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The University of Southern Mississippi

A COMPARISON OF PROSTATIC ACID PHOSPHATASE WITH TESTOSTERONE AND PROSTATE SPECIFIC ANTIGEN FOR THE SERODIAGNOSIS OF PROSTATE CANCER IN ADULT MALES

by

Hannah Rice

A Thesis

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Abstract

Cancer is a hyperplastic cellular malignancy that affected 1,436,000 people (newly diagnosed cases) in the United States last year. The top three most frequent forms of cancer were lung, prostate, and breast. Oncogenesis is associated with both genetic predisposition and environmental onslaught, with a mixture of the two being required for the malignancy to progress. Tumor markers, circulating serum factors, are used in the diagnosis of cancer. Prostate cancer is one of the most prevalent forms of this malignancy, affecting 230,110 men in 2004 alone. Diagnosis of prostate cancer is currently performed using results of an assay for prostate specific antigen (PSA). However, the theory has been advanced that either prostatic acid phosphatase (PAP) or testosterone may be a more accurate tumor marker than PSA. This study examines the efficacy of all three of these tests and specifically compares a PAP assay to the current standard test for PSA. A Diagnostics Automation, Inc. enzyme immunosorbent assay was used to measure prostatic acid phosphatase in 102 healthy adult males and 449 adult male patients. Predictive values were determined for PAP and compared with those of the PSA assay performed on the same samples. The results were as follows: diagnostic percent sensitivity was (20.73, PAP); (0.00, Testosterone); (30.12, PSA), the diagnostic percent specificity was: (80.38, PAP); (98.80, Testosterone); (91.29, PSA), and the diagnostic percent efficiency was: (71.51, PAP); (82.40, Testosterone); (81.73, PSA). From these data, it was concluded that the test for prostate specific antigen is the most accurate and efficient screen for prostate cancer. Finally, predictive values were determined for all three markers evaluated together.

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Introduction

Birindelli, et. al (2000) described cancer as "the result of circumvention of the apoptotic machinery, promotion of cell division and cell proliferation, loss of cell differentiation pathways, and disruption of cell-cell communication and interaction." (p.45) The process of carcinogenesis is composed of many steps involving specific genes prone to producing such a state and signals provided and controlled by the products thereof (Birindelli, Aiello, Lavarino, et. al, 2000). The resulting disease state produces malignant tissue that invades and destroys nearby tissue and can metastasize to other areas of the body (Cook, 1996). There is a large genetic component to cancer since it arises from alterations in cellular DNA or in the transcriptional or translational processes that produce abnormalities in gene expression (Loescher, Whitesell, 2003). Although all cancer is not hereditary, over 200 hereditary cancer syndromes have been described, and an individual's risk for cancer has been seen to be increased if multiple family members are afflicted (Hunt, 2005).

Cancer is aggressive, degenerative and affects many people worldwide. There were 565,600 cancer deaths in 2008 in the United States alone, and 1,437,200 new diagnoses (IARC, 2010). In 1999, there were an estimated 8,100,000 cases diagnosed worldwide in that single year (Alberts, Hess, 2005). Cancer is classified into three categories: sarcomas, affecting bone and fibrous tissue (muscle, blood vessels); carcinomas, affecting tissues such as the epithelium, lungs, breast, and colon; and leukemias and lymphomas, affecting the cells of the bone marrow and lymph nodes (Rosenbaum, 1983).

Prostate cancer is a carcinoma involving the epithelial cells of the prostate, a gland in the lower abdomen of males, just below the bladder and in front of the rectum, wrapping around the urethra. It is normally about 1.5" in diameter and produces prostatic fluid (a thick fluid that is part of semen) while simultaneously acting as a valve to allow sperm and urine to flow in the correct direction. Masses of abnormally proliferating cells swell the size of the prostate in malignant conditions and if they breach the fibrous membrane surrounding this organ, they can quickly circulate to other tissues to produce aggressive metastasis (Bostwick, MacLennan, Larson, 1996). Prostate cancer most often metastasizes to the lymph nodes, pelvic bones and spine or vertebrae, axial skeleton and proximal long bones, lungs, liver, bladder, and rectum (Cook, 1996).

The incidence of prostate cancer in 2008 was 186,320 in the United States (IARC, 2010). Prostate cancer is the most common cancer diagnosed in North American men (Alberts, Hess, 2005). It also affects many thousands more in other countries worldwide. Thus, the problem of prostate cancer is both widespread and significant.

