability to form new blood vessels to support their growth, and to allow invasion of surrounding tissues and distant metastasis [309]. Therefore assessment of angiogenesis and vascularity of the tumor has been examined as a potential predictor of cancer development, progression and patient survival in various types of malignancies, including prostate cancer [310-314].

How one quantifies angiogenesis remains a critical question. One of the methods used to quantify neovascularization in human samples is microvessel density (MVD), i.e., counting the number of vessels per tumor area. The first clinical study which used MVD as a potential marker of prostate cancer biology was performed by Weidner et al. and demonstrated that higher MVD correlated with increased efficiency of local and distant metastasis [315]. This finding suggested that assessment of MVD may have utility as a biomarker of disease progression and patients’ survival.
**Figure 2.6:** Functional capabilities tumors need to acquire during their development and progression. Figure taken from Hanahan et al 2000 [29].
2.8 Methods used to assess angiogenesis in prostate cancer

**Immunohistochemical staining of the vessels**

One of the methods commonly used to identify biomarkers of angiogenesis is immunohistochemical (IHC) staining visualizing the blood vessels within the tumor tissue with some form of quantitation. Antibodies used to detect blood vessels target the Factor VIII (FVIII) which is also called the von Willebrand factor (VWF), CD34 and CD31/Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) found on the surface of the endothelial cells. Despite differences in structure and function of the target proteins, IHC staining for FVIII/VWF and CD31/PECAM-1 detect mature blood vessels due to biological functions of those two factors [316-318]. Unlike the previous two stains, CD34 is a marker of immature cells and allows for visualization of newly formed and not fully differentiated blood vessels [319, 320]. Comparison of FVIII/VWF and CD31 against CD34 revealed that CD34 detected a higher number of vessels in prostate cancer tissue per high power field (x200) than either FVIII or CD31 [321, 322]. This difference is most likely due to the fact that CD34 targets the walls of small and immature blood vessels, whereas FVIII and CD31 detect mature and fully formed blood vessels. Examples of different stains used to visualize vasculature are show in Figure 2.7.
Figure 2.7: Immunohistochemical staining of the blood vessels using different types of antibodies. Sample images represent high power fields (200x) of a) staining against FVIII, b) staining against CD34 captured in our lab and c) CD31/PECAM-1 staining as presented in a study by Rogatsch H et al. [323].
Quantitation methods

There is no single approach or validated method used to quantify angiogenesis in prostate cancer. The original study by Weidner et al. evaluated vascularity using the hotspot method [315]. This method identifies the area in the tumor with the highest estimated vascularization, called “hotspots”, by examining the entire tumor area at a low power field (x 40) and only the hotspots are used to count the number of vessels per the high power field (commonly x 200 is used) [315]. The so called hotspot method is often criticized for being very bias-prone since it is dependent on the observers’ skills and experience. However, this method sometimes with small modifications has been widely used to assess vascularity due to its simplicity and the fact that it does not require any additional equipment or software.

Since 1993 there have been numerous attempts to improve the objectivity of the hotspot method. Barth suggested measuring the vascular surface density (VSD), which quantifies the vessel area per volume of the tumor tissue [324] using a square lattice with 121 points composed of 11 horizontal and 11 vertical lines. This grid was superimposed over a random high power field of a tissue section. Number of vessel walls which crossed the lines of the lattice were counted, volume of the stroma was assessed by counting the number of locations where line in the lattice crossed over stromal tissue. Even though this method permits comparison of tumors composed of differing percentages of stroma epithelium and glandular lumen [324], it involves time consuming calculations and may be inefficient when evaluating a large number of cases.
The first computerized method assessing angiogenesis in prostate cancer was introduced by Bostwick et al. and calculated the “optimized microvessel density” (OMVD) [325]. Each field was digitally captured twice to get an enhanced image of the tissue and the vascularity of the histological specimen. A computer algorithm was used to adjust for pixel size and true vessel and tissue area was calculated using a conversion factor. The images were then processed electronically and the total microvessel count and total tissue area were used to calculate the [326, 327]. Initial OMVD analyses performed using this method were promising [325], but later studies failed to establish any correlations between OMVD and clinical outcomes [326, 327]. Moreover, this method required image capture capabilities and software processing which could limit its use in clinical setting, especially in smaller medical centers.

Finally, Offersen et al. used a 25-point Chalkley’s eyepiece graticule projected onto a hotspot and oriented to maximize the number of stained vessels coinciding with 25 random dots [322]. Only vessels coinciding with the random dots counted used to calculate MVD. The Chalkley’s count for each tumor was reported as an average of three separate hotspot counts of blood vessels and allowed for an estimate of the blood vessels area. Despite its wide use for assessment of vascularity in other malignancies [328, 329], Chalkey’s method has not gained acceptance as a standard method for evaluation of angiogenesis in prostate cancer.

Careful examination of all the quantitation methods reveals that regardless of the method of choice used by the researchers most followed similar rules when evaluating
the MVD. MVD was assessed in a several non-overlapping, non-necrotic areas. Positive staining of a single cell (or a cluster of cells) was considered a vessel even if lumen was not noticeable. Red blood cells were not used to categorize structure as a vessel. MVD counts were presented as number of vessels within a single high power field (commonly x 200). MVD values were usually reported as a Mean, Median, Maximal or Minimal MVD and those numbers were then used to establish cut of points to classify patients into categories of high and low MVD or quartiles. If vessel area was calculated the MVD may be reported as % of total volume mean vascular area per measured field (MVAF) [330]. Those rules allowed for elimination of personal bias and standardization of all measurements. More importantly, this also permitted for comparison of MVD measurements performed using different methods.

2.9 MVD and clinical markers of prostate cancer progression

In order for MVD to be a useful biomarker it needs to correlate with survival and previously established biomarkers of prostate cancer progression. Commonly used biomarkers/predictors of prostate cancers progression include the following: involvement of seminal vesicles and regional lymph nodes; positive surgical margins [331]; extraprostatic extension; abnormal prostate specific antigen (PSA) level (>4 ng/ml) [2]; tumor stage based on either TNM or ABC tumor staging systems [332]; and Gleason score (Figure 2.8) or World Health Organization (WHO) grade [333-335].
Figure 2.8: Schematic diagram of the Gleason grading system courtesy of Dr. D.F. Gleason, Minneapolis, Minnesota. Integrated design created by Pittsburgh Supercomputing Center.
MVD and tumor stage

A correlation between tumor stage and MVD was hypothesized since increased vasculature supports tumors’ ability to invade local tissues and metastasize. Regardless of the type of antibody used to visualize tumor microvessels, majority of the studies reported a positive correlation between the microvessel density and the stage of the tumor [321-324, 329, 336-342] (Table 2.3). However there are several studies which failed to observe any significant association between the microvessel density and disease advancement [326, 343-347]. Based on current literature review there are no reports on a negative relationship between MVD and tumor stage.
Table 2.3: Relationship between microvessel density and stage of prostate cancer. MVD – microvessel density; Cont - means MVD was considered as a continuous variable; pT1, T1 – represent cancer stages.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>N</th>
<th>Stain</th>
<th>Cut off</th>
<th>Stage and MVD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[324]</td>
<td>41</td>
<td>FVIII</td>
<td>Cont</td>
<td>pT4&gt;pT3&gt;pT2&gt;control</td>
<td>pT4, pT3&gt;pT2&gt;control</td>
</tr>
<tr>
<td>[336]</td>
<td>149</td>
<td>CD34</td>
<td>90</td>
<td>T1b = 50 % high MVD</td>
<td>T3,4 = 77 % high MVD</td>
</tr>
<tr>
<td>[338]</td>
<td>221</td>
<td>FVIII</td>
<td>43</td>
<td>T1a = 4% high MVD</td>
<td>T&gt;2 = 58% high MVD</td>
</tr>
<tr>
<td>[337]</td>
<td>71</td>
<td>CD34</td>
<td>90</td>
<td>pT2a-b = 50% high MVD</td>
<td>pT3ab = 87% high MVD</td>
</tr>
<tr>
<td>[339]</td>
<td>32</td>
<td>FVIII</td>
<td>Cont</td>
<td>Localized disease = 80.2CI (71.4-91.0) Skeletal metastasis = 154.6CI (92.3-216.9)</td>
<td>?</td>
</tr>
<tr>
<td>[340]</td>
<td>25</td>
<td>FVIII</td>
<td>60</td>
<td>T1 = 50% high MVD</td>
<td>T3 = 30 % high MVD</td>
</tr>
<tr>
<td>[322]</td>
<td>51</td>
<td>CD34</td>
<td>Cont</td>
<td>↑MVD w/ ↑M-classification</td>
<td></td>
</tr>
<tr>
<td>[323]</td>
<td>46</td>
<td>CD31</td>
<td>Cont</td>
<td>pT2 = 26.0 ± 6.1</td>
<td>pT3 = 49.3 ± 11.7</td>
</tr>
<tr>
<td>[341]</td>
<td>98</td>
<td>FVIII</td>
<td>Cont</td>
<td>pT2 = 22 ± 10</td>
<td>pT3 = 31 ± 13</td>
</tr>
<tr>
<td>[342]</td>
<td>57</td>
<td>FVIII</td>
<td>Cont</td>
<td>pT2 = 23.9 ± 8.5</td>
<td>pT3 = 31.8 ± 9.9</td>
</tr>
<tr>
<td>[321]</td>
<td>102</td>
<td>CD31,</td>
<td>85</td>
<td>pT2 60 low vs 7 high MVD</td>
<td>pT3 25 low vs 10 high MVD</td>
</tr>
</tbody>
</table>

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MVD and tumor Grade

Gleason grade is an effective and commonly used predictor of prostate cancer progression and recurrence, and thus it was essential to establish whether MVD correlates may be associated with grading. Numerous studies described the association between Gleason grade or tumor differentiation and MVD [315, 321, 322, 327, 336, 337, 340, 342, 348-352] (Table 2.4). Not all published reports were able to detect a link between the MVD and Gleason score [326, 343-346, 353-356]. However, none of the studies published thus far detected a negative correlation between those two factors.

Prior to introduction of Gleason scoring system, tumor differentiation was assessed by the norms established by the WHO which in addition to the glandular architecture examined the nuclear morphometry of the tumor cells [357]. A few studies which employed that grading system demonstrated that MVD correlated with the level of tumor differentiation as evaluated by the norms established by WHO [338, 358, 359]. There has been a single study reporting no association between tumor differentiation and MVD [360].
<table>
<thead>
<tr>
<th>Ref.</th>
<th>N</th>
<th>Stain</th>
<th>Cut off</th>
<th>Grade and MVD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[336]</td>
<td>146</td>
<td>CD34</td>
<td>90</td>
<td>Grade 3-4</td>
<td>40% high MVD 8-10 76% high MVD</td>
</tr>
<tr>
<td>[337]</td>
<td>71</td>
<td>CD34</td>
<td>90</td>
<td>Grade 2-4</td>
<td>100% low MVD 5-6 51% high MVD ≥7 100% high MVD</td>
</tr>
<tr>
<td>[327]</td>
<td>211</td>
<td>FVIII</td>
<td>Cont</td>
<td>Grade ≤6 Median = 61.5 7 Median = 63.7 ≥8 Median = 62.4</td>
<td>0.06</td>
</tr>
<tr>
<td>[340]</td>
<td>25</td>
<td>FVIII</td>
<td>Cont</td>
<td>Grade 2-4 = 49 ±08.5 5-7 = 53.1 ±27.9 8-10 = 101.7 ±31.2</td>
<td>0.006</td>
</tr>
<tr>
<td>[348]</td>
<td>126</td>
<td>CD31</td>
<td>1-45 low 46-90 moderate 91-131 high</td>
<td>Grade &lt; 7 80% low MVD ≥7 20% low MVD &lt;7 30% high staining ≥7 70% high staining</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>[349]</td>
<td>80</td>
<td>CD31</td>
<td>Cont</td>
<td>Grade &lt; 7 mean = 22.48 ≥7 mean = 32.62</td>
<td>0.057</td>
</tr>
<tr>
<td>[352]</td>
<td>40</td>
<td>CD34</td>
<td>Cont</td>
<td>Grade 3 mean = 66.85±7.61 4 mean = 103.06±13.5 5 mean = 138.86±6.41</td>
<td>0.04</td>
</tr>
<tr>
<td>[322]</td>
<td>51</td>
<td>CD34</td>
<td>Cont</td>
<td>CD34↑MVD w/↑WHO grade FVIII↑MVD w/↑Gleason score</td>
<td>0.04</td>
</tr>
<tr>
<td>[342]</td>
<td>57</td>
<td>FVIII</td>
<td>Cont</td>
<td>Grade 1 median = 15 ± 5 2 median = 25 ±12 3 median = 37 ±14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>[321]</td>
<td>102</td>
<td>CD34</td>
<td>105</td>
<td>Grade &lt; 7 45 cases with low MVD vs 4 with high MVD Grade ≥7 40 cases with low MVD vs 13 with high MVD</td>
<td>0.027</td>
</tr>
<tr>
<td>[315]</td>
<td>74</td>
<td>FVIII</td>
<td>Cont</td>
<td>Grade 4-5 mean = 34 ± 16.6 6-7 mean = 56 ± 28.6 ≥8 mean = 83 ± 44.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>[351]</td>
<td>96</td>
<td>CD31</td>
<td>Cont</td>
<td>Grade &lt; 6 mean = 48.8 ≥7 mean = 73.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Table 2.4:** Relationship between microvessel density and grade of prostate tumor.
MVD and PSA

Preoperative PSA is another tool used as a predictor of prostate cancer risk of progression and recurrence, but data on its relationship with MVD are very limited. Research focused on the correlation between preoperative PSA levels and MVD detected positive correlation between those two factors [340, 348, 359, 361] (Table 2.5). However, investigations by Stroymeyer did not discover correlation between MVD and PSA even though MVD was related to tumor stage [341]. Similarly, research by Gettman did not relate MVD to either PSA, or tumor grade and stage [326]. There was even one report on a reverse relationship between PSA and MVD [355] (Table 2.5). This lack of straightforward association between PSA and MVD is not surprising considering the fact that PSA is not always synthesized by the prostatic tumors. This is especially true for the very poorly differentiated prostate cancer that is rapidly progressive and unresponsive to therapy. These tumors frequently lose some of the characteristics of prostate tissue (become de-differentiated), and thus are no longer able to synthesize PSA.

2.10 MVD as a predictor of local invasion

The ability of the tumor to invade and metastasize is thought to be dependent, at least in part, on angiogenesis. Therefore, tumor vasculature was assessed in relation to parameters indicative of invasion and local metastasis such as extraprostatic extension, positive surgical margins, and invaded seminal vesicles.
**Table 2.5:** Relationship between microvessel density and PSA levels  
PSA\(_{\text{pre-treat}}\) — prostate specific antigen level prior to any treatment; PSA\(_{\text{pre-op}}\) — prostate specific antigen level before radical prostatectomy

<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Stain</th>
<th>Cut off</th>
<th>PSA level and MVD</th>
<th>p-value</th>
</tr>
</thead>
</table>
| [361]     | 68 | CD34  | Cont        | PSAdensity correlated w/ MVD,  
\[ r = 0.785 \text{ in advanced PCa} \]               | 0.007    |
| [340]     | 25 | FVIII | Cont        | PSA\(_{\text{pre-treat}}\) ≤ 4 ng/ml = 43.3 ± 11.1  
≥ 4 ng/ml = 96.1 ± 33.1 | <0.0001  |
| [359]     | 104| FVIII | Cont        | PSA\(_{\text{pre-op}}\) ≤ 4 ng/ml mean MVD=142  
4.1-10.0ng/ml mean MVD=103  
10.1-20.0ng/ml mean MVD=137  
> 20.1 ng/ml mean MVD=140  | 0.031    |
| [348]     | 126| CD31  | 1-45 low    | PSA ≤ 10  
low MVD 52%, high MVD 48%  
PSA ≥ 10  
Low MVD 25%, high MVD 75% | <0.01    |
|           |    |       | 46-90 moderate|                                     |          |
|           |    |       | 91-131 high |                                     |          |
| [355]     | 60 | CD31  | Cont        | Low PSA, mean MVD = 31 ± 17  
High PSA, mean MVD = 21 ± 11 | 0.02     |
Current literature provides contradictory reports on association of MVD with prostate cancer invasion into the surrounding tissues. Results of study by Offersen et al. indicate that increased MVD is associated (almost significantly) with detection of extraprostatic extension (EPE) [322]. However, this observation was not supported by the findings from study by Rubin et al. who observed almost significant negative association between EPE and MVD [344] (Table 2.6). Study completed by De la Taille et al. did not observe any correlation between MVD and positive surgical margins but it reported on a positive correlation between presence of local invasions into seminal vesicles (SV) and increased MVD [321] (Table 2.6). The correlation between SV invasion and MVD was not observed in two other studies [344, 353]. The inconsistency of the results among individual studies may result form many factors including the limited size and statistical power of most studies, varying techniques among investigators, and different populations from which tumor samples are obtained. More work, using precisely defined techniques, is required to provide answers to these critical questions.

2.11 MVD as a predictor of metastasis

Lastly, relationship between distal metastasis and MVD was evaluated. Current research indicated that increased number of blood vessels was positively correlated with presence of metastasis in lymph nodes and distal sites [315, 322, 324, 349] (Table 2.6). This supports the idea that neovascularization is essential not only to allow tumor growth but also to promote tumor metastasis through the lymphatics or blood.
<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Stain</th>
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<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[322]</td>
<td>51</td>
<td>CD34</td>
<td>Cont</td>
<td>EPE0 with MVD mean = 57</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EPE+ with MVD mean = 71</td>
<td></td>
</tr>
<tr>
<td>[344]</td>
<td>100</td>
<td>CD31</td>
<td>90</td>
<td>EPE0 high MVD in 53%</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EPE+ high MVD in 32%</td>
<td></td>
</tr>
<tr>
<td>[321]</td>
<td>102</td>
<td>CD31,</td>
<td>85</td>
<td>24 % SV+, with MVD ≥ 85</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5% SV+, with MVD &lt; 85</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Stain</th>
<th>Cut off</th>
<th>Metastasis and MVD</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>[324]</td>
<td>41</td>
<td>FVIII</td>
<td>Cont</td>
<td>pN0 mean MVD = 94.1 ± 41.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pN+ mean MVD = 115.4 ± 24.5</td>
<td></td>
</tr>
<tr>
<td>[349]</td>
<td>80</td>
<td>CD31</td>
<td>Cont</td>
<td>pN0 mean MVD = 26.90</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pN+ mean MVD = 44.26</td>
<td></td>
</tr>
<tr>
<td>[322]</td>
<td>51</td>
<td>CD34</td>
<td>Cont</td>
<td>M0 mean MVD = 56</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M1 mean MVD = 71</td>
<td></td>
</tr>
<tr>
<td>[315]</td>
<td>74</td>
<td>FVIII</td>
<td>Cont</td>
<td>M0 mean MVD = 50.7 ± 20.8</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M1 mean MVD = 104.7 ± 43.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6: Relationship between microvessel density and prostate cancer invasion and metastasis. SV+ - tumor invaded into seminal vesicles, SV0 – no invasion into seminal vesicles; EPE+ – extraprostatic extension of the tumor, EPE0 – no extraprostatic extension; pN+ - positive lymph nodes, pN0 negative lymph nodes; M0 – no metastasis, M1 – metastasis detected.
2.12 MVD measured in biopsy vs. prostatectomy specimen

Information provided by MVD would be particularly valuable if it could be obtained at the time of biopsy and prior to surgical resection. Usually, biopsy samples are taken from patients who present with an abnormal digital rectal exam (DRE) and abnormal PSA level. If MVD measurements derived from biopsy samples were predictive of disease progression, evaluating MVD at this early stage of the disease would aid in selection of the most appropriate therapy. Another issue is whether MVD evaluated in biopsy sample is representative of the vascularization in other areas of the tumor as a whole.