Of primary importance in the study of prostate cancer is the method of diagnosis. In addition to medical history, physical examination, and visual and tactile (such as a rectal examination) methods of tumor determination, an accurate screening test must be developed to increase early detection, efficacy of treatment, and survival rates. Alterations of genes associated with cancer provide products that can be used as molecular markers to indicate a cancer disease state (Birindelli, Aiello, Lavarino, et. al, 2000). Prostate specific antigen (PSA) is currently accepted as the most accurate screening test for the detection of prostate cancer (Haese, Becker, Diamandis, et. al, 2002). However, prostatic acid phosphatase was used for many years as "the most

valuable enzyme marker for the diagnosis of prostate cancer," (p.52) because of its characteristic antigenic properties that are unlike other acid phosphatases (Lee, Li, Jou, et. al, 1982). Testosterone has also been theorized to have similar properties.

All three markers (PSA, PAP, and testosterone) will be assayed in patient samples, some of which are cancerous and others which are not. The number of false positives and negatives and true positives and negatives will be calculated to determine the percent specificity and sensitivity of each test. The tests will then be compared by these means to determine which is the most accurate for the diagnosis of prostate cancer. The objective of this study is to compare the diagnostic efficacy of PAP with that of two other markers (PSA and testosterone). It is hypothesized that PAP will prove superior to PSA and testosterone for the diagnosis of prostate cancer.

Literature Review

Cancer

History

Cancer seems to be a disease of modern times. However, it actually has a lengthy recorded history. According to Harrington, Bristow, Hill, and Tannock (2005), even Egyptian mummies have been found with osteosarcomas. Ancient writings and pictures have been discovered that document cases of malignant tumors. Over the years, cancer has been attributed to many different causes. Hippocrates wrote that he believed cancer to be the result of an imbalance of the bodily "humors": the black humor (from the spleen), blood, bile, and phlegm. The first acknowledgement of possible environmental causes of cancer occurred in the Middle Ages (Harrington, L., Bristow, R., Hill, R., Tannock, I., 2005).

One of the most influential studies of cancer was published by Percivall Pott in 1775. Pott studied the relationship between testicular cancer in chimney sweeps and the coal that remained on their skin due to infrequent bathing. As a result of his publication, a Danish worker's guild began to advise bathing daily. By 1892, the incidence of cancer in that region had greatly decreased compared to others (Friedberg, E., 1985). Another implication of Pott's study is the introduction of the idea that cancer can become malignant after a period of latency, that is, exposure to a carcinogenic agent or event may occur years before the production of a tumor. The chimney sweeps in the study were sweeps as young boys, but the cancer did not develop until years later (Harrington, L., Bristow, R., Hill, R., Tannock, I., 2005). Studies such as Pott's firmly identified cancer as the result of environmental causes.

In the nineteenth century, a scientist named Virchow observed the cellular nature of cancer. Virchow postulated that "every cell is born from another cell" (Harrington, L., Bristow, R., Hill, R., Tannock, I., 2005), true of both normal cells and cancerous ones. One notable aspect of cancer cells is their ability to continue growth beyond the point at which normal cells would cease proliferation. Cairns (1975) defined tumors as "groups of abnormally proliferating cells."

Much of the recent study of cancer has focused on genetics and heredity. Studies that focus on this aspect must be performed on inbred populations, such as the Mormons of Utah. As a result, cancer has been recognized as a genetic disease. Not only that, but cancer has been associated both with inherited factors such as mutations of DNA and problems with DNA repair. Research into the genetics of cancer has also suggested that cancer is one disease with multiple causes as opposed to the old belief that each cancer represented a separate disease. Results have also shown that multiple changes in the genetic material are required for tumors to occur and the cell's ability to respond to signals indicating mutations or lack thereof and consequent persistence of the mutation may be among the primary causes of cancer (Harrington, L., Bristow, R., Hill, R., Tannock, I., 2005). This and the recent discovery of the role of vascularization in the early stages of the proliferation of cancer cells has provided strength to arguments of proponents for treatments aimed at the molecular level of cancer.