Thus far, there have been only a few studies examining the predictive value of biopsy samples. Two studies failed to observe any correlation between MVD evaluated in biopsies and grade, stage or patient survival [343, 346]. Contrary to the above results, Hall et al. showed that MVD measured in biopsies was significantly associated with stage, grade, and biochemical failure [340]. Interestingly, MVD measured in biopsy samples was not only related to tumor stage and grade but also was associated with the blood flow measured by Doppler ultrasonography [351]. Furthermore, MVD measured in repeated biopsies was significantly correlated with disease progression [346].

There are a few studies assessing reliability of biopsy samples in comparison to the radical prostatectomy measurements. These data reveal that MVD measurements...
performed in biopsies reflected those made in radical prostatectomy specimens and were equally predictive of stage, Gleason grade, disease behavior [323, 325, 352, 362], extraprostatic extension and PSA failures [325] as prostatectomy samples. The number of studies examining the predictive value of the biopsies is still limited, as are the number of cases in each study. Thus there is a need for a larger more comprehensive trial that will definitively answer the question whether MVD measured in biopsies provides reliable and useful predictive prognostic information.

2.13 MVD correlates with increased perfusion of the tumor tissue

Although microvessel density is a surrogate measure of angiogenesis, there are questions as to whether it reflects the actual functionality of the vessels - that is whether it provides information on tumor perfusion. Tissue perfusion can be assessed by power/color Doppler ultrasonography and contrast enhanced magnetic resonance (MRI). There are a few studies examining correlation between tissue perfusion detected by power/color Doppler ultrasonography and MVD evaluated in histological samples from transurethral resection of the prostate (TURP) or radical prostatectomy [351, 356, 363]. Results of this research indicate that increased pixel density of Doppler ultrasonography corresponds to increased microvessel density detected in prostatectomy or biopsy specimens. Dynamic contrast-enhanced MRI measurements of prostate tumor established that tissue specific transport parameter ($k_{21}$) was in direct relation to the number of vessels and to the percent of the area composed by vessels [330, 364]. This indicates that MVD is a valid method of assessing functional prostate tumor
angiogenesis, which reflects increased tissue perfusion and higher vascular permeability within the tumor mass.

2.14 MVD correlates with expression of biomarkers of angiogenesis and inflammation

Increased neuvascularization of the tumor tissue is associated with molecular changes, which support more angiogenic profile of the prostatic tissue such as presence of neuroendocrine cells (NEs), glomeruloid microvascular proliferation (GMPs) and increased VEGF expression.

NEs are a population of cells derived from the neural crest normally present in the glandular epithelium of the prostate. They release a variety of endocrine and paracrine factors thus may regulate the function of the gland. Studies revealed that some populations of NEs are able to express PSA, which suggests that they were derived from re-differentiated epithelial cells in the prostate [365]. NEs are often found in primary prostatic adenocarcinomas and are associated with progression of prostate cancer to androgen independence [365]. NEs do not proliferate themselves but secrete numerous growth factors, mitogenic hormones and pro-angiogenic factors such as VEGF [358]. Thus NEs’ secretions lead to increased proliferation of surrounding cancer cells and promote formation of new blood vessels. Research by Borre et al. indicates that increased MVD is correlated with presence of NEs and higher expression of secreted
VEGF [358]. Correlation between the presence of NEs and increased MVD supports their relationship to the disease progression.

Increased number of microvessels is also associated with expression and activation of numerous pro-angiogenic factors. One of the most potent activators of neovascularization is VEGF, which promotes endothelial cell proliferation and increases permeability of the blood vessels [309, 366]. Secretion of VEGF is essential for formation of new blood vessels. Subpopulations of tumor cells, such as previously mentioned NEs, are able to secrete VEGF (among other growth factors) which promotes tumor growth in an anutocrine and/or paracrine fashion. As expected, a strong correlation between VEGF secretion and high MVD was observed in a number of studies [356, 358, 367].

Glomeruloid Microvascular Proliferation (GMP) is focal proliferative budding of epithelial cells which resemble renal glomerulus [309, 366]. Initially, this structure was observed in glioblastomas and was associated with increased aggressiveness of the tumor [368]. Sunberg and Nagy demonstrated that local expression of VEGF promotes formation of GMP structures in nude mice [368, 369]. Straume et al. were able to demonstrate that GMPs can be found in prostate, breast and endometrial tumors [368]. Presence of this early angiogenic structure was also associated with increased number of blood vessels in the tumor tissue and more aggressive angiogenic signature [368]. This supports the theory that GMPs are precursor of new blood vessels and their presence signals increased angiogenesis in the tumor tissue.
Platelet-derived endothelial cell growth factor (PD-ECGF), also known as thymidine phosphorylase (TP), is another potent angiogenic factor often found in prostatic tumor. TP/PD-ECGF was found to promote chemotaxis and proliferation of endothelial cells [354]. Recent studies revealed a positive correlation between higher MVD and increased expression of TP/PD-ECGF [354, 360, 370]. Moreover, the ability of TP to promote vascularity may contribute to more invasive character and faster progression of prostate cancer [354, 370].

Thrombospondin-1 (TSP-1), a matrix adhesion glycoprotein, is a well known inhibitor of angiogenesis and metastasis [31]. Based on the functions of TSP-1 a negative correlation is expected between MVD and TSP-1 expression. Mehta et al. observed that higher MVD was associated with decreased expression of TSP-1 and increased levels of mutated p53 [371]. These results and previous studies suggest that TSP-1 is regulated by p53, thus mutation in p53 prevents up-regulation of TSP-1 and creates pro-angiogenic environment [31]. Similar investigations performed by Grossfeld et al. showed there was no significant association between TSP-1 expression and MVD [353]. Further research is needed to elucidate the involvement of tumor suppressor genes in the process of tumor angiogenesis and conclusively establish whether correlation with MVD observed by Mehta et al. was real or coincidental.

Episialin (also called MUC1) is a glycoprotein normally expressed in the apical membrane of the glandular epithelium but during carcinogenesis its expression is
depolarized and MUC1 can be found throughout the entire cytoplasm of the epithelial cells [355]. Some studies report that higher levels and depolarized expression of MUC-1 are associated with simultaneous secretion of variety of angiogenic factors [372] and more aggressive disease [373]. Expression of MUC-1 is also related with increased microvessel count (P = 0.02) [355]. This suggests that MUC-1 may assist in regulation and promotion of carcinogenesis.

The link between inflammation and prostate cancer progression is supported by the expression patterns of IL-8 [352]. IL-8 is a chemokine associated with increased angiogenesis, tumorigenicity and metastasis in animal models due to its ability to increase expression and secretion of the metaloproteinases [374]. Epidemiological studies show that patients diagnosed with prostate cancer have higher levels of IL-8 than their healthy counterparts [375]. Study by Murphy et al. revealed that cytoplasmic expression of IL-8 correlated with increased blood vessel density and higher Gleason score [352]. These observations suggest that IL-8 may affect cancer progression by promoting inflammation and angiogenesis.

Observations mentioned above imply that increased microvessel density of prostate cancer is associated with presence of angiogenic and inflammatory characteristics of the tumor tissue and increased aggressiveness of the disease.
2. 15 MVD correlates with molecular biomarkers of prostate cancer development

Uncontrolled cell proliferation and decreased cell apoptosis are some of the hallmarks of cancer. Tumor suppressors such as RB, p53 and oncogenes like bcl-2 which regulate those processes are frequently mutated during cancer development and progression [376]. It is yet to be established whether genetic mutations in those genes play a role in the process of neovascularization.

Some studies demonstrate a significant correlation between the presence of mutated p53 and increased vascular density [371], especially in tumors with higher Gleason score [350]. Majority of the studies, though not all [367], report that higher MVD and presence of mutated p53 were also associated with more advanced disease and decreased patient survival [350, 353, 371, 377]. Based on those results it appears that expression of mutated p53 not only provides cancer cells with resistance to apoptosis but also with signals promoting angiogenesis.

Expression of RB and p53 in relation to MVD was examined by Krupski et al. [377]. Results of this study showed that MVD was correlated with higher Gleason score but it did not contribute significantly as a predictive factor when RB and p53 status were considered for disease specific survival [377]. These data suggests that loss of either one of those two tumor suppressors promotes tumor angiogenesis by deregulation of VEGF and TSP-1 expression [31]. When MVD and levels of bcl-2 expression were examined, it was observed that both parameters correlated with increasing tumor stage and disease relapse [348]. Despite the relatively small size of this study, these findings support the role of bcl-2 in promotion of angiogenesis in prostate cancer progression. Further
research is necessary to solidify the information on relationship between tumor suppressors, oncogenes and how specific defects lead to alterations in prostate tumor MVD.

One of the most lethal steps in human prostate carcinogenesis is gaining independence from the androgens [24, 26, 27]. This may occur through a variety of mechanisms [378] including mutations or amplification of androgen receptor [24]. Mydlo et al. examined the hypothesis that reduced expression of a functional androgen receptor would be associated with increased MVD but was unable to find any association between MVD and AR status [350]. Another proto-oncogene associated with poor prognosis is HER-2/neu, whose overexpression is correlated with development of hormone resistance in prostate cancer [379]. Since MVD is associated with more advanced and aggressive prostate cancer, it was anticipated to correlate with overexpression of HER-2/neu. Evaluation of HER-2/neu expression and MVD in tissue samples from radical prostatectomies revealed no relationship between those two parameters [350]. According to Mydlo et al., expression patterns of either AR or HER-2/neu were not associated with either Gleason or score or p53 status [350].

Cyclooxygenase-2 (Cox-2) overexpression is thought to be associated with prostate cancer progression by down-regulating apoptosis, promoting inflammation and tumor angiogenesis thus increasing tumor invasiveness and progression [380]. Previous research suggests that MVD may be correlated with Cox-2 expression. In a study performed by Di Lorenzo et al., higher MVD was associated with increased expression of
Cox-2 in histological samples but the relationship was not statistically significant. Higher MVD and Cox-2 overexpression were associated with Gleason score and predictive of biochemical failure [348]. These results were not supported by findings from a recent study by Mukherjee et al. who observed a negative correlation between IHC staining for Cox-2 and microvessel density and did not notice any correlation between Gleason score and MVD or Cox-2 [343]. Cox-2 expression is notoriously difficult to validate by IHC and seems to be quite sensitive to processing techniques. Thus these contradictory results suggest that more research is needed to clarify the relationship between Cox-2, inflammation and angiogenesis, as well as MVD in prostate cancer.

2.16 MVD and cell proliferation and apoptosis

Newly developed blood vessels supply necessary nutrients and remove metabolites allowing the tumor to proliferate. Without sufficient blood supply tumor are unable to grow beyond a 1 mm in diameter because the number of proliferating cells equals that of cells undergoing apoptosis or necrosis [309]. Previous research reported that increased expression of cyclin D1, which is a cell cycle regulatory protein, was associated with increased MVD and higher expression of Ki-67 (a marker of proliferation) [352]. These findings were not supported by Matsushima et al. who did not observe any correlation between Ki-67, MVD or Gleason score [349]. Apoptotic cells can be detected by transferase-mediated dUTP nick end labeling (TUNEL) assay and their number can be reported as the apoptotic index (AI), i.e., the ratio of TUNEL
positive (apoptotic) cells to the total number of cells in the high power field. Matsushima et al. reported that AI was higher in areas with lower MVD in patients who did not receive treatment [349]. However in tissues collected from patients treated with neoadjuvant hormonal therapy increased AI co-localized with areas of higher vascularization in the tumor tissue [349]. This would indicate that functional blood supply provides access of the treatment agent to the tumor tissue resulting in increased tumor cell death. Some insight into the relationship between proliferation, apoptosis, and angiogenesis can be derived from our laboratory is precise work with rodent prostate tumors [381]

2.17 MVD and genetic damage

Previous studies suggest that genetic instability provides grounds for alterations leading to prostate cancer progression [382]. Flow cytometry is a simple qualitative method to evaluate DNA ploidy. It employs propidium iodine labeled DNA to detect overall DNA content in each cell, and count and separate cells into fractions. Comparative genomic hybridization (CGH) is used to obtain more detailed data on DNA ploidy. It is a more sophisticated method which distinguishes specific locations in each of the chromosomes where aberrant DNA ploidy was detected. In a study by Hall et al., DNA aberrations were measured using flow cytometry correlated with increasing MVD counts. That is, diploid tumors had the lowest MVD and non-diploid tumor cells, which are detected in more advanced cancer, the highest MVD [340]. Using the CGH method Strohmeyer et al. was able to detect specific locations of chromosomal aberrations
associated with MVD. They reported that low MVD was correlated with losses on chromosome 13q, and increased MVD was correlated with losses on chromosomes 4p, 6q, 8p, 8q, 10q and with gains on chromosomes 2q, 7q, and Xq. Evaluation of microsatellite DNA revealed a significant correlation between instability at locus 171 and increased number of MVD [342]. These findings suggest that genetic alterations may affect prostate cancer progression by promoting changes leading to increased tumor neovascularization.

2.18 Effects of chemopreventive agents and therapy on MVD

It is important to determine whether therapeutic or preventive interventions can modify the density of the blood vessels thus affecting tumor progression. Thus far there have been some trials examining the affects of drugs on the number of blood vessels and VEGF expression in the prostate tissue. Keledjian et al. evaluated the long-(6-17 months) and short-term (1 week to 6 months) effects of Terazosin on vascularity in prostate tissue of patients with benign prostate hyperplasia (BPH) [383]. Terazosin is a quinazoline-derived alpha-1 adrenoreceptor antagonist used to relief symptoms of BPH, hypertension and shown to induce apoptosis prostate stroma smooth muscles. Tissue samples of prostate cancer were collected during prostatectomies from patients treated with terazosin and control group consisting of age-matched patients with localized disease. Compared to controls terazosin-treated patients had higher AI but no change in cell proliferation was observed. Terazosin reduced microvessel density regardless of the duration of the treatment which co-localized with increased AI. However, treatment with
terazosin had not evident effects on expression of VEGF expression in prostate tissue. These results suggest that terazosin may be used as a potential anti-angiogenic treatment against advanced prostate cancer.

Three studies [384-386] examined a sample of patients with BPH who were treated with finasteride prior to undergoing prostate surgery. Finasteride is an inhibitor of the $5\alpha$-reductase, an enzyme responsible for conversion of testosterone to its more active derivative dinydrotestosterone (DHT) [387]. Finasteride has been shown to induce atrophy, apoptosis and reduce prostate volume [388], thus it may be suitable for treatment and chemoprevention of prostate cancer [389]. Population of the studies by Hochberg et al. and Pareek et al. consisted of patients scheduled for the surgery (n = 12) treated daily with 5mg finasteride for at least 6 weeks and age-matched controls [384, 385]. The finasteride treated group demonstrated significant decrease in expression of VEGF and reduced MVD in prostate tissue, as compared to the control group [385]. A study by Donohue et al. was a randomized, placebo-controlled trial, that examined the effects of two-week long finasteride treatment on the vascularity of a BPH after a TURP. Consistent with previous results, finasteride treated group had lower MVD than the placebo group, which correlated with reduction of VEGF expression [386]. Results of this research indicate that finasteride reduces expression of VEGF and thus affects the vascularity of the prostate tissue.

Dutasteride is a $5\alpha$-reductase inhibitor reported to be more effective at decreasing DHT formation than finasteride [390]. It was evaluated in a randomized, double-blind
placebo controlled trial [391], in which patients scheduled for radical prostatectomy (Gleason 7 or less) were given 10mg of dutasteride or the placebo for 7 days followed by 5mg of the drug or the placebo for another 6 to 10 weeks. Prostate tissue from dutasteride treated patients demonstrated higher apoptotic index and reduction in the number of blood vessels after 45 days of treatment, as compared to the placebo-treated group. There was also a significant trend toward decreasing MVD and increased treatment time [391].

Current number of studies investigating potential effects of drugs on MVD is limited, but stimulating and encouraging. Further research is necessary to evaluate effects of agents commonly used in prostate cancer prevention or treatment and to establish mechanism whereby they affect vascularity of the prostate and prostate cancer.

2.19 MVD as a predictor of biochemical and clinical recurrence

Because an effective and valuable biomarker in diagnostic tissue samples or surgically resected prostates should accurately predict cancer recurrence and patient survival, MVD was tested for its ability to predict those clinical outcomes. Patients may experience either biochemical or clinical prostate cancer recurrence following a radical prostatectomy. Biochemical recurrence is diagnosed when blood levels of PSA, which drop dramatically after radical prostatectomy and/or radiation treatment [392], begin to rise despite the lack of any other measurable disease.
Increasing PSA indicates that cancer has returned either locally or as a distant metastasis although patient is not experiencing any other symptoms. This situation may persist for months or years before cancer can be detected by any other measures [393, 394]. Clinical recurrence signifies that aside from rising PSA the patient also demonstrates other disease symptoms correlating with radiographic measurable disease, such as bone pain and urinary obstructive symptoms [395]. Clinical recurrence can be confirmed with a computer tomography or with a bone scan.

The current literature review reveals that MVD effectively predicts reduced time to biochemical and/or clinical recurrence [321, 326, 336, 337, 340, 341, 348, 353, 359, 396] (Table 2.7). A few of the reviewed studies reported that in multivariate survival analyses increased MVD was an independent predictor of prostate cancer progression [336, 341, 345, 371] (Table 2.7). This suggests that MVD, in association with other predictors of prostate cancer, provides additional information and thus improves accuracy of the diagnosis and treatment selection.
<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Cut off</th>
<th>Risk</th>
<th>95% CI</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>[337]</td>
<td>71</td>
<td>90</td>
<td>RR = 2.55</td>
<td>1.18 – 5.52</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>[336]</td>
<td>149</td>
<td>90</td>
<td>RR = 2.4</td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>[396]</td>
<td>66</td>
<td>Extreme Quintiles</td>
<td>HR = 4.36</td>
<td>1.753 – 10.845</td>
<td>0.0015</td>
</tr>
<tr>
<td>[326]</td>
<td>147</td>
<td>Between Quartiles</td>
<td>RR = 1.07 OMVD</td>
<td>0.77 – 1.48</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RR = 1.13 AWMVD</td>
<td>0.88 – 1.44</td>
<td>0.14</td>
</tr>
<tr>
<td>[353]</td>
<td>85</td>
<td>111.5</td>
<td>↑58%±10.1% for high MVD</td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td>[340]</td>
<td>25</td>
<td>60</td>
<td>86% for high MVD</td>
<td></td>
<td>0.0003</td>
</tr>
<tr>
<td>[359]</td>
<td>104</td>
<td>122</td>
<td>HR = 4.8</td>
<td>1.9 – 12.0</td>
<td>0.0003</td>
</tr>
<tr>
<td>[348]</td>
<td>126</td>
<td>Between Tertials</td>
<td>HR = 1.26</td>
<td>0.6 – 2.6</td>
<td>0.54</td>
</tr>
<tr>
<td>[371]</td>
<td>98</td>
<td>65</td>
<td>High MVD w/ ↑progression risk (adjusted for grade)</td>
<td></td>
<td>0.001</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>High MVD w/ ↑progression (adjusted for stage)</td>
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<td>0.046</td>
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<tr>
<td>[345]</td>
<td>109</td>
<td>127</td>
<td>HR = 3.3</td>
<td>1.7-6.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>[341]</td>
<td>98</td>
<td>40</td>
<td>Low MVD 81.3 % w/o progression</td>
<td></td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High MVD 70.7% w/o progression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[321]</td>
<td>102</td>
<td>85</td>
<td>OR = 2.62</td>
<td>1.20 – 5.67</td>
<td>0.0149</td>
</tr>
</tbody>
</table>

**Table 2.7:** Microvessel density as predictor of disease progression, biochemical and/or clinical recurrence. MVD – microvessel density; OMVD – optimized microvessel density; AWMVD – area weighted microvessel density.
2.20 MVD as a predictor of patient survival

Since angiogenesis promotes growth and progression of cancer, higher MVD may be expected to have negative impact on patients’ prognosis and survival. This hypothesis was examined in a number of studies which revealed that, as expected, increased MVD was inversely related to disease-non-specific survival [322, 336, 345, 371, 397], as well as to disease-specific survival [338, 358, 377, 398] (Table 2.8). Furthermore, two of those studies also established that the MVD was an independent predictor of survival relationship [371, 398]. Research presented above is supporting the hypothesis that MVD would be effective biomarker, and a predictor of disease recurrence and patients’ survival. It also suggests that MVD may facilitate selection and timing of treatment modalities to maximize the effects of therapy and minimize its side effects.
<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Cut off</th>
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<th>95% CI</th>
<th>P-values</th>
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<td>[336]</td>
<td>149</td>
<td>90</td>
<td>Low MVD 51.4% disease-free (at 60 ms)</td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High MVD 71.2% disease-free (at 60 ms)</td>
<td></td>
<td></td>
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<tr>
<td>[338]</td>
<td>221</td>
<td>Cont</td>
<td>RR = 1.03</td>
<td>1.02-1.05</td>
<td>&lt; 0.0001</td>
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<td></td>
<td>221</td>
<td></td>
<td>RR = 3.84</td>
<td>2.17-6.82</td>
<td>&lt; 0.0001</td>
</tr>
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<td>[358]</td>
<td>221</td>
<td>Cont</td>
<td>RR = 1.01</td>
<td>1.01-1.02</td>
<td>0.0004</td>
</tr>
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<td>[377]</td>
<td>42</td>
<td>Extreme quartiles</td>
<td>RR = 1.30</td>
<td>1.02-1.66</td>
<td>0.077</td>
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<td>[398]</td>
<td>98</td>
<td>135</td>
<td>RR = 2.7</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>[371]</td>
<td>98</td>
<td></td>
<td>RR = 3.237</td>
<td>1.435-7.302</td>
<td>0.005</td>
</tr>
<tr>
<td>[397]</td>
<td>79</td>
<td>Tertials</td>
<td>RR = 1.73</td>
<td>1.13-2.65</td>
<td>0.010</td>
</tr>
<tr>
<td>[322]</td>
<td>51</td>
<td>66</td>
<td>Low MVD 30 ± 9 ms</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High MVD 16 ± 7 ms</td>
<td></td>
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**Table 2.8:** Microvessel density as a predictor of patient survival
Measurement of tumor vascularity as a biomarker of angiogenesis may clearly have a role in helping to understand prostate carcinogenesis and as a predictor of clinical outcomes. However, much more work needs to be accomplished to define the optimal approach to measuring angiogenesis in human tissue. A unified and standardized methodology needs to be established for MVD to be recognized as a standard cancer biomarker. Future studies need to examine whether the hotspot method is not only useful because of its simplicity but also its accuracy in assessing tumor vasculature. To eliminate or reduce the impact of examiners’ bias and improve reproducibility, standardized computer software should be developed. The relationship between MVD and other, already established biomarkers of carcinogenesis (p53, Rb, Ki-67, AI, etc.) needs to be reassessed and fully understood. Further studies are necessary to determine whether assessment of MVD in biopsies is as predictive of tumor behavior as measurements made in radical prostatectomy specimens. If biopsy, as opposed to prostatectomy, MVD is equally effective in predicting disease progression and patients’ survival we need to assess if it can be used in determining the diagnosis and treatment selection. Finally, there is a need for larger clinical trials examining the effects of therapy and/or chemoprevention on MVD of the tumor, disease progression and patient prognosis.
2.22 Summary

The literature review presented in this chapter provided a brief overview of the data on prostate cancer development and progression, angiogenesis as an integral part of prostate carcinogenesis, and selected dietary factors and their potential effects on prostate cancer. We have demonstrated that dietary energy restriction may affect proteins in the IGF-I system and thus may affect prostate cancer proliferation and apoptosis in rodent models. Previous animal studies in our laboratory have shown that dietary restriction is correlated with a reduction in MVD in prostate cancer.

Therefore, we wanted to test whether dietary energy restriction mediates its affects through insulin-like growth factor system to affect tumor angiogenesis by altering expression of vascular endothelial growth factor. This hypothesis was tested in a rat and cell culture models as the first study in this thesis. We also chose to evaluate effects of selected nutrients on prostate cancer angiogenesis in human samples. There is need to improve the methods allowing for examination of vasculature in human prostate cancer. We chose to examine vascular architecture (vessel size and shape) in addition to traditional vessel counting. Our laboratory has the good fortune of being a part of the HPFS study and we have access to the HPFS prostate cancer tissue bank. This allowed us to use samples from the tissue bank to assess whether image analyses of tumor vascular architecture and microvessel density is an objective and reliable tool for evaluating prostate cancer angiogenesis. We then examined whether measurements of vascular architecture and microvessel density are predictive of the presence of more
aggressive disease and increased risk of death from prostate cancer in men from the Health Professionals Cohort. Once we validated that analyses of vascular architecture and microvessel density are objective and reliable methods for assessing angiogenesis in prostate cancer, we were able to ask how angiogenesis is affected by dietary habits of men diagnosed with prostate cancer in the Health Professionals Follow-up Study cohort.

2.23 References


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220. Maragoudakis, M.E., M. Sarmonika, and M. Panoutsacopoulou, *Antiangiogenic action of heparin plus cortisone is associated with decreased collagenous protein


CHAPTER 3
INTERRELATIONSHIPS BETWEEN DIETARY ENERGY INTAKE, IGF-I AND VEGF IN RATS WITH ADENOCARCINOMA OF THE PROSTATE

3.1 Abstract

Epidemiological studies suggest that positive energy balance and obesity may promote prostate carcinogenesis. Studies in animal models show that diet restriction or reduction in energy intake from carbohydrates or lipids attenuates tumor growth and reduces tumor vasculature. Calorically restricted diet leads to changes in a number of regulatory hormones, including insulin-like growth factor 1 (IGF-I). We propose that the endocrine changes associated with energy restriction may influence critical processes in the developing tumor, such as angiogenesis, that result in an inhibition of growth. The present study examines changes in IGF-I expression caused by dietary restriction and their association with the expression of vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis. Weanling male Copenhagen rats were randomized into two diet groups: ad libitum or 40% restriction (n=5). After 8 weeks rats were implanted with rat prostate adenocarcinoma cells (AT6.3) which are known to express VEGF. Two weeks later plasma, normal prostate and liver, and prostate tumor samples were collected for analyses. Diet restriction reduced plasma concentrations of IGF-I and
increased IGF-binding protein 3 (IGFBP3). Lower IGF-I concentrations correlated with reduced IGF-I mRNA expression in the liver, the primary organ of IGF-I synthesis. Dietary restriction also affected expression of IGF-I and its receptor in the prostate tumor tissue. In parallel, tumors from diet restricted rats also showed significantly lower VEGF mRNA and protein concentrations in prostate tumors. These findings suggest that IGF-I may be one factor regulating tumor VEGF expression and angiogenesis. An *in vitro* study employing AT6.3 cells assessed the ability of IGF-I to modulate VEGF expression. Indeed, IGF-I induced a dose- and time-dependent increase in the secretion of VEGF. These results suggest that diet restriction may reduce tumor growth, in part, by alterations in the IGF-I axis and its signaling in prostate cancer cells leading to lower expression of angiogenic growth factors such as VEGF.

### 3.2 Introduction

The incidence of prostate cancer differs vastly between countries, which can only partially be explained by differences in screening and detection [1, 2]. In general, high prostate cancer rates are observed in North America, Western Europe, and Northern Europe with intermediate rates observed in Mediterranean nations, and lower rates observed in Africa and many parts of Asia [3-5]. Results of migration studies indicate that as populations move from a low risk area to an area of higher risk the prostate cancer incidence will increase, particularly if migration occurred at a younger age [6, 7]. Ecological studies, known as descriptive epidemiologic investigations, indicate that a “Western” dietary pattern is strongly associated with increased risk of prostate cancer.
However, which components of a "Western" diet, either singly or in combination, are responsible for the change in risk have not been clearly elucidated.

This section of the thesis will focus on one of many hypotheses regarding nutrition and prostate cancer risk. A role for "energy balance" or obesity in prostate carcinogenesis has evolved from a number of studies in rodent models and from human epidemiology. It is known that countries exhibiting the "Western" dietary pattern also have a higher prevalence of obesity [8, 9]. Epidemiological studies suggest that excessive energy intake and associated obesity may increase the overall risk of prostate cancer [10], may be related to a higher stage of prostate cancer at the time of diagnosis [11], and may be associated with a higher risk of recurrence after radical prostatectomy [12]. Although none of these studies are definitive and some studies have not observed these associations [13-15], there are two rodent studies that also support a role for energy intake in prostate cancer. The first study, using the well-differentiated Dunning R3327-H androgen-dependent rat prostate adenocarcinoma, showed that 20 to 40% dietary restriction caused a dose-dependent reduction in tumor growth [16]. The authors also reported that 30% energy restriction by removal of either carbohydrate or fat calories each similarly reduced tumor growth [16]. Another study employed the androgen-NMU carcinogenesis model in rats and showed a significant improvement in survival in rats consuming a 30% restriction of total diet [17]. Together, these studies support the contention that control of obesity by improving the relationship between energy intake and expenditure may reduce prostate cancer risk.
The precise mechanisms whereby dietary energy balance may alter prostate cancer remain to be clearly elucidated. Indeed, it is likely that the beneficial effects may involve many interacting mechanisms that vary during different phases of the life-cycle [13-15, 18-20]. The endocrine system is critical to orchestrating adaptations to variations in dietary intake in order to maximize the performance and health of the host [21]. For example, rodent studies conducted by our group show that dietary energy intake alters concentrations of hormones such as testosterone, prolactin, growth hormone (GH), and insulin-like growth factors (IGFs), all of which can regulate growth and function of normal and malignant prostate cells [16, 22]. Interestingly, recent studies in non-human primates [23] and humans [24-26] placed on energy restricted diets show similar patterns of endocrine changes.

This project focused on IGF-I based upon the accumulating evidence that higher concentrations of biologically active hormones are implicated in the risk of prostate cancer in several large epidemiologic studies [27, 28]. The IGF system includes the two insulin-like growth factors IGF-I and IGF-II, their cell surface receptors (IGF-IR and IGF-IIR), six IGF binding proteins (IGFBP 1 - 6), IGFBP-related proteins (IGFBP-rPs), insulin-receptor-related receptor (IRR), and a group of IGFBP proteases [29, 30]. IGF-I can be synthesized in virtually any tissue but the circulating form of IGF-I is synthesized and secreted by the liver [29-31]. Nearly 90% of IGF-I circulating in plasma is bound to IGFBP3, another protein primarily produced in the liver [30, 31]. In general, the greater the proportion of IGF-I bound to binding proteins, the lower its biological activity [32, 33]. Recent studies revealed that IGF-I can also act in a paracrine and autocrine fashion
within tissues to promote local cell growth, proliferation, and transformation, as well as inhibit cell differentiation and apoptosis [29, 30, 34, 35]. The two IGF-I receptors are present on the cell membrane in variety of tissues and differ in their structure and function. IGF-IR is a tetramer composed of two alpha and two beta subunits with 60% homology to the insulin receptor [33, 36]. Despite this homology, IGF-I has low affinity to insulin receptor and may activate it only in pharmacological concentrations [36]. The majority of IGF-I functions are mediated through the IGF-IR [37]. IGF-IIR is a monomer with an extracellular ligand binding domain which may bind IGF-II or mannose-6-phosphate-containing molecules. IGF-IIR has no tyrosine kinase activity and upon binding IGF-II degrades it through the lysosomal pathway, therefore serving as its antagonist [33].

As noted above, we have reported that restriction of energy intake reduces circulating IGF-I [16, 22]. This has been confirmed in other laboratories [38, 39]. The present study was designed to extend our findings on IGF-I by further elucidating the expression of IGF-I and IGFBP3 in the liver, as well as simultaneously evaluating expression in the normal prostate and a developing prostate adenocarcinoma. These simultaneous measurements provide a more comprehensive understanding of how energy balance may influence the IGF-I axis, both as a hormone and an autocrine/paracrine growth factor.

A second question evaluated by the my study is the relationship of energy restriction to VEGF expression. One mechanism underlying the ability of energy restriction to inhibit tumor growth focuses upon tumor angiogenesis. This hypothesis
emerged from an earlier study from our laboratory that showed a significant change in prostate tumor architecture [40] and biomarker expression [16] within the tumors from rats fed energy restricted diets. We observed that vascularity was dramatically reduced in parallel with increased tumor cell apoptosis and lower tumor grade. Thus, we have proposed that the endocrine changes may influence angiogenesis by altering the expression of key angiogenic growth factors. Angiogenesis is the process of new blood vessel formation, and is one of many essential steps in tumor growth and progression. The new blood vessels provide the tumor with oxygen and essential nutrients, while providing for the removal of toxic metabolites [41], and providing a conduit for local invasion and distant metastasis [42]. Vascular endothelial growth factor (VEGF) is one of the major promoters of angiogenesis in cancers [43] that has been shown to be over-expressed in prostate cancer tissue samples [44-46], and is serving as a target for therapeutic agents designed to reduce VEGF expression or signaling [47-50].

Our next goal was to define an experimental system where we could simultaneously evaluate the IGF-I hormonal axis and the expression of VEGF in prostate cancer. After a review of the options, we chose to employ male Copenhagen rats randomized into groups of *ad libitum* feeding and 40% dietary energy restriction with inoculation of subcutaneously implanted Dunning AT6.3 syngeneic prostate adenocarcinoma cells. One advantage is that the rat model allows us to accurately measure and control food intake, which is more difficult in mice. In addition, we desired a prostate cancer cell line that expressed VEGF and could be examined both *in vivo* and *in vitro*. Finally, we have previously demonstrated that AT6.3 cells are a rat prostate
adenocarcinoma cell line which expresses IGF-I Receptor (IGF-IR) and signaling networks and thus is responsive to IGF-I stimulation [35].

In summary, our goals were to examine the following: (1) the hypothesis that dietary energy restriction affects the IGF-I axis by altering expression of both endocrine and autocrine/paracrine components of the system; (2) the hypothesis that VEGF expression is reduced in prostate tumors from energy restricted rats; (3) the hypothesis that changes in IGF-I expression correlate with altered VEGF expression \textit{in vivo}; and, that (4) IGF-I regulates the expression of IGF-I \textit{in vitro}. The objective of this study was not to examine tumor growth, as this has been accomplished in previous studies [16, 17], but rather to examine tumors very early after inoculation when the size remains modest and the expression of genes is not compromised by necrosis, a common occurrence in rapidly growing transplantable tumors.

3.3 Materials and Methods

Reagents

Recombinant IGF-I was purchased from R &D Systems (Minneapolis, MN) and used to prepare stock solutions of 100 µg/mL. Working concentrations of IGF-I used in experiments ranged from 12.5 -150 ng/ml. Cobalt Chloride (CoCl$_2$) was purchased from Sigma (Saint Luis, MI) and a stock solution of 25mM CoCl$_2$ was prepared and filtered through non-pyrogenic 0.22µm$^2$ filters (Corning Inc, Corning, NY). The working concentration of CoCl$_2$ used to treat cells was 50 µM.
Animals and Housing

Weanling male Copenhagen rats (n=10) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed individually in standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-hour light/dark cycles). After one week of adjustment, the body weight of each animal was recorded and rats were randomized into two diet groups: *ad libitum* feeding (Control) or 40% diet restriction (Restricted) with 5 rats per treatment. Rats in both groups were fed the AIN-76A rodent diet (1.0 g precision pellets) daily. Body weight and food consumption were recorded twice a week. Eight weeks after initiation of the respective diets, rats were subcutaneously inoculated with syngeneic AT6.3 cells (10^6 cells in 0.2 ml of sterile PBS) in both rear flanks. Dietary treatments were continued for two weeks after tumor implantation. Figure 3.1 shows the study design. Calipers were used to document that tumors were successfully inoculated and growing. Two weeks after tumor implantation, rats were killed and tumors, normal prostate and liver samples were resected and portions immediately frozen in liquid N₂ for subsequent RNA and ELISA analysis. Blood samples were collected and processed for analysis of IGF-1, IGFBP3, growth hormone and VEGF.

Prostate adenocarcinoma tumor cell line

An androgen-independent Dunning rat prostate adenocarcinoma cell line, AT6.3, was provided by Dr. John T. Isaacs (Johns Hopkins University, Baltimore, MD). The cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 250 nM/L of Dexamethasone, 2 mM/L L-glutamine,
20,000 IU/L penicillin and 20 mg/L streptomycin, in a 5% CO2 humidified atmosphere at 37°C. Prior to implantation in the rats, the cells were seeded in T-150 culture flasks at 5x10^5 cells per flask, and incubated for 48 hours until they reached 60 - 70% confluency. Cells were then harvested, counted using a hemocytometer, and washed twice with sterile PBS. All cell culture materials were purchased from Gibco BRL Life Technologies (Grand Island, NY).
Figure 3.1: Experimental protocol for the rat study.

Rats were randomized into two groups and fed control or 40% energy restriction diet. At week 8 rats were bilaterally, subcutaneously inoculated with AT6.3 prostate cancer cells (106 cells in 0.2 ml PBS). After 10 weeks rats were killed, blood and tissue samples were collected.
In vitro study of the effects of IGF-I and Hypoxia in AT6.3 cells

For in vitro measurements, AT6.3 cells were seeded and maintained by serial passage. To assess whether IGF-I treatment results in VEGF secretion in a time-dependent manner, cells were exposed to 50ng/ml IGF-I in serum-free media for 12 to 72 hours. In order to evaluate whether IGF-I induces secretion of VEGF in a dose dependent manner, AT6.3 cells were plated in 6-well plates at a density of 2.5 x 10^4 cells per well, incubated overnight and subsequently treated with IGF at concentrations ranging from 12.5 to 150ng/ml. Treated cells were then harvested and counted, while conditioned media cell supernatants were collected from each well for quantitation of secreted VEGF by ELISA. All analyses were repeated in triplicate and all studies repeated 3 times.

After completing the above studies, we were interested to determine how IGF-I may interact with low oxygen to stimulate VEGF expression. We also conducted a study using CoCl_2 exposure, which has emerged as an approach to simulate hypoxic conditions in vitro. Cells were plated at 5 x 10^5 cells/well in 6-well plates and incubated overnight. Subsequently, cells were exposed to IGF-I in serum-free media with or without CoCl_2. After 24 hours of treatment, cells were harvested and counted while conditioned media from each well was used to measure concentrations of VEGF secreted by the cells. Measurements of in vitro secreted VEGF were normalized to the number of cells in each well.
Total RNA extraction, reverse transcription and real time PCR

Total RNA was extracted from rat tissues and harvested cells using the RNeasy Kit (Qiagen, Valencia, CA) following manufacturer’s instructions. RNA concentration and purity was evaluated using a spectrophotometer (Beckman DU 530 Life Science UV/Vis Spectrophotometer, Fullerton, CA). Reverse transcription reactions were performed using 2 µg of total RNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Expression of VEGF, IGF-I, IGF-IR and IGFBP3 in total RNA extracted from tumor and liver tissues as well as treated cells was evaluated by quantitative real time PCR. All reactions were performed on the Applied Biosystems 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). A commercially available TaqMan Gene Expression Assay kit (Applied Biosystems) was used to perform all real time reactions. The kit includes Universal MasterMix and TaqMan Gene Expression Assay containing a set of primers (proprietary of the manufacturer) and a set of probes. Reactions were carried out in total volumes of 25 µl following the manufacturer’s protocol. Briefly, cDNA samples were diluted 10 times with DEPC water (Ambion, Austin, TX), and 2 µl of each diluted cDNA sample were then dispensed in triplicate onto a 96-well real time PCR plate (Applied Biosystems). The reaction mix was prepared by combining Universal Master Mix with TaqMan Gene Expression Assay and DEPC water in appropriate volumes. After mixing, 23 µl of prepared reaction mix were dispensed onto the 96-well plate. For each reaction, the target gene was run along with ribosomal 18s (Applied Biosystems) as an endogenous control for normalization purposes. Gene expression was calculated using $C_T$ values and
expressed as relative values assuming the gene expression in the control group equals 1 (or 100%).

Protein extraction from tissues

In order to extract protein from tissues, frozen tissue samples were pulverized in liquid nitrogen then homogenized in T-PER Tissue Protein Extraction Reagent buffer with the Halt Protease Inhibitor Cocktail Kit from Pierce Biotechnology (Rockford, IL) following manufacturer’s protocol. Protein concentrations for each sample were determined using the DC Protein Assay (BioRad, Hercules, CA) and a spectrophotometer (Beckman).

ELISA analysis of IGF-I, IGFBP3 and GH

Active Rat IGF-I EIA Assay (DSL-10-2900, Diagnostic Systems Laboratories, Webster, TX) was used to assess IGF-I concentration, Active IGFBP3 ELISA Kit (DSL-10-6600) was used to measure the concentrations of IGFBP3, and Active Mouse/Rat GH EIA (DSL-10-72100) assay was used to assess GH concentration in serum samples collected from the rats. The assays were performed according to the manufacturer’s guidelines. Briefly, standard, controls and samples containing rat IGF-I, or IGFBP3, or GH were pipetted into 96-well plates and detected using a horseradish peroxidase (HRP)-linked antibody and a tetramethylbenzidine (TMB) substrate. The addition of an acidic stopping solution terminated the reaction and substrate turnover was measured using a microplate reader with dual absorbance at 450 nm and 570 nm. IGF-I, IGFBP3 and GH concentrations in the unknown samples were calculated based on the standard curves
prepared using standards provided by the manufacturer. ELISA analyses were performed in triplicate.

**VEGF ELISA analyses**

VEGF concentrations were analyzed in tissue, blood samples and in media collected during *in vitro* experiments on AT6.3 cells exposed to IGF-I. Protein was extracted from tumor tissues as described above and VEGF protein concentrations were measured using the Quantikine M Murine VEGF ELISA kit (R&D Systems, Minneapolis, MN). Tissue protein lysates were diluted 50-fold and serum blood samples were diluted 5-fold. Measurements of VEGF concentrations in tissues were normalized to protein concentrations and expressed as pg/µg of extracted protein (pg/ml / µg/ml = pg/µg). *In vitro* measurements were adjusted to the cell number. All measurements were performed in triplicate.

**Statistical analysis**

A two-tailed unpaired t-test was performed to compare rat body and organ weights. To evaluate differences in blood concentrations of IGF-I, IGFBP3, GH, and VEGF between the two treatment groups, a two-tailed unpaired t-test was performed. The expression of IGF-I, IGFR, IGFBP3, and VEGF mRNA, assessed by real time experiments, was statistically analyzed using a two-tailed paired t-test. The tissue concentrations of soluble VEGF protein were analyzed by a two-tailed unpaired t-test. Trend analyses were performed for dose and time response to IGF-I treatment and a P-for trend was reported. VEGF protein measured in the cell culture study was evaluated using
one-way ANOVA followed by Tukey’s post hoc analyses to detect differences between each pair of columns. A minimum of three experiments was performed for each test. Results were considered significant if $P < 0.05$.

3.4 Results

Diet restriction, rat body weight and organ weight

Rats consuming a diet restricted by 40% had a final body weight 27 % lower than the controls fed *ad libitum* ($P < 0.0001$). It is important to note that rats fed the restricted diet increased body weight throughout the 8 week period (+ 47g), although certainly at a much slower rate than the controls that gained 82.7 grams during this period (Figure 3.2). We also analyzed liver and prostate tissue weight in the rats after 8 weeks. Absolute weights of liver and prostate from rats on 40% restriction were significantly lower than organs collected from controls. However, when the mass of each organ was adjusted for the total body weight of the rat, differences in organ weights were no longer significant (Table 3.1), and thus the organs remained in proportion to host weight. As stated in the objectives, this was not a study designed to evaluate tumor weight due to the short time between inoculation and harvest. Our goal was to examine tumor tissue early in the growth phase prior to developing areas of hypoxia and necrosis. Furthermore, the ability to quantitate tumor weight was compromised due to the very invasive behavior of the tumor with extension into subcutaneous space and flank muscle. Thus, estimates of tumor weight are not reported.
IGF-I, IGFBP3 and GH circulating concentrations

We examined the effects of the diet on circulating IGF-I, IGFBP3 and GH. We used ELISA assay to measure circulating concentrations of IGF-I. Diet restricted rats showed 35% lower concentrations of circulating IGF-I than *ad libitum* fed rats (P = 0.0469, Figure 3.3A). We observed a dramatic (more than 7-fold) increase in the level of IGFBP3 in the diet restricted rats as compared to the *ad libitum* fed controls (P = 0.0001, Figure 3.3B). The ratio of IGF-I to IGFBP3 was dramatically higher in rats fed *ad libitum* than in diet restricted controls (P = 0.0255, Figure 3.3C). When GH concentrations were examined, we observed great variability consistent with the known diurnal and pulsatile variation of GH, and no significant differences between the rats in the two groups (Figure 3.3D). Previous studies showing changes in GH following dietary intervention required documentation of changes over multiple blood samples during a 24 hr period [22].
Figure 3.2: Body weight of the rats fed control or 40% restricted diet. Tumor inoculation was performed at 8 weeks. The difference between treatment groups became significant at week 5 (P < 0.001). Values are mean ± SD, n = 5/treatment. Arrows indicate tumor inoculation.
<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>Control</th>
<th>Restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>176.8 ± 5.8</td>
<td>178.6 ± 12.7</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>308.4 ± 21.9</td>
<td>225.3 ± 7.3 *</td>
</tr>
<tr>
<td>Prostate weight (g)</td>
<td>2.6 ± 0.53</td>
<td>1.9 ± 0.41 *</td>
</tr>
<tr>
<td>Prostate weight (mg)/body weight (g)</td>
<td>8.56</td>
<td>8.43</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>7.4 ± 0.8</td>
<td>6.1 ± 0.73 *</td>
</tr>
<tr>
<td>Liver weight (mg)/body weight (g)</td>
<td>24.05</td>
<td>27.07</td>
</tr>
</tbody>
</table>

* Significantly different from control, P < 0.05.

**Table 3.1:** Body weight and tissue weights of rats fed *ad libitum* or restricted diets for 10 weeks. Values are means ± SD, n = 5/treatment.
IGF-I and IGFBP3 mRNA expression in liver

The liver is the major source of circulating IGF-I and IGFBP3. Thus, we examined the effects of dietary treatment on IGF-I and IGFBP3 mRNA expression using quantitative real time PCR technology. We detected a 50% reduction in the concentration of IGF-I mRNA expression in the diet restricted rats as compared to the *ad libitum* fed rats (P = 0.0051, Figure 3.4A). IGFBP3 mRNA expression was also examined but we did not observe a statistically significant difference in mRNA concentration (Figure 3.4B).

IGF- I and IGF-1R mRNA expression in prostate tumor tissue

IGF- I mRNA and IGF- IR mRNA expression in tumor tissue was examined using real time PCR. Dietary restriction reduced the expression of IGF-I in the prostatic tumor by 45% (P = 0.0242, Figure 3.5A). Similarly, when we examined the expression of IGF-IR in the tumor tissue, diet restriction reduced the concentration of IGF- IR mRNA by 40% (P = 0.0083, Figure 3.5B).
Figure 3.3: Effects of dietary restriction on circulating concentrations of IGF-I, IGFBP3 and GH. Dietary restriction lowered plasma concentrations of IGF-I (A); increased plasma concentrations of IGFBP3 (B); and dramatically lowered the ratio of IGF-I to IGFBP3 (C). Diet restriction did not affect serum concentrations of GH (D). Each bar represents mean ± SD n = 5/treatment. * Indicates a significant difference between the restricted and control groups (P < 0.05).
Figure 3.4: Effects of dietary restriction on the hepatic expression of IGF-I and IGBP3. (A) Dietary restriction reduces hepatic IGF-I mRNA expression compared to the ad libitum fed rats (p = 0.0051). (B) Dietary restriction had no statistically significant effect on hepatic expression of IGFBP3 mRNA. Bar graphs represent mean ± SD of three separate real-time PCR experiments per rat.
VEGF expression in tumor tissue

We evaluated the effects of dietary restriction on VEGF mRNA expression and the concentrations of extracted VEGF protein in prostate cancer tissue. Real time PCR analyses showed that diet restriction reduced the VEGF mRNA expression by 30% as compared to the *ad libitum* fed rats (*P* = 0.0176, **Figure 3.6A**). We observed 33% lower VEGF content in tumors from diet restricted rats as compared to *ad libitum* fed rats (*P* = 0.0003, **Figure 3.6B**). Diet restriction had no significant effect on VEGF protein extracted from normal prostate tissue (**Figure 3.7A**). In this study, we observed no significant effect of diet restriction on serum VEGF concentrations (**Figure 3.7B**).
Figure 3.5: Effects of dietary restriction on IGF1 and IGFIR expression in prostate tumors. (A) Dietary restriction lowered expression of IGF-I mRNA in prostate tumor tissue compared to the \textit{ad libitum} fed rats ($p = 0.0242$). (B) Dietary restriction lowered expression of IGF-IR mRNA in prostate cancer tissue ($p = 0.0083$). Each bar graph represents mean $\pm$ SD of three separate quantitative real time PCR experiments per rat.
Figure 3.6: Effects of dietary restriction on VEGF mRNA and protein in prostate tumors. (A) Dietary restriction reduced expression of VEGF mRNA in prostatic tumors as measured by quantitative Real time PCR (p = 0.0176). (B) Dietary restriction reduced concentrations of secreted VEGF protein (sVEGF) in prostatic tumors by 33% (P = 0.0003). Bar graphs represent mean ± SD of three separate replicates per rat (n=5).
Figure 3.7: Dietary restriction had no significant effect on VEGF protein in normal prostate or circulating concentrations of VEGF. (A) Extracted VEGF protein content in the normal prostate. (B) The concentration of circulating VEGF was not altered by diet. Bar graphs represent mean ± SD of three separate replicates per rat.
In vitro study examining the VEGF secretion in response to IGF-I and hypoxia

Based upon the above findings, we proposed that IGF-I may directly influence the regulation of tissue VEGF in prostate cancer cells. Thus, we performed in vitro studies using AT6.3 cells to evaluate whether IGF-I can directly induce expression of VEGF. We exposed the cells to increasing doses of IGF-I (12.5 – 150 ng/ml) in serum-free media for 24 hours and used ELISA to measure concentrations of VEGF in the media. Trend analyses revealed that AT6.3 cells secreted increasing amounts of soluble VEGF protein in response to increasing doses of IGF-I (Figure 3.8; \( P = 0.0008 \)). We also examined whether IGF-I is able to induce VEGF protein release in time-dependent manner and treated the cells with 50 ng/ml IGF-I in serum-free media for 12 – 72 hours. We observed a significant increase in the concentrations of soluble VEGF protein over time, with the highest concentrations measured at 72 hours of treatment. Trend analyses revealed that the observed changes were statistically significant (\( P < 0.0001 \)) and that IGF-I induced VEGF secretion in time-dependent manner (Figure 3.9).

Finally, we used cobalt chloride as a mimetic of hypoxia and examined the interactions with IGF-I on VEGF expression. We simulated hypoxic conditions by treating cells with 50 \( \mu \text{M} \) of CoCl\(_2\) then we added IGF-I at 50 ng/ml in serum-free media for 24 hours, and measured concentrations of VEGF protein secreted by the AT6.3 cells into the media. We observed that IGF-I significantly increased secretion of VEGF compared to cells exposed to the serum-free media alone. In the presence of CoCl\(_2\), a further increase in VEGF secretion was observed in all treated groups. Cells treated with IGF-I secreted similar amounts of VEGF protein as did the cells exposed to complete media in hypoxic conditions (Figure 3.10; ANOVA \( P = 0.0046 \)). The results of those
experiments indicate that IGF-I can directly induce VEGF secretion in a prostatic adenocarcinoma cell line in both normoxic and in hypoxic conditions.

3.5 Discussion

Dietary restriction, growth, and obesity.

Dietary restriction reduced the weights of the rats and slowed down their rate of growth. The rats in the restricted group continued to grow throughout the duration of the entire study which indicates that 40% dietary restriction does not cause profound undernourishment. As has been report over many decades in rodent studies, the diet restricted rats appeared healthy and vigorous [9]. Upon necropsy all organs appeared healthy. Although organ weights were lower in restricted rats compared to controls, there were no differences in the organ weights after adjusting for the weight of the rats. In addition, we observed that rats in the ad libitum group had dramatically enlarged large fat pads than rats in the restricted group. These results indicate that with 40% dietary restriction, lean body mass was retained and the only changes were observed in the amount of fat. Thus, our model of 40% dietary restriction prevents obesity in the laboratory animals.

As previously stated, this study was designed to terminate soon after establishment of tumor growth so as to avoid necrosis that is common using rapidly growing tumor model, which would complicate our interpretation of the data. Thus, tumor growth was limited to a mere two weeks. In addition, the AT6.3 cells implanted subcutaneously proved to be locally very aggressive in vivo. We were able to observe
local invasion of the tumor into the adjacent muscle/bone tissues, as well as extending tentacles into subcutaneous spaces, which effectively prevented us from dissecting the entire tumor tissue specimen without contamination. We harvested only that part of the tumor that we felt was free of normal tissues of analysis. Previous studies form our laboratory have documented the tumor inhibiting effects of total diet restriction or selective removal of carbohydrate calories or fat calories in models of prostate carcinogenesis [16, 17].

Dietary restriction alters IGF-I.

Our study demonstrates that 40% dietary energy restriction reduced circulating concentrations of IGF-I and increased concentrations of IGFBP3. These changes resulted in dramatic reduction of the IGF-I/IGFBP3 ratio which is considered to be an indicator of the free/active IGF-I available to interact with receptors in various tissues. Our results are supported by previous studies which reported that dietary energy restriction is associated with reduced IGF-I concentrations [16, 51, 52]. Furthermore, these findings support findings from epidemiological studies where reduced IGF-I concentrations were associated with lower risk of prostate cancer [27, 28, 34, 53-55].
**Figure 3.8:** IGF-I induces VEGF secretion *in vitro* in AT6.3 prostate adenocarcinoma cells. Concentrations of secreted VEGF were measured in the supernatants after 24 hours of treatment. Trend analyses revealed a significant trend of VEGF secretion in response to increasing doses of IGF-I (P = 0.0008). Bar graph represents mean values ± SD, n = 3/treatment.
**Figure 3.9:** IGF-I induces VEGF protein secretion in a time dependent manner. AT6.3 prostate adenocarcinoma cells were treated with 50ng/ml of IGF-I and concentrations of secreted VEGF were measured in the supernatants after 12 - 72 hours of treatment. The bar graph represents means ± SD, n = 3/treatment. Trend analyses revealed a significant trend towards higher concentrations of VEGF in the media in response increasing incubation time (P < 0.0001). Bars with the same letters are not significantly different from each other.
Figure 3.10: Effects of the interactions of IGF-I and cobalt chloride on the in vitro secretion of VEGF protein by AT6.3 prostate adenocarcinoma cells. The concentration of secreted VEGF was measured in the supernatants after 24 hours of treatment. All columns marked with the same letter are not significantly different from each other (ANOVA analyses P = 0.0046). Graphs represent mean ± SD of three separate experiments.
In this study we also examined IGFBP3, which we had not previously evaluated in rodent studies. The role of IGFBP3 in prostate cancer remains uncertain. First, IGFBP3 is a critical binding protein in the circulation and higher concentrations are thought to reduce the biologically active concentrations of IGF1 [32, 33]. Furthermore, IGFBP3 has been recently shown to induce, in an IGF-I independent manner, pro-apoptotic and anti-proliferative effects in cancer cells [56-58]. There is speculation as to whether IGFBP3 induces those changes through a cell membrane receptor [32, 36, 59]. Research by Lee et al. [58] suggests that IGFBP3 is able to recruit retinoid X receptor alpha from the nucleus, translocate into the mitochondria and initiate apoptotic cascade. Future research is necessary to establish the existence of IGFBP3 receptors and or cofactors and mechanism by which IGFBP3 directly affects cancer cells, independently of its role in binding circulating IGF-I.

Epidemiological data on the relationship between IGFBP3 and prostate cancer has not been thoroughly evaluated. In the study by Chan et al. [34] the relationship between IGFBP3 and risk of prostate cancer was examined in men from the Physicians Health Study. They observed that increased concentrations of IGFBP3 was inversely associated with risk of prostate cancer, after adjusting for the IGF-I concentrations RR = 0.4 95% CI – 0.2 -1.0 between the top and bottom quartiles of IGFBP3, P =0.09)[34]. Later studies from the same group demonstrated that the highest IGFBP3 concentrations were associated with reduced risk of advanced prostatic disease (RR= 0.2, 95%CI = 0.1-0.6 top vs bottom quartiles of IGFBP3) [28]. Similar results were reported in a meta-analysis, where the comparison of cohort trials and case-control studies revealed that higher
IGFBP3 concentrations are associated with a reduced prostate cancer risk (OR=0.88, 95% CI =0.61-1.28) [54]. These beneficial effects of higher concentrations of IGFBP3 were not observed by others [27, 55]. For example, a study using the HPFS cohort reported that increased concentrations of IGFBP3, adjusted for IGF-I, were correlated with a slightly increased risk of prostate cancer (OR = 1.40, 95% CI = 0.80-2.44, P-trend = 0.56). In the study nested within the Northern Sweden Health and Disease Cohort Study, prostate cancer risk was not associated with IGFBP3 concentrations (P-trend = 0.44) [55]. Further research is necessary to establish whether circulating IGFBP3 concentrations are related to risk of prostate cancer.

Some studies suggest that risk of cancer is best assessed by considering the free IGF-I, whose concentrations are best determined by calculating the ratio of circulating IGF-I to IGFBP3 [28]. The relationship of the IGF-I / IGFBP3 ratio on risk of prostate cancer was evaluated in several studies [28]. One striking example reported that men with the highest quartiles of IGF-I and the lowest quartiles of IGFBP3 had 9.5 times higher risk of developing prostate cancer than those with low IGF-I and high IGFBP3 concentrations [28]. Our findings support this observation since we find that diet restriction dramatically reduced the ratio of IGF-I to IGFBP3, which in turn, is correlated with a reduction in prostate cancer incidence and progression. It is important to note that few studies have looked at this ratio in controlled dietary animal studies. For example, the study by Goya et al. [52] reported that 65 % (under-nutrition) caloric restriction of female rats, resulted in a significant reduction in circulating concentrations of both IGF-I by 50% (P < 0.05) and IGFBP3 by approximately 80% (P < 0.05). Another study of a
carcinogen-induced model of mammary carcinogenesis with 40% dietary energy restriction again observed reduction in circulating concentrations of IGF-I (P<0.01) and IGFBP3 (P < 0.01) [51]. Both studies reported a significant reduction rats body weight and in the IGF-I concentrations, which was also observed in this study. However, contrary to our results, they also detected reduction in the IGFBP-3 concentrations. Further studies and the use of optimized methodology will help evaluate effects of dietary energy restriction on concentrations of those two proteins.

We did not observe a significant change in the circulating concentrations of growth hormone in this experiment. Growth hormone concentrations are pulsatile and vary dramatically depending on the time of day. It ranges from low values observed during the fed and awake state, to a rapid release of this hormone resulting in high concentrations observed in fasted and sleeping state [60]. Our study was not designed to optimize measurements of growth hormone. Thus, due to the time of the rat necropsy, we were not able to assess the status of GH accurately. However, in a variety of animal models, diet restriction ranging from 20 – 66% results in a reduction of circulating GH [22, 51, 61].

Dietary restriction hepatic IGF-I / IGFBP3 gene expression

Both IGFBP3 and IGF-I can be synthesized in variety of tissues, although their circulating forms are predominantly derived from the liver [30]. As observed in several previous publications, hepatic expression IGF-I mRNA was decreased in response to 40 % dietary restriction [16, 51, 52]. However, the expression of hepatic IGFBP3 was not
altered. Similarly, a study by Takenaka et al. reported that circulating concentrations of IGFBP3 in male rats were reduced in response to protein restriction while the concentrations of hepatic IGFBP3 expression were unaffected [62]. A more recent study examining the effects of under-nutrition on hormonal homeostasis found a similar response in female Wistar rats [52]. This may suggest that hepatic regulation of IGF-I expression is the critical regulatory adaptive step in responding to changes in energy balance. However, our data is consistent with the hypothesis that changes in circulating IGFBP3 may be due to other tissues increasing expression and secretion [31]. Alternatively, the increased plasma concentrations of IGFBP3 may be a result of reduced clearance of the protein from the circulation by hepatic or peripheral tissues. A study by Arany et al. in a rat model demonstrated that if IGFBP3 is not complexed with acid-labile subunit protein, it is very rapidly removed from the circulation by organs such as liver, kidneys and stomach [63].

Diet restriction and the autocrine and paracrine IGF-I network in the normal and malignant prostate.

It has been well documented that the IGF-I system promotes prostate carcinogenesis by increasing proliferation and providing survival signals, as well as assisting in the progression towards androgen independent disease [64-68]. A recent study suggested that IGF-I can promote VEGF expression independent of hypoxia, thus supporting the process of tumor angiogenesis [69]. In our model of prostate cancer we observed a reduction in the expression of IGF-I and IGF-IR mRNAs in the tumor tissue in response to diet restriction. This would suggest that diet restriction is able to affect a
tumor’s autocrine and/or paracrine growth factor signaling. Previous studies suggest that inhibition or down-regulation of IGF-I signaling through its IGF-IR receptor results in increased apoptosis and reduced proliferation [35]. Our data suggest that diet restriction may affect tumor progression by potentially affecting tumor cell proliferation, apoptosis and angiogenesis.

**Diet restriction and VEGF expression**

Previous research suggests that diet restriction may affect angiogenesis through altering the expression of VEGF and FLK-1, which is one of the VEGF receptors [70-72]. VEGF is one of the most potent angiogenesis promoters and its expression has been shown in variety of tumors including prostate [44]. In accordance with those studies, we observed down-regulation of VEGF mRNA and protein secretion in tumor tissues in response to dietary treatment. Interestingly, we did not observe any changes in the vasculature of the implanted tumors, which may be due to the model we used. AT6.3 cells are rat prostate adenocarcinoma cells which upon implantation formed very invasive and locally aggressive tumors. This model gives us an opportunity to examine an IGF-I responsive cell line both in the *in vitro* and *in vivo*. The effects of the diet were observed in the tumor tissue and we did not observe any changes in gene expression the normal prostate or circulating concentrations of VEGF protein. This indicates that modest dietary restriction may affect tumor angiogenesis without having any detrimental effects on the normal prostate.
**In vitro studies of IGF-I and VEGF expression**

The results of the *in vivo* study suggested that diet restriction mediates its effects through altering IGF-I signaling which alters prostate angiogenesis by reducing VEGF expression. We performed *in vitro* studies using the AT6.3 cell line to examine the direct effects of IGF-I on the expression of this angiogenic promoter. Our results indicate that IGF-I was able to induce VEGF secretion in dose- and time-dependent manners in this prostate cancer cell line. Our finding is supported by a study done in an *in vitro* model of human pancreatic cancer where IGF-I promoted the expression of VEGF. Furthermore, an *in vivo* inhibition of IGF-IR signaling in a mouse orthotopic model of pancreatic cancer resulted in a reduction of VEGF expression and decreased angiogenesis [69]. Together these results suggest that IGF-I signaling not only promotes proliferation and attenuates apoptosis, but also promotes angiogenesis through upregulation of VEGF expression. Expression of VEGF was further enhanced by hypoxic conditions simulated by the addition of cobalt chloride. Cobalt ions simulate hypoxic conditions by binding to the hypoxia inducible factor 1α (Hif-1α) within its degradation domain. This binding prevents Hif-1α interaction with von Hippel-Lindau (VHL) protein and ubiquitination of Hif-1α, which results in increased transcriptional activation of variety of genes normally regulated by the Hif complex in hypoxic conditions [73]. Cobalt ions are very effective at mimicking hypoxia in both *in vivo* and *in vitro* systems. This method avoids potential problems associated with use of a hypoxic chamber such as the effects of excessive pressure and leaks in the seal. However, since cobalt only interacts with Hif-1α, it will not cause a hypoxic response in Hif-1 degradation independent systems and thus may not be appropriate when examining expression of other proteins [74, 75]. These *in vitro*
findings agree with our *in vivo* observations that IGF-I may induce VEGF expression in the prostate tumor tissue.

In summary, our results suggest that 40% diet restriction reduces circulating concentrations of IGF-I and IGFBP3 and lowers expression of IGF-I and IGF-IR in the tumor tissue, which in turn is associated with the IGF-dependent expression of VEGF and attenuate tumor angiogenesis. Our findings are consistent with epidemiological observations suggesting correlation between the increased risk of prostate cancer and excessive energy consumption and associated obesity. Also, dietary down-regulation of IGF-I is supported by observational studies, which suggest a direct relationship between increased levels of IGF-I and risk of prostate cancer. Finally, the results of our *in vitro* studies demonstrate that IGF-I is able to directly induce VEGF expression in rat prostate adenocarcinoma.

3.6 References


CHAPTER 4

VASCULAR ARCHITECTURE AS P predictor OF AGGRESSIVE PROSTATE CANCER IN MEN FROM THE HEALTH PROFESSIONALS FOLLOW-UP STUDY COHORT

4.1 Abstract

Prostate cancer is characterized by a wide range of clinical behavior from indolent disease to rapid progression. In addition to traditional prognostic factors such as staging and Gleason grade, novel biomarkers are needed to help define prognosis and provide optimal therapy tailored for each individual. Accumulating evidence suggests that tumor angiogenesis plays a critical role in prostate cancer growth, invasion and metastasis. Currently, there are few objective and qualitative methods for the assessment of angiogenesis that have been validated for use in predictive models of disease outcome. The present study was conducted to assess the relationship of prostate tumor vascular density and architecture to risk of progression.

We examined prostate cancer microvessel density (MVD) and architecture (vessel size and shape) in a cohort of prostatectomy patients nested within the Health
Professionals Follow-up Study (HPFS) of the Harvard School of Public Health. The HPFS is a prospective epidemiologic study of over 50,000 men that began in 1986. For each man reporting a prostate cancer diagnosis and undergoing prostatectomy between 1986 and 2004, we requested archived tissue blocks and obtained adequate tissue specimens from 591 men. Histologic sections were immunostained for two endothelial cell markers, CD34 and Factor VIII. Three non-overlapping 200x fields within the cancer were evaluated for vascular density, vessel size and shape using digital image analysis. Cox regression models were used to assess angiogenesis in relation to grade, stage, and clinical outcomes.

Higher grade tumors (Gleason 7-10 vs. 2-6) showed greater microvessel density, smaller vessel diameter, and more irregular shaped vessels based on Factor VIII and CD34 staining (all P<0.001). Advanced stage at prostatectomy and risk of subsequent death from prostate cancer (mean 7.6 yrs of follow-up) was also associated with greater vascular density, and smaller, more irregular shaped vessels (all P<0.009). Tumors with higher microvessel density, based on quartiles, were associated with a 7-fold greater risk of cancer death. Irregular vessel shape was also a significant predictor of mortality.

Image analysis of CD34 or Factor VIII immunostained prostate cancers specimens provides an objective tool for quantifying angiogenesis. Vascular density as well as vessel size and shape, may serve as valuable biomarkers to distinguish cancer with indolent biology from those with aggressive potential.
4.2 Introduction

Prostate cancer is a very heterogeneous disease and its clinical behavior ranges from indolent disease to rapid progression. PSA testing allows for more frequent detection of localized disease [1], allowing for earlier detection and treatment. However, approximately 20% of patients who receive treatment for localized disease experience recurrence [2-4]. The challenge is to distinguish patients who may need more aggressive treatment because of their quickly progressing disease from those who may not need the same type of treatment because their disease is progressing slower. Currently used predictors of disease progression and recurrence after prostatectomy include involvement of seminal vesicles and regional lymph nodes, positive surgical margins, extraprostatic extension, abnormal PSA level (>4 ng/ml), as well as the two most important factors – TNM tumor staging and Gleason grading system. All of the above factors are very useful predictors of survival in the clinical setting, however accurate assessment of the disease becomes more difficult when two-thirds of patients present with Gleason score 5-7 and PSA 4-10ng/ml [5]. Different patients with the same stage of cancer may not have the same prognosis. Novel biomarkers are needed to improve the ability to distinguish disease with more aggressive biology from the more indolent forms.

A growing amount of data indicates that solid tumors depend on the ability to develop new blood vessels (angiogenesis) to support their growth, invasiveness and to
allow formation of distant metastasis [6, 7]. Angiogenesis can be evaluated using immunohistochemical (IHC) staining to detect endothelial cells, assess microvessel density (MVD), and to visualize vascular architecture. The most common antibodies used to evaluate MVD target CD34, Factor VIII (FVIII), or CD31/PECAM-1 proteins [8]. A classic study by Weidner et al [9] demonstrated that increased MVD was predictive of local invasiveness and formation of distal metastasis in prostate cancer, thus it may be an effective predictor of tumor biology and progression. Currently, there are few objective and quantitative methods for the assessment of angiogenesis that have been validated for use in predictive models of disease outcome.

The goals of the present study were as follows: 1) To establish whether image analysis of CD34 and FVIII stained prostate cancer tissues is an objective and quantitative tool for assessment of vascular architecture in prostate cancer. 2) To confirm that vascular architecture differs between normal and prostate cancer tissue. 3) To assess the relationship between prostate tumor vascular architecture and the risk of more aggressive disease and survivorship, using the population nested in the HPFS cohort.

4.3 Materials and Methods

Study population

The Health Professionals Follow-up Study is an ongoing prospective cohort study of 51,529 male U.S. health professionals who were aged 40-75 at the time of recruitment.
in 1986. All men were asked to fill out a questionnaire soliciting information on age, marital status, height and weight, disease history, medications, smoking, physical activity and diet. Follow-up questionnaires mailed out every two years asked participants if they were diagnosed with a variety of diseases, including prostate cancer during the previous two years. Individuals identified as being diagnosed with prostate cancer through follow-up questionnaires were requested to provide confirmatory medical records. Clinical features such as grade, clinical stage, pathologic stage and lethality are reported. Paraffin-embedded tissue blocks and accompanying pathology reports from the prostatectomies were requested. Upon arrival at OSU, the individual cases were recoded into the OSU computerized database and stored in the tissue bank until analysis. From 1986 until 2002 we have received over 1000 cases and analyzed 591 prostate tissue samples from the HPFS cohort participants who were diagnosed with prostate cancer, underwent prostatectomy and authorized us to use their tissue specimens for our study. Within that subgroup of 591 men 27 men died by the year 2003.

**Antibodies and Reagents**

Rabbit polyclonal antibody against Factor VIII (FVIII) (# N1505) and monoclonal mouse antibody against CD34 (# 236) used for IHC were purchased from Dako DakoCytomation Carpinteria, CA. Other reagents used for IHC staining include DakoCytomation EnVision + Dual Link System Peroxidase (cat # K4061), DakoCytomation Dual Endogenous Enzyme Block (S2003), DakoCytomation Liquid 3,3’-diaminobenzidine (DAB)+ Substrate Chromogen System (K3468), Mayer’s
Hematoxilin Solution (MHS32-1L) from Sigma-Aldrich Inc. (St. Louis, MO), Surgipath Micromount Mounting media from Surgipath Medical Industries, Inc. (Grayslake, IL), Antigen Retrieval Citra Plus Solution and concentrated-Reagent Diluent were purchased from Biogenex (San Ramon, CA). Unless otherwise stated, all reagents were purchased from Dako DakoCytomation, (Carpinteria, CA).

H&E staining

Paraffin-embedded tissue blocks were sectioned and transferred to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Following re-hydration, slides were stained with Hematoxylin and Eosin (H&E) on an automated slide stainer (Leica Microsystems Inc. Deerfield, IL). The slides were removed from the autostainer and coverslipped. The slides were reviewed for grade and stage and high-resolution images recorded for our digital histopathology bank and CD storage.

General immunohistochemical staining procedure

All immunohistochemical staining was performed on an OptiMax Automated Cell Staining System (BioGenex Laboratories, San Ramon, CA), which allows for staining of up to 40 slides simultaneously under precisely controlled conditions, thus eliminating a significant amount of variation associated with staining by hand.

We examined tumor Microvessel Density (MVD) with antibodies directed against CD43 and Factor VIII, which are early and late markers of angiogenesis respectively. Briefly, sections were deparaffinized, rehydrated and washed prior to staining. Sections
stained with FVIII were pre-treated with heated citrate buffer for antigen retrieval. To eliminate nonspecific binding, sections were treated with peroxide for 5 minutes. Subsequently, sections were exposed to primary antibodies directed against either FVIII (1:2000) or CD34 (1:200) for 30 minutes (DakoCytomation) followed by a peroxidase labeled polymer conjugated to goat anti-rabbit or anti-mouse immunoglobulins (DakoCytomation). To visualize CD34/FVIII staining, sections were treated with DAB chromogen solution (DakoCytomation) and counterstained with hematoxylin (Sigma-Aldrich Inc). Slides/sections were then dehydrated and coverslipped. During the staining process, sections were washed with phosphate buffered saline (PBS) three times before and after each of staining step. Unless otherwise stated, all incubations were done at room temperature. All sections were counterstained with hematoxylin, dehydrated and mounted.

Digital imaging and analysis

All sections were examined by a pathologist, under light microscopy, for characterization of tumor morphology and cytological features. Tumor areas were identified and outlined in up to three representative sections per case. The outlined tumor areas were visualized under bright field microscopy using a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY). Representative images of three non-necrotic and non-overlapping high power fields (200x) were captured from each section using a RT Slider Spot Camera (Diagnostic Instruments, Sterling Heights, MI) mounted onto the microscope. Captured images were analyzed using Image ProPlus 4.5 software.
Images were evaluated for the presence of angiogenic markers (CD34 and FVIII) based on the following parameters:

- Blood vessel number,
- Total vessel area (sum of all the vessels, \( \mu m^2 \)),
- Area per vessel (\( \mu m^2 \)),
- Diameter (mean, \( \mu m \)),
- Perimeter (mean, \( \mu m \)),
- Roundness (mean, \( \mu m \)),
- Mean percent area per 2x2 \( \mu m^2 \) per vessel,
- Percent of all vessels per 2x2 \( \mu m^2 \) area,

Branching structures were counted as a single vessel, the lumen did not have to be noticeable for a structure to be considered a vessel and red blood cells were not used to categorize a structure as a blood vessel. Slides were evaluated without knowledge of the Gleason grade or staging of the tumor.

Comparison of vascular architecture between normal prostate and prostate cancer tissue

Of the 591 prostate tissue samples stained for Factor VIII we selected 100 representative cases with Gleason scores ranging from under 4 to over 8. On each of the prostate tissue sections we located benign stromal and glandular tissues at least 5 mm from the tumor area outlined by the pathologist. We captured images of the normal tissue and analyzed MVD and vascular architecture parameters described above. We then
compared the measurements made in the normal prostate tissue to those made in the corresponding tumor areas.

Statistical analyses

Data from image analyses performed with Image ProPlus were exported into an Excel file and the average for each case was calculated. Other basic statistical analyses were performed allowing us to correlate prostate cancer stage and grade with vascular architecture. All gathered data were sent to the Harvard School of Public Health for further analyses using the HPFS database. Cox regression models adjusted for age and Gleason score were used to assess prostate death in relation to the angiogenesis biomarkers. To compare vasculature in normal prostate and cancer tissue we performed simple two-tailed t-tests on MVD, mean vessel area, total vessel area, % area per vessel, diameter, perimeter and shape.

4.4 Results

Study Cohort

Of the 51,529 men in the HPFS cohort, we obtained tissue samples from 591 men who were diagnosed and underwent radical prostatectomy between 1986 and 2002. In this subpopulation of subjects, the mean age at the time of diagnosis was 62 years (SD ± 6.3 years). 172 (31.7%) men demonstrated advanced prostate cancer (C1/C2/D) and 370 (66.3%) had localized disease (A2/B) at the time of diagnosis. 449 (77.1%) subjects
presented with a lower grade disease with combined a Gleason score of less than 7 and
133 (22.9%) had tumors with a Gleason score of 7 or higher. Of the men who were
diagnosed with prostate cancer and provided tissue samples, 27 (4.6%) died of the
disease through 2003. The mean follow-up of the study cohort was 7.3 years (SD ± 3.1
years) (Table 4.1).
### Characteristics of the Study Population

<table>
<thead>
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<th>Characteristics</th>
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<tbody>
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<td><strong>Tumor Stage, N (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Localized (A/B)</td>
<td>370 (66.3 %)</td>
</tr>
<tr>
<td>Advanced (C1/C2/D)</td>
<td>171 (31.7 %)</td>
</tr>
<tr>
<td><strong>Tumor Grade, N (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Low Grade (&lt; 7)</td>
<td>449 (77.1 %)</td>
</tr>
<tr>
<td>High Grade (≥ 7)</td>
<td>133 (22.9 %)</td>
</tr>
<tr>
<td><strong>Prostate Cancer Death, N (%)</strong></td>
<td>27 (4.6 %)</td>
</tr>
<tr>
<td><strong>Age at Diagnosis, Mean (SD)</strong></td>
<td>62 yrs (6.3)</td>
</tr>
<tr>
<td><strong>Follow-up, Mean (SD)</strong></td>
<td>7.3 yrs (3.1)</td>
</tr>
</tbody>
</table>

*Table 4.1: Characteristics of the 591 men from the HPFS Prostate Cancer Cohort who were included in our analyses. The study population was followed from 1986 till 2004.*
Based on FVIII staining, we noticed that normal prostate had a significantly lower MVD than prostate cancer tissues. Specifically, mean MVD in the normal prostate was $11.67 \pm 5.878$ while mean MVD in prostate cancer tissue was $32.25 \pm 17.27$ (P < 0.0001; Figure 4.1A). We evaluated the mean vessel area and observed that normal prostate had significantly larger mean area per vessel ($6546 \mu m^2 \pm 7682 \mu m^2$) than prostate cancer ($1826 \mu m^2 \pm 1348 \mu m^2$, P < 0.0001, Figure 4.1B). Similarly, the mean percent area per vessel in normal prostate was significantly greater ($0.3269 \% \pm 0.2846 \%$) than in prostate cancer ($0.1202 \% \pm 0.0889 \%,$ P < 0.0001; Figure 4.1D). Based on our analyses there was no difference in the total percent area comprised by vessels between normal and prostate cancer tissue ($47,870 \mu m^2 \pm 26697\mu m^2$ and $43,390 \mu m^2 \pm 18338 \mu m^2$, respectively; Figure 4.1C). We observed a difference between the mean vessel diameter and perimeter between normal prostate and cancer tissue. Specifically, normal prostate was characterized by vessels of larger diameter ($75.25 \mu m \pm 31.4 \mu m$) and perimeter ($318 \mu m \pm 144.9 \mu m$) than vessels observed in prostate cancer ($40.57 \mu m \pm 12.03 \mu m$ in diameter and $181.8 \mu m \pm 54.81 \mu m$ in vessel perimeter, P<0.0001 for both parameters measured). A comparison of the two parameters is shown in Figures 4.2A and 4.2B. Lastly, the shape of the vessels was evaluated using a scoring system which assumes that perfectly round vessel is scored as 1 and the more irregular the shape of the vessel the higher the score. The shape of the vessels observed in normal prostate was significantly more regular ($2.335 \pm 0.4668$) than the shape of vessels in prostate cancer ($2.592 \pm 0.4556$, P < 0.0001; Figure 4.2C). Overall, these analyses indicate that prostate cancer
tissue is characterized by significantly higher MVD and that vessels are significantly smaller (diameter, perimeter and area) and more irregular in shape than vessels observed in normal prostate.

**Vascular architecture and advanced prostate cancer**

Based on image analyses of both CD34 and FVIII staining, we were able to identify the characteristics of an angiogenic profile within the tumor tissue. Regardless of the type of stain, more angiogenic tumors had a higher microvessel density (MVD) per high power field. More angiogenic tissues usually contained vessels characterized by a smaller mean area per vessel, smaller diameter and perimeter, as well as a more irregular vessel shape (details for CD34 in Table 4.2, details for FVIII in Table 4.3). Examples of staining for CD34 and FVIII are presented in Figure 4.3A and 4.3B. Measurements for both stains were very comparable. Therefore we report on results for CD34 as representative of both stains. Within the group of subjects diagnosed with localized disease, the mean MVD is $75.1 \pm 3.45$ and while the MVD in advanced disease is $83 \pm 9.0$. There is a trend toward a higher MVD with more advanced disease but it is not statistically significant ($P = 0.068$; Figure 4.4A). The mean area of the vessels is significantly larger in the subjects with localized disease compared to those with advanced disease. The mean vessel area [$\mu m^2$] for localized disease is $557 \mu m^2 \pm 51.5 \mu m^2$ while in advanced disease, the mean vessel area is $450 \mu m^2 \pm 25.5 \mu m^2$ ($P = 0.0017$; Figure 4.4B). Similarly, the diameter of the blood vessels was greater in localized tumors that in those with distant metastasis. The mean vessel diameter for
localized disease is 25.6 μm ± 0.45 μm compared to 23.5 μm ± 1.1 μm for advanced disease (P = 0.0014; Figure 4.4C). The shape of the vessels was also examined in the advanced and localized tumors using a previously defined scoring system. It was observed that higher stage tumors were characterized with more irregularly shaped vessels than lower stage tumors. Localized tumors had more regular vessels, which were on average rated 3.96 ± 0.095, while advanced tumors had vessels of more irregular shape were, on average, rated 4.45 ± 0.25 (P = 0.0002; Figure 4.4D). These results demonstrate that more advanced and faster progressing tumors have a higher number of vessels with smaller vessel area and diameter as well as more irregularly shaped vessels.
Figure 4.1: Comparison of MVD and area of the vasculature between normal prostate and prostate cancer tissue. Individual graphs represent measurements of A) microvessel density, B) mean vessel area, C) total tissue area comprised of vessels, and D) % tissue area per vessel, in normal and cancer prostate tissues. Graphs report mean ± SD values of each measurement. * Indicates statistically significant differences between compared groups (P < 0.0001).
Figure 4.2: Comparison of vessel diameter perimeter and vessel shape between normal prostate and prostate cancer tissue. Individual graphs represent measurements of A) vessel diameter, B) perimeter and C) vessel shape in normal and cancer prostate tissues. Graphs report mean ± SD values of each measurement. * Indicates statistically significant differences between compared groups (P < 0.0001)
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<tr>
<th>Characteristics Measured</th>
<th>Range</th>
<th>More Angiogenic Profile</th>
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<tbody>
<tr>
<td>Microvessel Density</td>
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</tr>
<tr>
<td>Average Vessel Area</td>
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</tr>
<tr>
<td>Diameter of Blood Vessels</td>
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</tr>
<tr>
<td>Perimeter of Blood Vessels</td>
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<td>Shape of the Vessels</td>
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<td>Irregular shape</td>
</tr>
<tr>
<td>Round = 1</td>
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</table>

**Table 4.2:** Measurements of vascular architecture in relation to angiogenic profile based on CD34 staining.

<table>
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<tr>
<th>Characteristics Measured</th>
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<th>More Angiogenic Profile</th>
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<tr>
<td>Microvessel Density</td>
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<tr>
<td>Average Vessel Area</td>
<td>104 – 34,134 µm²</td>
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<td>Diameter of Blood Vessels</td>
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<td>Perimeter of Blood Vessels</td>
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**Table 4.3:** Measurements of vascular architecture in relation to angiogenic profile based on FVIII staining.
Figure 4.3: Representative images of CD34 and FVIII immunohistochemical staining of prostate cancer tissue. A) represents tissue stained against CD34 at a x 200, and B) shows tissue stained against FVIII under x 200.
Vascular architecture in relation to Gleason grade

Within the group of subjects diagnosed with higher Gleason scores (combined Gleason score \( \geq 7 \)) the mean MVD was 97.8 ± 9.1 and for those with lower Gleason scores (combined Gleason score \( \leq 6 \)), it was 71.6 ± 3.1 (\( P < 0.0001 \), Figure 4.4A). There was no significant difference in the mean area per vessel between the groups of subjects with high and low Gleason scores. The mean area per vessel in the higher and lower Gleason score groups were 531 \( \mu \text{m}^2 \pm 52.5 \mu \text{m}^2 \) and 544 \( \mu \text{m}^2 \pm 25.5 \mu \text{m}^2 \), respectively (\( P = 0.68 \), Figure 4.4B). Similarly, there was no difference in the vessel diameter between the subjects with high and low Gleason score. The mean diameter of vessels in the tumors with higher Gleason score was 25.3 \( \mu \text{m} \pm 1.15 \mu \text{m} \) and the mean diameter of vessel in the tumors with lower Gleason score was 25.2 \( \mu \text{m} \pm 0.5 \mu \text{m} \) (\( P = 0.85 \), Figure 4.4C). Finally, the shape of the vessels was rated and the difference between the high and low Gleason groups was not statistically significant. On average, the shape of the vessels in the advanced Gleason group was scored as 3.86 ± 0.23 while in the low Gleason group the shape of the vessels was rated as 4.07 ± 0.095 (\( P = 0.069 \), Figure 4.4D). The evaluation of vascular architecture summarized above indicates that high grade tumors differ from low grade tumors only by the number of vessels and that the size and shape of vessels is not associated with higher grade.

Correlations between vascular architecture and MVD based on CD34 staining

Based on CD34 staining, which allows for detection of more immature vessels, we observed that tumors with higher microvessel density had vessels of smaller diameter,
perimeter and average area per vessel. There was an association between small vessel area and increased irregularity of the vessels. However, the vessels with smaller perimeters usually demonstrated a more regular shape. As anticipated, there was a strong correlation between vessel diameter, perimeter and mean vessel area. Age adjusted correlation coefficients are shown in Table 4.4, and all associations were statistically significant (P < 0.05) with the exception of the lack of correlation between vessel shape (roundness) and vessel number.

Relative risk of prostate cancer death associated with microvessel density and architecture

Cox regression models were used to assess the relative risk of prostate cancer death in relation to biomarkers of angiogenesis. Correlation analyses were adjusted for age at diagnosis and Gleason score since both of those factors are very strong predictors of prostate cancer survivorship. Based on quartiles, microvessel density alone was not an important predictor of prostate cancer death (RR = 0.9, 95% CI = 0.3-3.3, P_{trend} = 0.65, Table 4.5). Vessel area was significantly and inversely correlated with increased risk of death (P_{trend} = 0.011). Subjects who had the smallest vessels in tumor tissues (the fourth quartile) had 6.9 times higher relative risk of dying from prostate cancer than those with the largest vessels (RR=6.9, 95%CI = 0.9-5.3, for highest vs. the lowest quartile). The presence of irregular blood vessels is a strong predictor of prostate cancer risk. Subjects with the most irregularly shaped vessels in the tumor tissue (fourth quartile vs. first quartile) had 8 times higher relative risk of prostate cancer death, as compared to those
with more regular shaped vessels (RR = 8.0, 95%CI = 1.8-35, \(P_{\text{trend}} = 0.0027\)). Vessel diameter was an almost significant predictor of prostate cancer-related death. Patients whose tissue was characterized by the smallest diameter (top quartile) had 6 times higher relative risk of dying from prostate cancer than men with vessels of the largest diameter (RR = 6.0, 95%CI = 0.8-47, \(P_{\text{trend}} = 0.055\))
Figure 4.4: MVD and measurements of vascular architecture correlate with tumor stage and Gleason Score based on CD34 staining. Individual graphs represent the correlation between A) microvessel density, B) mean vessel area, C) mean vessel diameter and D) vessel shape and tumor stage and grade. Graphs report mean ± SD values of each measurement. * Indicates statistically significant differences between compared groups (P < 0.05)
<table>
<thead>
<tr>
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<th>Diameter</th>
<th>Perimeter</th>
<th>Shape</th>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Area</td>
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<tr>
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<td>-0.26</td>
<td>+0.20</td>
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Table 4.4: Age-adjusted correlation coefficients between MVD and vascular architecture based on CD34 staining. All correlations are statistically significant (P < 0.05) except for the correlation between number of vessels and vessel shape, marked by #.
<table>
<thead>
<tr>
<th>Quartiles</th>
<th>Deaths, N</th>
<th>Relative Risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microvessel Density</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4 – Higher</td>
<td>6</td>
<td>0.9 (0.3 – 3.0)</td>
</tr>
<tr>
<td>Q3</td>
<td>11</td>
<td>1.9 (0.7 – 5.2)</td>
</tr>
<tr>
<td>Q2</td>
<td>4</td>
<td>0.5 (0.1 – 2.0)</td>
</tr>
<tr>
<td>Q1 - Lower</td>
<td>6</td>
<td>Reference</td>
</tr>
<tr>
<td><strong>P for trend</strong></td>
<td></td>
<td><strong>0.65</strong></td>
</tr>
<tr>
<td><strong>Vessel area</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4 – Smaller</td>
<td>15</td>
<td>6.9 (0.9 – 53)</td>
</tr>
<tr>
<td>Q3</td>
<td>7</td>
<td>4.5 (0.5 – 36)</td>
</tr>
<tr>
<td>Q2</td>
<td>3</td>
<td>2.2 (0.2 – 21)</td>
</tr>
<tr>
<td>Q1 - Larger</td>
<td>2</td>
<td>Reference</td>
</tr>
<tr>
<td><strong>P for trend</strong></td>
<td></td>
<td><strong>0.011</strong></td>
</tr>
<tr>
<td><strong>Vessel shape</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4 – Irregular</td>
<td>17</td>
<td>8.0 (1.8 – 35)</td>
</tr>
<tr>
<td>Q3</td>
<td>8</td>
<td>5.3 (1.1 – 25)</td>
</tr>
<tr>
<td>Q2</td>
<td>2</td>
<td>Reference</td>
</tr>
<tr>
<td>Q1 – Regular</td>
<td>0</td>
<td>No Deaths</td>
</tr>
<tr>
<td><strong>P for trend</strong></td>
<td></td>
<td><strong>0.0027</strong></td>
</tr>
<tr>
<td><strong>Vessel diameter</strong></td>
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<td></td>
</tr>
<tr>
<td>Q4 – Smaller</td>
<td>13</td>
<td>6.0 (0.8 – 47)</td>
</tr>
<tr>
<td>Q3</td>
<td>7</td>
<td>4.0 (0.5 – 32)</td>
</tr>
<tr>
<td>Q2</td>
<td>5</td>
<td>3.7 (0.4 – 32)</td>
</tr>
<tr>
<td>Q1 – Larger</td>
<td>2</td>
<td>Reference</td>
</tr>
<tr>
<td><strong>P for trend</strong></td>
<td></td>
<td><strong>0.055</strong></td>
</tr>
</tbody>
</table>

**Table 4.5:** Relative risk of prostate cancer death associated with angiogenic biomarkers. All correlations were adjusted for Gleason score and age at diagnosis.
4.5 Discussion

Our study examined 591 cases of prostate cancer and currently represents the largest study evaluating vasculature in prostate cancer. We extended the scope of our analyses to two different immunohistochemical stains and measured not only microvessel density, commonly measured in previous studies, but also examined other parameters of vascular architecture such as vessel area, diameter, perimeter and shape.

To the best of our knowledge, previous reports examining the vasculature in prostate tissue focused mostly on prostate cancer not on normal tissue and measured solely microvessel density [10-14]. Thus this is the first study looking at vascular architecture in normal and malignant prostate tissue. We noticed that normal prostate tissue is characterized by lower MVD, having vessels of larger area, diameter and perimeter, as well as more regular shape than vessels observed in malignant prostate. These observations confirm that blood vessels in normal tissues have more regular vasculature than tumors [15]. These observations also support the idea that growing prostate tumors form numerous small and irregular vessels resulting in meandering, sometimes not fully functional, vasculature [16, 17]. These results also demonstrate that vascular architecture analyses are able to detect differences between malignant and normal prostate tissue. These observations encouraged our further investigations into the relationship between vascular architecture and tumor stage and grade.
As anticipated and based on previous research [9, 11, 18-27], we detected a strong correlation between the higher MVD and increased prostate cancer Gleason grade. Interestingly, the relationship between MVD and prostate cancer grade was not recorded in all previous reports [14, 28-35]. Furthermore, in contrast to many previous reports, we were not able to detect a significant association between advanced prostate cancer and MVD [10, 11, 18, 19, 22, 25, 26, 36-39]. Our results imply that MVD is related to the histological differentiation of prostate tumor tissue rather than advancement of the disease. That is, the more irregular the glandular architecture of the tumor the more dense its vasculature. This theory is supported by results of others who, despite observing a relationship between tumor grade and MVD, were unable to detect association between stage and MVD [28, 30, 32, 34, 35, 40].

Assessment of other parameters of vascular architecture revealed that reduced mean vessel area, smaller diameter and more irregular shape of the vessels are associated with more advanced disease. Age-adjusted analysis of correlations among these parameters confirmed the anticipated relationship between area, diameter and perimeter. It also pointed out that more irregular vessel shape was associated with smaller mean vessel area and diameter, but larger vessel perimeter. Surprisingly, in our study none of those parameters significantly correlated with prostate cancer Gleason score. This implies that histological differentiation of the cancer is not directly related to vascular architecture. Based on our data, we can hypothesize that prostate cancer characterized by smaller vessels (diameter and mean area) and more irregular vessel shape may be
undergoing a more rapid growth and have more aggressive biology [15, 16]. These observations would indicate that evaluation of the vessel architecture, in addition to commonly analyzed MVD, provides information on the aggressiveness of prostate cancer. If angiogenesis is truly more active in aggressive tumors, then it should be associated with higher levels of vascular endothelial growth factor (VEGF). Future studies examining the co-localization of VEGF expression cells and vasculature of prostate cancer will provide more information on the relationship between angiogenesis and prostate cancer progression.

We also examined the relationship between angiogenic biomarkers and prostate cancer survivorship using Cox regression models. Since Gleason grade and age at diagnosis are the most predictive factors of patient survivorship, we adjusted our correlations for both of those factors. Our previous analysis demonstrated that MVD was very closely related to Gleason score therefore, once we adjusted for grade, MVD was no longer a predictor of prostate cancer survivorship. This finding contradicts earlier reports by Lissbrant et al. and Mehta et al. [41, 42], which demonstrated that MVD was correlated with the increased risk of prostate cancer related death [11, 25, 37, 43-45] and other studies which indicate that MVD was an independent predictor of prostate cancer death (RR = 2.7, P = 0.02 and RR = 3.237, 95% CI = 1.13-2.65, P=0.005, respectively).

Finally, we evaluated the prognostic value of other parameters of vascular architecture as predictors of prostate cancer survivorship and observed that men with the
smallest mean vessel area had significantly higher risk of death from prostate cancer. Furthermore, men with the most irregularly shaped vessels had a very significant increased risk of prostate cancer death. These correlations support the previously described relationship between vascular architecture and increased risk of advanced disease. Overall, our analyses indicate that assessment of MVD in combination with other parameters of prostate cancer vascularity provides a useful biomarker for disease progression.

One of the limitations of this study is the lack of information on biochemical and/or clinical recurrence. This is partially due to the fact that this study was initiated in 1984, prior to introduction of prostate specific antigen (PSA) screening, thus information on PSA was not solicited. As a result, HPFS data base contains data only on the men who died of prostate cancer and we do not know how many of the men in HPFS cohort died of other causes after recurrence of prostate cancer. However, numerous previous studies which examined MVD as a risk factor for biochemical recurrence observed an increased risk of recurrence with higher MVD [10, 11, 34, 42]. For example, Silberman et al [34] observed that men with higher MVD (presented as a dichotomized variable) had a hazard ratio (HR) of 3.3 (95% CI = 1.7 – 6.5) of experiencing biochemical or clinical prostate cancer recurrence than men with lower MVD. Based on the correlation between vessel size and shape and more advanced prostate cancer described earlier, we could anticipate observing higher risk of recurrence in men who present with tumors
characterized by vessels of smaller diameter, smaller mean area and more irregular vessel shape.

Overall, this study demonstrates that image analyses of CD34 and FVIII immunostained tissues provide us with an objective and quantitative tool for assessment of prostate cancer vasculature. Furthermore, we were able to use microvessel density, as well as other parameters of vascular architecture as biomarkers, allowing us to identify disease with more aggressive potential. This information can be used in addition to Gleason grade and tumor stage to recognize patients who could benefit from additional treatment from those who may be spared its side effects. Additional studies will determine if treatment with therapeutic, chemopreventive agents or dietary factors have ability to alter prostate cancer vasculature and thus affect patients’ prognosis and survival.

4.6 References


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5.1 Abstract

Prostate cancer is one of the most commonly diagnosed visceral cancers and third most common cause of cancer-related death in American men [1]. There is a dramatic difference between prostate cancer incidence observed in Asian countries and in populations of the Western world (United States or Northwestern Europe) [2]. Western diet usually characterized by consumption of, fat especially from animal sources, dairy and calcium, excessive amounts of calories and lower intake of fruits and vegetables [2, 3].

Numerous studies describe the impact of the diet on the development of prostate cancer and its progression to metastatic disease. However, there are currently no clinical studies evaluating the effects of diet on angiogenesis, which is known to be an essential step in development of metastasis. Therefore, the goal of this study is to evaluate microvessel density (MVD) and vascular architecture of prostate cancer tissues collected from a population nested in the HPFS cohort and, using database containing information
collected through semi-quantitative food frequency questionnaires, assess the relationship of angiogenesis with selected dietary factors.

We examined prostate tumor MVD and architecture (vessel size and shape) in a cohort nested within the Health Professionals Follow-up Study (HPFS) of the Harvard School of Public Health. The HPFS is a prospective epidemiologic study of over 50,000 men that began in 1986. For each man reporting a prostate cancer diagnosis and undergoing prostatectomy between 1986 and 2004, we requested archived tissue blocks and obtained adequate tissue specimens from 396 men. Histological sections were immunostained for two endothelial cell markers, CD34 and Factor VIII. Three non-overlapping 200x fields within the cancer were evaluated for vascular density, vessel size and shape using digital image analysis. Pearson coefficients and multiple linear regression models were used to evaluate correlation between selected dietary factors and vascular architecture.

Higher BMI was correlated with reduced vasculature of the prostate cancer, for example it was correlated with MVD (-0.26, P<0.0001) and smaller total vessel area of prostate cancer tissue (-0.32, P<0.0001). Higher intake of vitamin D was associated with reduced microvessel density (-0.11, P=0.026) as well as smaller total vessel area (-0.102, P = 0.043).

We suspect that observed correlation between BMI and improved vascular architecture profile of the prostate cancer tissue is a result of bias built into our cohort. Beneficial effects of the vitamin D intake are supported by previous epidemiological
studies and encourage further investigations into the relationship between prostate cancer angiogenesis and vitamin D intake.

5.2 Introduction

Prostate cancer is one of the most commonly diagnosed visceral cancers and third most common cause of cancer-related death in American men [1]. There is a dramatic difference between prostate cancer incidence observed in Asian countries and in populations of the Western world (United States or Northwestern Europe) [2, 4]. One of the key differences between Asian and Western world are the dietary habits, which may have an impact on prostate cancer development and progression [5]. Western diet usually characterized by consumption of, fat especially from animal sources, dairy and calcium, excessive amounts of calories and lower intake of fruits and vegetables [2, 3].

Over the past few decades various studies evaluated the impact of those dietary factors on the risk of development and progression of prostate cancer (Figure 5.1). Giovannucci et al used the Health Professionals Follow-up Study (HPFS) cohort to assess the relationship between the risk of advanced prostate cancer and consumption of fat [6] and observed that high total fat consumption (especially saturated, monounsaturated fatty acids and alpha-linolenic acids) was related to the risk of advanced prostate cancer (RR = 1.79).

Consumption of red meat is high in western culture and was strongly associated with advanced prostate cancer (RR = 2.64; 95% CI = 1.21-5.77) [6]. In another study
increased consumption of red meat was associated with higher risk of metastatic prostate cancer (RR = 1.7, 95% CI = 1.0-2.5 between extreme quartiles) [7]. This correlation can be contributed in part to the content of saturated and monounsaturated fatty acids in red meat. However, other substances present in red meat or formed during its preparation (grilling, broiling and doneness of meat), such as heterocyclic and polycyclic amines may also increase risk of the disease (1.2-fold increase in risk, 95% CI, 1.01-1.48) [8].

Epidemiological studies evaluated dairy foods in relation to prostate cancer and observed increased consumption of milk products and calcium was positively associated with increased overall risk of disease [9-11] and metastatic disease [12-14]. For example in HPFS study, high calcium intake was associated with increased risk of advanced disease (RR = 2.97, intake ≥ 2000 mg/d vs. 500 mg/d) and metastatic prostate cancer (RR = 4.57) [14]. Some authors suggest that increased consumption of dairy results in higher calcium intake which in turn inhibits vitamin D pathway [9, 10, 12, 14, 15]. This hypothesis is supported by a recent study which showed that low vitamin D status measured within 10 years of diagnosis with prostate cancer, increased risk of prostate cancer in Finish and Norwegian men by 3.1 to 4.2 fold [16].
<table>
<thead>
<tr>
<th>Evidence</th>
<th>Reduced risk</th>
<th>No relation</th>
<th>Increased risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convincing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possible</td>
<td>Tomatoes</td>
<td>Alcohol</td>
<td>High body mass</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>Vitamin C</td>
<td>Total fat</td>
</tr>
<tr>
<td></td>
<td>Vitamin E</td>
<td>Coffee</td>
<td>Animal fat, especially</td>
</tr>
<tr>
<td></td>
<td>Vitamin D</td>
<td>Tea</td>
<td>saturated fat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Meat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Milk and dairy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>products</td>
</tr>
<tr>
<td>Insufficient</td>
<td>Total fruit and vegetable intake</td>
<td>High energy intake</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.1:** Strength of scientific evidence on dietary factors modifying risk of prostate cancer. Modified from the original published in the “Food, Nutrition and Prostate Cancer: a global perspective”, World Cancer Research Fund in association with American Institute for Cancer Research, 1997.
Lower incidence of prostate cancer observed in men in Asia may be in part contributed to their diet rich in soy products [17], as well as colorful fruits and vegetables [18]. A study employing the HPFS cohort demonstrated reduced prostate cancer incidence and lower risk of advanced disease in men who consumed high amounts of lycopene and tomatoes [19]. A multiethnic case control study conducted in men living in United States, Canada and British Columbia also showed reduced risk of advanced prostate cancer with higher consumption of yellow, orange and cruciferous vegetables [20].

Numerous studies point to obesity associated with western diet as one of the modifiable risk factors for prostate cancer [21-23]. Body mass index (BMI) is an indicator of optimal weight for health (based on the information from the National Institute of Health) and is used to categorize people as normal weight (BMI = 18.5-24.9), overweight (BMI = 25 – 29.9) and obese (BMI >30) based on the. Study conducted in men from the Cancer Prevention Study I and II (CPS I and CPS II) cohorts demonstrated that obese man had higher prostate cancer mortality rates than the non-obese ones (RR = 1.27; 95% CI = 1.04-1.56 in CPS-I; RR= 1.21; 95% CI = 1.07-1.37 in CPS-II) [23, 24]. There was also a positive association between obesity and higher grade and more advanced stage of the disease [25, 26] as well as shorter time to biochemical progression after prostatectomy (HR = 4.09, 95% CI = 1.67-10.01) [25].

All of the above mentioned studies describe the impact of the diet on the development of prostate cancer and its progression to metastatic disease. However, there
are currently no clinical studies evaluating the effects of diet on tumor angiogenesis, which is known to be an essential step in development of metastasis. In our previous study we have validated that evaluation of the microvessel density (MVD) and vascular architecture based on an immunohistochemical (IHC) staining of blood vessels is an objective method for quantifying angiogenesis.

The goal of this study was to evaluate MVD and vascular architecture of prostate cancer tissues collected from a population nested in the HPFS cohort and, using database containing information collected through semi-quantitative food frequency questionnaires, assess the relationship of angiogenesis with selected dietary factors.

5.3 Materials and Methods

Study cohort

The Health Professionals Follow-up Study is an ongoing prospective cohort study of 51,529 male U.S. health professionals who were aged 40-75 at the time of recruitment in 1986. All men were asked to fill out a questionnaire soliciting information on age, marital status, height and weight, disease history, medications, smoking, physical activity and diet. Follow-up questionnaires mailed out every two years asked participants if they were diagnosed with a variety of diseases, including prostate cancer during the previous two years. Individuals identified as being diagnosed with prostate cancer through follow–up questionnaires were requested to provide confirmatory medical records. Clinical features such as grade, clinical stage, pathologic stage and lethality are reported.
Paraffin-embedded tissue blocks and accompanying pathology reports from the prostatectomies were requested. Upon arrival at OSU, the individual cases were recoded into the OSU computerized database and stored until analysis in the tissue bank. From 1986 until 2002 we have received over 1000 cases and analyzed 396 prostate tissue samples from the HPFS cohort participants who were diagnosed with prostate cancer, underwent prostatectomy and authorized us to use their tissue specimens for our study.

**Antibodies and Reagents**

Polyclonal rabbit antibody against Factor VIII (FVIII) (# N1505) and monoclonal mouse antibody against CD34 (# 236) were used for IHC were purchased from Dako DakoCytomation Carpinteria, CA. Other reagents used for IHC staining include DakoCytomation EnVision + Dual Link System Peroxidase (cat # K4061), DakoCytomation Dual Endogenous Enzyme Block (S2003), DakoCytomation Liquid 3,3’-diaminobenzidine (DAB)+ Substrate Chromogen System (K3468), Mayer’s Hematoxilin Solution (MHS32-1L) from Sigma-Aldrich Inc. (St. Louis, MO), Surgipath Micromount Mounting media from Surgipath Medical Industries, Inc.(Grayslake, IL), Antigen Retrieval *Citra Plus* Solution from BioGenex (San Ramon, CA), and concentrated-Reagent Diluent from Biogenex. Unless otherwise stated, all reagents were purchased from Dako DakoCytomation, (Carpinteria, CA).

**H&E staining**

Paraffin-embedded tissue blocks were sectioned and transferred to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Following re-hydration, slides were stained
with Hematoxylin and Eosin (H&E) on an automated slide stainer (Leica Microsystems Inc, Deerfield, IL). The slides were removed from the autostainer, and coverslipped. The slides were reviewed for grade and stage and high-resolution images recorded for our digital histopathology bank and CD storage.

**General immunohistochemical staining procedure**

All immunohistochemincal staining was performed on an OptiMax Automated Cell Staining System (BioGenex Laboratories, San Ramon, CA), which allows for staining of up to 40 slides simultaneously under precisely controlled conditions, thus eliminating a significant amount of variation associated with staining by hand.

We examined tumor Microvessel Density (MVD) with antibodies directed against CD43 and Factor VIII, which are early and late markers of angiogenesis respectively. Briefly, sections were deparaffinized, rehydrated and washed prior to staining. Sections stained with FVIII were pre-treated with heated citrate buffer for antigen retrieval. To eliminate nonspecific binding, sections were treated with peroxide for 5 minutes. Subsequently, sections were exposed to primary antibodies directed against either FVIII (1:2000) or CD34 (1:200) for 30 minutes (DakoCytomation) followed by a peroxidase labeled polymer conjugated to goat anti-rabbit or anti-mouse immunoglobulins (DakoCytomation). To visualize the CD34/FVIII staining, sections were treated with DAB chromogen solution (DakoCytomation) and counterstained with hematoxylin (Sigma-Aldrich Inc). Slides/sections were then dehydrated and coverslipped. During the staining process, sections were washed with phosphate buffered saline (PBS) three times before and after each of staining step. Unless otherwise stated, all incubations were done
at room temperature. All sections were counterstained with hematoxylin, dehydrated and mounted.

**Digital imaging and analysis**

All sections were examined by a pathologist, under light microscopy, for characterization of tumor morphology and cytological features. Tumor areas were identified and outlined in up to three representative sections per case. The outlined tumor areas were visualized under bright field microscopy using a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY). Representative images of three, non-necrotic, non-overlapping high power fields (200x) were captured from each section using a RT Slider Spot Camera (Diagnostic Instruments, Sterling Heights, MI) mounted onto the microscope. Captured images were analyzed using Image ProPlus 4.5 software (Media Cybernetics, Silver Spring, MD) designed specifically for histological analysis. Images were evaluated for the presence of angiogenic markers (CD34 and FVIII) based on the following parameters:

- Blood vessel number,
- Total vessel area (sum of all the vessels, \( \mu \text{m}^2 \)),
- Area per vessel (\( \mu \text{m}^2 \)),
- Diameter (mean, \( \mu \text{m} \)),
- Perimeter (mean, \( \mu \text{m} \)),
- Roundness (mean, \( \mu \text{m} \)),
- Mean percent area per 2x2 \( \mu \text{m}^2 \) per vessel,
- Percent of all vessels per 2x2 \( \mu \text{m}^2 \) area,
Branching structures were counted as a single vessel and lumen did not have to be noticeable for a structure to be considered a vessel. Red blood cells were not used to categorize structure as a blood vessel. Slides were evaluated without knowledge of the Gleason grade or staging of the tumor.

Statistical analyses

Data from image analyses performed with Image ProPlus were exported into an Excel file and the average for each case was calculated. All gathered data were sent to the Harvard School of Public Health for further analyses using the HPFS database. Statistical analyses performed include Pearson correlation analyses and linear regression analyses to evaluate relationship between the selected dietary factors and vascular architecture.

5.4 Results

Study cohort

Of the 51,529 men in the HPFS cohort, we obtained tissue samples from approximately a thousand men who underwent radical prostatectomy and for the purposes of this study we have stained and analyzed 396 cases. In this subpopulation 84.6% men have been diagnosed with localized disease (stage C1 or lower), while 15.4 % of them were diagnosed with advanced disease (C2, D; Table 5.1). Majority of the men in our study population (54%) had Gleason score of less then 7, and 46% of the men had Gleason score 7 or higher and 54 %. Men in this in our cohort were diagnosed with
prostate cancer between 1986 and 2002 with almost 24% of them being diagnosed between 1992 and 1994 which correlates with introduction of routine prostate specific antigen (PSA) screening (Table 5.1).

CD34 and FVIII staining in relation to Gleason grade

Based on the image analyses of both CD34 and FVIII we examined MVD and key vascular architecture parameters in relation to Gleason grade (Table 5.2). Gleason grade was assigned to each captured tissue image by a pathologist. Tissue graded from 1 to 3 was classified as low Gleason grade, and tissue graded from 4 to 5 was classified as high Gleason grade. Both CD34 and FVIII staining provided similar results therefore they will be discussed together. We noticed that regardless of the type of staining used, higher MVD was observed in tissue of higher Gleason grade (P < 0.0001). The average area per vessel was smaller in tumors with higher Gleason score compared to lower grade tumors (P < 0.0001). There was no significant difference between the total vessel areas observed in the low or high grade prostate tumors (P = 0.074 for CD34 and P = 0.069 for FVIII).

The average diameter and average perimeter of the vessels were significantly lower in tumor tissues with higher Gleason grade than in those with lower Gleason grade (P < 0.0001 for diameter and P = 0.003 for perimeter). Finally, using the Image ProPlus software we examined the shape of the vessels based on the assumption that a perfectly round vessel is rated 1 and the more irregular the vessel the higher the score assigned. Using this scoring system we observed that tumor tissue with higher Gleason grade was characterized by more irregularly shaped vessels than those present in lower Gleason grade (P = 0.001).
<table>
<thead>
<tr>
<th>Stage of the Tumor</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized A, B, C1</td>
<td>335 (84.6)</td>
</tr>
<tr>
<td>Advanced C2, D</td>
<td>61 (15.4)</td>
</tr>
<tr>
<td>Gleason Score</td>
<td></td>
</tr>
<tr>
<td>Low grade (&lt; 7)</td>
<td>197 (54.0)</td>
</tr>
<tr>
<td>High grade (≥ 7)</td>
<td>168 (46.0)</td>
</tr>
<tr>
<td>Gleason of image</td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>270 (81.6)</td>
</tr>
<tr>
<td>4-5</td>
<td>61 (18.4)</td>
</tr>
<tr>
<td>Diagnosis date</td>
<td></td>
</tr>
<tr>
<td>1986-1988</td>
<td>13 (3.3)</td>
</tr>
<tr>
<td>1988-1990</td>
<td>25 (6.3)</td>
</tr>
<tr>
<td>1990-1992</td>
<td>60 (15.1)</td>
</tr>
<tr>
<td>1992-1994</td>
<td>98 (24.8)</td>
</tr>
<tr>
<td>1994-1996</td>
<td>77 (19.4)</td>
</tr>
<tr>
<td>1996-1998</td>
<td>38 (9.6)</td>
</tr>
<tr>
<td>1998-2000</td>
<td>75 (18.9)</td>
</tr>
<tr>
<td>2000-2002</td>
<td>10 (2.5)</td>
</tr>
</tbody>
</table>

**Table 5.1:** Clinical characteristics of the study population nested in the Health Professionals Follow-up Study cohort.
CD34 and FVIII staining in relation to Tumor stage

We examined the differences between vessel number and vascular architecture in advanced and localized tumors (Table 5.3). We classified localized tumors as those staged A-C1 and advanced tumors were staged as C2 – D. CD34 and FVIII produced similar results, therefore unless discrepancy between the two stains was observed, result are discussed together. The MVD was higher in advanced tumors as compared to the localized ones (P = 0.004 for CD34; P<0.0001 for FVIII). The average vessel area was smaller in advanced tumors as compared to the localized ones (P =0.002 for CD34; P = 0.033 for FVIII). There was no significant difference between the total area of the vessels between localized and advanced tumors (P = 0.32 for CD34; P = 0.09 for FVIII). Advanced tumors were also characterized by vessels with smaller diameter than that of the localized tumors (P = 0.0015 for CD34; P = 0.047 for FVIII). No significant differences were observed between vessel perimeter in advanced and localized tumors (P = 0.052 for CD34; P = 0.25 for FVIII). Finally, we examined the shape of the vessels using the scoring system described earlier. We noticed that advanced tumors had more irregular shape vessels than localized tumors (P = 0.0003 for CD34; P = 0.009 for FVIII). Since CD34 and FVIII were gave very similar results, dietary analyses below are reported for CD34 staining only as representative of both stains.
<table>
<thead>
<tr>
<th>Mean (SE)</th>
<th>CD34</th>
<th></th>
<th>Factor VIII</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gleason 4-5 N = 61</td>
<td>Gleason 1-3 N = 270</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>Microvessel Density</td>
<td>84.7 (3.7)</td>
<td>57.0 (1.1)</td>
<td>&lt;0.0001</td>
<td>84.0 (3.8)</td>
</tr>
<tr>
<td>Total vessel area μm²</td>
<td>27,951 (1659)</td>
<td>24,971 (695)</td>
<td>0.074</td>
<td>27,951 (1659)</td>
</tr>
<tr>
<td>Average area per vessel, μm²</td>
<td>340.8 (15.2)</td>
<td>475.8 (14.7)</td>
<td>&lt;0.0001</td>
<td>340.8 (15.2)</td>
</tr>
<tr>
<td>Average vessel diameter, μm</td>
<td>21.0 (0.4)</td>
<td>24.4 (0.3)</td>
<td>&lt;0.0001</td>
<td>21.8 (0.4)</td>
</tr>
<tr>
<td>Average vessel perimeter, μm</td>
<td>117.7 (2.5)</td>
<td>128.4 (1.6)</td>
<td>0.003</td>
<td>122.8 (2.5)</td>
</tr>
<tr>
<td>Average vessel shape 1= round</td>
<td>5.0 (0.1)</td>
<td>4.6 (0.05)</td>
<td>0.001</td>
<td>5.0 (1.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.2:** CD34 and Factor VIII staining in relation to Gleason grade. Data is based on the grade per image.
<table>
<thead>
<tr>
<th>Mean (SE)</th>
<th>CD34</th>
<th></th>
<th>Factor VIII</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Advanced N = 61</td>
<td>Localized N = 335</td>
<td>p-value</td>
<td>Advanced N = 54</td>
</tr>
<tr>
<td>Microvessel Density</td>
<td>83.1 (4.9)</td>
<td>69.4 (1.8)</td>
<td>0.004</td>
<td>74.6 (4.7)</td>
</tr>
<tr>
<td>Total vessel area (\mu m^2)</td>
<td>30909 (2011)</td>
<td>33675 (1134)</td>
<td>0.32</td>
<td>27785 (1730)</td>
</tr>
<tr>
<td>Average area per vessel, (\mu m^2)</td>
<td>413.2 (26.1)</td>
<td>518.4 (13.7)</td>
<td>0.002</td>
<td>390.8 (24.3)</td>
</tr>
<tr>
<td>Average vessel diameter, (\mu m)</td>
<td>22.7 (0.6)</td>
<td>25.1 (0.3)</td>
<td>0.0015</td>
<td>22.1 (0.6)</td>
</tr>
<tr>
<td>Average vessel perimeter, (\mu m)</td>
<td>123.2 (2.9)</td>
<td>129.8 (1.4)</td>
<td>0.052</td>
<td>122.6 (3.2)</td>
</tr>
<tr>
<td>Average vessel shape (L=) round</td>
<td>4.8 (0.12)</td>
<td>4.3 (0.05)</td>
<td>0.0003</td>
<td>4.96 (0.11)</td>
</tr>
</tbody>
</table>

**Table 5.3:** CD34 and FVIII staining in relation to advanced and localized prostate cancer.
Impact of selected dietary factors on MEAN MVD and vascular architecture in prostate tumors

Tumor vasculature may be influenced by variety of dietary factors but it was not our goal to describe effects of all of them, instead we focused only on a few selected ones: body mass index (BMI) and intake of total calorie (reported in kcal) and vitamin D. Age-adjusted Pearson correlation analyses revealed that higher BMI was correlated with lower mean MVD, smaller total vessel area and smaller diameter of the vessels (Table 5.4). Simultaneously, higher BMI was also positively correlated with more irregular vessel shape. Overall vitamin D intake was correlated with reduced mean MVD, but this relation was almost statistically significant (P = 0.066). Vitamin D was also non-significantly correlated with reduced mean value of total vessel area and increased irregularity of the vessels. When supplement use was deducted from the dietary analyses vitamin D was significantly correlated with increased irregularity of the vessels in the tumor (+ 0.12, P = 0.022).
Table 5.4: Relationship between dietary factors and mean values of MVD and vascular architecture in prostate cancer. Table presents age-adjusted Pearson correlation coefficients (and p-values) for relation between nutrient and mean values of MVD and total vessel area, diameter and vessel shape prostate cancer specimens. Bolded values are statistically significant or almost statistically significant.
Impact of selected dietary factors on MAXIMUM value of MVD and vascular architecture in prostate tumors

Similar results were observed when Pearson correlation coefficients were calculated for maximum number of vessels and vascular architecture parameters (Table 5.5). Higher BMI was very significantly (P < 0.0001) correlated with lower maximal value of MVD, reduced maximal value of total vessel area and maximal vessel diameter. Simultaneously, increased BMI was positively correlated with more irregular vessels. Total vitamin D intake (including supplements) was significantly correlated with reduction in the maximal MVD and reduced maximal total vessel area. There was also a negative correlation between vitamin D intake and average maximal diameter of the vessels but it was not significant. Finally, positive correlation was observed between vitamin D intake and increased maximal irregularity of the vessels. When supplement intake was excluded from the analyses vitamin D intake was almost significantly correlated with higher maximal irregularity of the vessels and reduced maximal MVD.
<table>
<thead>
<tr>
<th>Nutrient factors before cancer diagnosis</th>
<th>Microvessel Density Correlation (P-value)</th>
<th>Total Vessel Area Correlation (P-value)</th>
<th>Average Vessel Diameter Correlation (P-value)</th>
<th>Average Vessel Shape Correlation (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D</td>
<td>-0.11 (0.024)</td>
<td>-0.102 (0.043)</td>
<td>-0.013 (0.80)</td>
<td>+0.023 (0.65)</td>
</tr>
<tr>
<td>Vitamin D w/o supplement</td>
<td>-0.092 (0.068)</td>
<td>-0.057 (0.26)</td>
<td>-0.002 (0.96)</td>
<td>+0.093 (0.064)</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.26 (&lt;0.0001)</td>
<td>-0.32 (&lt;0.0001)</td>
<td>-0.20 (&lt;0.0001)</td>
<td>+0.16 (0.001)</td>
</tr>
</tbody>
</table>

Table 5.5: Relationship between dietary factors and maximal values of MVD and vascular architecture in prostate cancer. Table presents age-adjusted Pearson correlation coefficients (and p-values) for relation between nutrient and maximal values of MVD and total vessel area, diameter and vessel shape prostate cancer specimens. Bolded values are statistically significant or almost statistically significant.
<table>
<thead>
<tr>
<th>Microvessel Density</th>
<th>Total Vessel Area</th>
<th>Average Vessel Diameter</th>
<th>Average Vessel Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q4 vs. Q1 Beta (p-value)</td>
<td>Q4 vs. Q1 Beta (p-value)</td>
<td>Q4 vs. Q1 Beta (p-value)</td>
<td>Q4 vs. Q1 Beta (p-value)</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Vitamin D</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>-9.8 (0.043)</td>
<td>-4510 (0.11)</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
<td>Vit D w/o supplements</td>
</tr>
<tr>
<td>BMI*</td>
<td>BMI*</td>
<td>BMI*</td>
<td>BMI*</td>
</tr>
<tr>
<td>-38.8 (&lt;0.0001)</td>
<td>-29022 (&lt;0.0001)</td>
<td>-4.9 (0.002)</td>
<td>+0.84 (0.003)</td>
</tr>
</tbody>
</table>

**Table 5.6:** Dietary factors alter mean MVD and vascular architecture in prostate cancer. Values presented in the table are based on results of linear regression analyses comparing extreme quartiles of mean of measures. Analyses were adjusted for age at diagnosis. *BMI compares >30.0 versus <18.5. Note: all BMI >18.5 showed similar effects.
Table 5.7: Dietary factors alter maximal MVD and vascular architecture in prostate cancer. Values presented in the table are based on results of linear regression analyses comparing extreme quartiles of maximal of measures. Analyses were adjusted for age at diagnosis. * BMI compares >30.0 versus <18.5. Note: all BMI >18.5 showed similar effects.

<table>
<thead>
<tr>
<th>Microvessel Density</th>
<th>Total Vessel Area</th>
<th>Average Vessel Diameter</th>
<th>Average Vessel Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q4 vs. Q1 Beta (p-value)</td>
<td>Q4 vs. Q1 Beta (p-value)</td>
<td>Q4 vs. Q1 Beta (p-value)</td>
<td>Q4 vs. Q1 Beta (p-value)</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Vitamin D</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>-15.2 (0.031)</td>
<td>-6260 (0.08)</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Vit D w/o supplements</td>
<td>----</td>
<td>----</td>
<td>Vit D w/o supplements</td>
</tr>
<tr>
<td>-10.3 (0.15)</td>
<td>----</td>
<td>----</td>
<td>+0.24 (0.09)</td>
</tr>
<tr>
<td>BMI*</td>
<td>BMI*</td>
<td>BMI*</td>
<td>BMI*</td>
</tr>
<tr>
<td>-60.2 (&lt;0.0001)</td>
<td>-37103 (&lt;0.0001)</td>
<td>-6.8 (0.003)</td>
<td>+0.94 (0.006)</td>
</tr>
</tbody>
</table>
Comparison of extreme quartiles of BMI as well as vitamin D and total calorie intake in relation to MVD and vascular architecture

Multivariate linear regression analyses were used to evaluate the relationships between extreme quartiles of BMI, total energy intake and vitamin D intake and mean and maximum measurements of MVD and vascular architecture (Table 5.6 and Table 5.7, respectively). Comparison of men in the highest quartile of BMI (BMI > 30) to those with the lowest BMI (<18.5), revealed that men with higher BMI had significantly lower MVD, total vessel area, as well as average vessel diameter (both mean and maximal values of those parameters). At the same time, men with BMI > 30 had more irregular vessels than men with BMI <18.5.

Men with the top quartile of the total vitamin D intake had significantly lower mean and maximal MVD than men who consumed the lowest amounts of vitamin D (P = 0.043 for mean MVD, P = 0.031 for max MVD). Men who consumed highest amounts of vitamin D had also smaller mean and maximal total vessel area than the men who consumed the lowest amounts of vitamin D, though this relationship was not statistically significant (P = 0.11 and P = 0.08 respectively). Excluding supplementation men who consumed highest amounts of vitamin D had lower maximal MVD (P = 0.08) than those in the lowest quartile of vitamin D consumption.
5.5 Discussion

Relationship between microvessel density and vascular architecture and grade and stage

Image analyses of CD34 and FVIII immunostained prostate cancer tissues were previously established to be a valid tool in examining vascular architecture. Our previous study demonstrated that microvessel density, as well as vascular architecture parameters such as vessel diameter, shape, and mean vessel area were related with more advanced prostate cancer and higher Gleason grade. We confirmed our previous observations in the current set of samples and observed that men diagnosed with more advanced prostate cancer had tumors with higher MVD, lower average area per vessel, vessel with smaller diameter and more irregular shape. Similarly we were able to observe that Gleason grade of the prostate cancer tissue was associated with increased MVD, lower mean vessel area, smaller vessel diameter and more irregular shape of the vessels. These results correspond with previous studies demonstrating that increased MVD was related to more advanced [16, 27-37] and higher grade of prostate cancer [28, 29, 32, 33, 36-44]. MVD was also associated with increased risk of prostate cancer recurrence after radical prostatectomy [28, 29, 32, 33, 35, 38, 45-48]. For example, in a study by Bono et al men with higher MVD (cut off point = 90) had increased relative risk of having recurrence, than men who had lower MVD (RR=2.55, 95% CI= 1.18-5.52, P < 0.05) [29]. Previous studies and our results support the hypothesis that irregular vascular architecture and higher microvessel density predict more aggressive prostate cancer biology.
Obesity (BMI) affects vascularity of the prostate cancer

Ecological studies point out that obesity is associated with increased risk of more advanced disease and higher grade of prostate cancer [49, 50]. Furthermore, BMI > 30 was also correlated with increased risk of biochemical progression [26, 51, 52] and higher risk of death from prostate cancer [53]. Interestingly, men who were lean in their twenties and then rapidly gained weight between 25 years of age and the time of diagnosis, had significantly shorter time to prostate cancer progression (17 ms vs. 39 ms in those who gained weight slower, \( P_{\text{trend}} = 0.005 \) [54].

Obesity was also reported to alter hormonal status of the body, in fact obese man were shown to have lower PSA than the non-obese ones (OR = 1.8, 95% CI = 1.2-2.3) [55]. Baillargeon et al [56] observed that PSA decreased proportionally to increasing BMI even after adjusting for ethnicity and age. These studies suggest that obesity may mask presence of cancer by lowering PSA and contributes to detection/diagnosis of more advanced disease. Overall, majority of the studies, though not all [57-59] demonstrate that obesity is associated with increased risk of prostate cancer. Moreover, study by Silha et al [60] indicates that obese individuals have higher circulating levels of vascular endothelial growth factor (VEGF; correlation coefficient = 0.21, \( P = 0.042 \)) which may contribute to tumor angiogenesis and increase risk of metastasis.

Aside from somewhat controversial epidemiological studies, there is increasing amount of research in animal models which used energy restriction to prevent obesity. Results of that research clearly states that 20-40 % reduction in dietary energy intake is
associated with reduced tumor incidence (65% vs 79%) [61], increased survival (HR = 0.68) [61], reduced the tumor size (P < 0.001) [62] and increased tumor cell apoptosis [62]. Finally, 20-40% reduction of dietary energy intake also resulted in decreased expression of VEGF thus dietary energy restriction attenuated tumor angiogenesis (P < 0.003) [62]. This data implies that obesity should have a promoting effect on prostate cancer angiogenesis. Overall, observations made in animal models support the idea that obesity promotes prostate carcinogenesis.

Based on the outcomes of the earlier studies we anticipated to see a positive correlation between increasing BMI and more angiogenic profile in the prostate tissue. Surprisingly, results of our analysis indicate that BMI was negatively correlated with MVD and total vessel area. This suggests that higher BMI is associated with less angiogenic pattern of prostate cancer tissue. We also observed that BMI was directly correlated to increasing irregularity of the vessels and decreasing average diameter, both of which were predictive of more vascular and aggressive prostate cancer. Data from the linear regression analyses examining the extreme quartiles of BMI (BMI <18.5 vs. BMI > 30) revealed that men with higher BMI had very significantly reduced MVD and smaller total vessel area even though their vessels had smaller diameter and more irregular shape. Overall, these results imply that obesity has protective effects against prostate cancer angiogenesis.

There are at least a few possible explanations for this seemingly protective effect of obesity. Firstly, we need to keep in mind that some studies suggested that obesity may
in fact have protective effects against prostate cancer. Since larger waist and higher BMI are associated with reduced blood levels of testosterone ($P < 0.05$) [63], which is known to promote growth of hormone-dependent prostate cancer, thus being obese leads to lower testosterone and lower risk of prostate cancer. Study by Robinson supported this theory as he noticed that men who were obese as teenagers and went through delayed puberty had lower risk of prostate cancer (OR = 0.79, 95% CI = 0.46-1.38) [64]. He contributed this protective effect of obesity to lower androgen activity during this crucial time in prostate development. This hypothesis is contradicted by Okasha [53], whose study reported that obesity in young adulthood is associated with increased risk of death from prostate cancer. Furthermore, a Swedish case-control study examining the early lifestyle factors on development of prostate cancer did not observe any relationship between weight during puberty and risk of prostate cancer (OR = 1.0) [65].

It is possible that this unexpected correlation between BMI and improved angiogenic profile may be due to a bias in our study population. This study is nested in the HPFS cohort and includes only men who were diagnosed with prostate cancer and underwent radical prostatectomy. As implied by the name of the surgery the purpose of radical prostatectomy is removal of the entire intact organ, which may be challenging in obese men. Studies suggest that surgeons may be less likely to perform radical prostatectomy in men with BMI>30 because of technical difficulties associated with such operation, specifically higher odds of risk of capsular incision, which is considered “less than ideal operation” (OR = 1.57) [66]. Furthermore, overweight and obese patients commonly present with co-morbidities such as hypertension, diabetes and heart disease.
Ahlering [67] also points out that even with recently introduced robotic prostatectomy obese patients had more complications than the non-obese ones (26.3% vs. 4.9%, \( P = 0.01 \)) and did not recover as quickly and well as the non-obese men (7 vs 4.3 weeks, respectively). Finally, since obesity has been shown to increase risk of prostate cancer recurrence [25, 26, 52], it forces clinicians to conduct more intense follow-up [52]. All of these factors contribute to treatment selection and mean that obese patients frequently forgo prostatectomy and are encouraged to receive other treatments. Hypothesized treatment selection in relation to patient’s BMI is presented in Figure 5.2. Those who do choose to undergo radical prostatectomy most likely do not have other co-morbidities and probably have smaller tumor volume of lower Gleason score, thus have better prognosis. As a result, our study population is biased and inadequately represents obese men. This may lead to the observed results where obese patients have seemingly better prognosis and less angiogenic profile than the non-obese ones. Further studies are needed to establish whether our observation was a result of a bias or actual protective effects of obesity associated with a novel mechanism.
Figure 5.2: Impact of patients’ BMI on the treatment selection in prostate cancer therapy. XRT – external beam irradiation therapy.
Effects of vitamin D intake on vascularity of the prostate cancer

Vitamin D intake was another dietary factor significantly affecting the vascular architecture and MVD in our study. Even though overall dairy intake was not a factor, higher intake of vitamin D (both from food sources and supplements) was associated with reduced MVD and reduced total vessel area. Similarly, men in the highest quartile of total vitamin D intake had significantly lower MVD than men in the lowest intake quartile. These results imply that increased vitamin D intake reduces angiogenic profile of the prostate cancer tissue and thus may reduce its aggressiveness.

Our finding is supported by numerous epidemiological studies which indicate that below median levels of vitamin D (25 hydroxyvitamin D (25-OH D) were associated with 70% increased prostate cancer, especially in men < 52 years old (adjusted odds ratio = 3.5) [68]. Recent study examining the HPFS cohort revealed that increasing vitamin D levels by an increment of just 25 nmol/L was associated with significant reduction in total cancer risk (RR = 0.83, 95% CI = 0.74-0.92) and lower total cancer mortality (RR = 0.71, 95% CI = 0.60-0.83) [69]. It is known that vitamin D synthesis is induced by solar UV radiation and studies done both in Europe and in US recorded a geographical pattern of prostate cancer incidence associated with sun exposure [70-72]. In the southern areas on North America and Europe, where men are exposed to higher UV radiation, lower rates of prostate cancer and cancer death are observed. Inversely, higher incidence and mortality are reported in the northern part of the continents where solar UV radiation is lower [21, 70-73]. For example, study by Luscombe et al [73] demonstrated that low
exposure to UV rays was associated with higher risk of prostate cancer development (OR= 3.03, 95% CI = 1.59-5.78).

Potential mechanisms of beneficial effects of vitamin D are currently under investigation. Cell culture studies suggest that vitamin D mediates its effects by binding to the nuclear vitamin D receptor (nVDR), thus inhibiting cell proliferation and promoting differentiation in prostate cancer cells (for example it up-regulates expression of PSA) [74-76]. Furthermore vitamin D inhibited anchorage dependent growth, decreased prostate cancer cell motility and chemotaxis, and down-regulated activity of metalloproteinase-9 (MMP-9) and -2 (MMP-2) thus decreasing prostate cancer invasiveness [74]. Interestingly, vitamin D was found to up-regulate expression of its own receptor (VDR) as well as promote expression of and retinoid-X receptor alpha and androgen receptor [74]. These results are especially promising, when associated with the recent finding that prostate cancer cells express 25-hydroxylase which is the enzyme responsible for metabolizing circulating form of vitamin d (25-OH-D) to the active hormone - 25(OH)D3 (calcitriol) [75]. Finally, in vitro studies show effects of vitamin D on cancer angiogenesis. Vitamin D analogues have been shown to reduce expression of previously mentioned MMP-9 and MMP-2, as well as down-regulate expression of VEGF in lung carcinoma cells (LLC) [77]. Moreover, in a recent study calcitriol inhibits prostate cancer cell induced human umbilical vein endothelial cell migration and tube formation [78]. The mechanism of this inhibition involves suppression of interleukin-8 signaling by inhibiting NF-kappa B nuclear DNA binding [78]. These studies provide
potential mechanism behind the benefits of increased vitamin D intake observed in the present study.

Overall, these data support our finding that shows correlation between vitamin D intake and reduction in prostate cancer microvessel density and suggest that vitamin D supplementation would be beneficial for prevention of this disease. However, we need to keep in mind that excessive intake of vitamin D and increased blood levels of calcitriol promote inactivation vitamin D by 24-hydroxylase [16]. Furthermore, due to its role in regulation of calcium metabolism [79], consumption of excessive amounts of vitamin D may result in bone loss (it promotes calcium re-absorption from the bones). This may be especially pronounced in prostate cancer patients who underwent androgen ablation therapy and may be at particularly high risk of osteoporosis [80]. Future studies need to establish optimal levels of UV exposure and/or dietary intake of vitamin D for optimal bone health and anticancer benefits.

In conclusion, data presented in this study indicate that dietary factors are able to affect prostate cancer vascular architecture and microvessel density. It remains to be established whether observed apparently protective effects of obesity on prostate cancer angiogenesis are result of a bias built into the study cohort, or a true protective effect obesity mediated through a yet undetermined mechanism. Relationship between vitamin D and prostate cancer vasculature supported by previous findings begs further investigation into chemopreventive effects of this hormone. Furthermore, it also forces us to evaluate current intake of this vitamin and establish new norms of vitamin D intake.
to improve bone health and optimize its cancer-preventive benefits. Special attention should be paid to populations at higher risk for osteoporosis.

5.6 References


Prostate cancer is one of the most commonly diagnosed visceral cancers in men in the United States. It is characterized by a wide range of clinical behavior from an indolent disease to very rapid progression. Increasing amounts of data from epidemiological, animal and \textit{in vitro} studies suggest that prostate cancer development and progression may be affected by dietary and lifestyle factors such as energy imbalance and obesity. Effects of the diet are mediated by changes in circulating hormones which then affect various stages of prostate carcinogenesis, including angiogenesis.

This dissertation consists of studies which have enhanced our understanding of the impact of dietary factors on the process of prostate cancer angiogenesis. The first study suggests that dietary energy restriction can affect the process of prostate tumor angiogenesis by changing the hormonal status of the body. In our rat model we observed that diet energy restriction can affect the process of prostate cancer angiogenesis by reducing expression of VEGF, a major promoter of this process. Effects of the diet were at least in part mediated through changes in the bioavailability of IGF-I at the blood and tissue level. These direct effects of IGF-I on VEGF expression were then confirmed in
our *in vitro* model. The fact that energy restriction alters prostate cancer angiogenesis also implies that it may affect tumors ability to invade local tissues and form distant metastasis.

Our second study validates that image analyses of CD34 and FVIII immunostained prostate cancer tissue provide an objective and quantitative tool for analyses of tumor vasculature. Despite numerous publications evaluating microvessel density in relation to prostate cancer, the use of this biomarker has still not been validated as a predictor of prostate cancer progression and patient survival. Our study demonstrates that prostate tumor vasculature can be quantitated in an objective manner and that it predicts more aggressive tumor biology and higher grade disease. Furthermore, our results establish that evaluation of vascular architecture can be used, in addition to previously established biomarkers, as a predictor of patient survival.

Finally, our third study indicates that diet and lifestyle factors can affect prostate cancer vasculature. Using previously validated biomarkers we examined the relationship between obesity and vitamin D intake and prostate cancer vascularity. Although we have observed a protective effect of obesity on prostate vasculature, we suspect it was a result of a bias present in our cohort. The bias comes from the selection of candidates for prostatectomy which discriminates against obese patients who present a technical challenge for surgery. As a result overweight men may undergo prostatectomies only if they have very good prognostic features. Our data also suggests that men consuming higher levels of vitamin D have lower microvessel density in prostate cancer and thus are
predicted to have less aggressive disease. This finding may also explain how vitamin D attenuates prostate cancer development and progression, not only by inhibiting prostate cancer proliferation and increasing apoptosis, but also by reducing angiogenesis. Our studies demonstrate that vascular architecture is a valuable marker of a more aggressive potential of prostate tumor thus provide basis for further investigations evaluating effects of therapy and/or chemopreventive agents. Future studies are necessary to confirm or disprove the effects of obesity on prostatic tumor vasculature. Finally, the relationship between vitamin D intake and prostate cancer demands additional exploration in order to establish the optimal intake of this hormone for optimal bone health and cancer prevention.


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