Epidemiology

McLaughlin and Gallinger (2005) define epidemiology as "the study of the distribution and determinants of disease in human populations."(p.4). The International Agency for Research on Cancer's (IARC) most recent statistics (2010) indicate that the

yearly incidence of cancer in the United States is 1,436,000, with 745,200 male cases and 692,000 female cases. In that year there were 565,600 cancer deaths. The risk of contracting cancer before the age of 75 was calculated to be 33.5% for males and 26.7% for females (total 29.9%), and the risk of dying from cancer before age 75 is 12.8% for males and 9.8% for females (total 11.2%) (IARC, 2010).

Major Types

The International Agency for Research on Cancer (IARC, 2010) determined the five most frequent cancers among both sexes to be lung, prostate, breast, colorectal, and bladder cancers. For women, the most frequent are breast, lung, colorectal, corpus uteri, and non-Hodgkin lymphoma cancers. For men, the top five are prostate, lung, colorectal, bladder, and non-Hodgkin lymphoma cancers. These statistics exclude non-melanoma skin cancers, which are typically the most frequent in both sexes.

Lifestyle Choices

Rosenbaum (1983) stated, "We have control over 70-80% of the causes of cancers." (p.8). Many lifestyle choices have been related to an increased risk of some cancers, such as smoking and lung cancers. Thirty-two percent of cancers are caused by smoking tobacco and 4% by excessive alcohol ingestion (Rosenbaum, 1983). Eighty-seven percent of all lung cancers have been attributed to cigarette smoking, as well as 30% of cancer deaths (Cook, 1996). Alcohol and smoking combined cause even more cancer. Alcohol disturbs the function of the immune system and increases the body's susceptibility to illness and malignancies that cause cancer. It is considered a cocarcinogen that increases the tumor causing ability of other carcinogens (Rosenbaum, 1983).

There is a well-established link between diets high in meat, sugar, and dairy products and increased incidence of breast cancer. Additionally, salted and pickled foods have been linked to stomach cancer in Japan. Overall, increased fat and caloric intake, obesity, meat consumption, and decrease in grains have been observed to correlate with high incidences of breast cancer, colon cancer, and uterine cancer (Rosenbaum, 1983). Even so, carcinogenesis is still a complex multistep process that depends on many other factors such as promoters, genetics, previous damage, and individual resistance.

Oncogenes and Tumor Suppressor Genes

Basic knowledge of oncogenes and tumor suppressor genes is necessary to understand cancer on a molecular level, as these are the primary entities involved in carcinogenesis. All cancers have a genetic component whether somatic or inherited (Loescher, Whitesell, 2003). Oncogenes arise from proto-oncogenes that regulate cells' signaling pathways. When a mutation occurs in a proto-oncogene that activates it to oncogene status, production of the protein produced by the transcription thereof is either increased or the protein itself is altered in structure or function (Hunt, 2005). These types of genes normally encode proteins that act to promote cellular proliferation by participating in signaling pathways that relay growth stimulating signals through cells and are essential to many normal cell functions. The complexity of the regulatory processes controlling the expression of proto-oncogenes has two implications for neoplastic (cancerous) cells: 1) the large number of components involved provides a large number of potential mutation targets, and 2) multiple regulatory pathways ensure that mutation/carcinogenesis must occur in multiple proto-oncogenes to be effective. Growth

factors and growth factor receptors are two types of oncogene products (Loescher, Whitesell, 2003).

Tumor suppressor genes are those whose protein products negatively regulate cell growth by blocking the action of growth promoting proteins (Loescher, Whitesell, 2003). Some have been seen to directly antagonize the action of proto-oncogenes in growth regulation (Fearon, 1998). Some of these genes are normally active transcription factors within the cell nucleus. Abnormal repression of tumor suppressor genes results in deregulation of the cell cycle (excess cellular proliferation by prolonging proliferation signals) or cellular disorganization (Loescher, Whitesell, 2003). Tumor suppressor genes require a germline mutation and a somatic mutation or two somatic mutations to initiate carcinogenesis (Hunt, 2005). This theory is referred to as Knudson's Two-Hit Hypothesis and is based on Knudson's study of the autosomal dominant inheritance witnessed in epidemiological studies of retinoblastoma (Fearon, 1998).

Activation of multiple oncogenes and inactivation of several growth suppression genes is required for the acquisition of a completely neoplastic phenotype (Park, 1998). This is the result of the incorporation of oncogenes and tumor suppressor genes in signaling pathways and cellular regulation (Kalderon, 2000).

Growth Factors, Receptors, and Signaling

Growth factors are protein products that stimulate cells in the resting state to enter the cell proliferative cycle in a process that occurs over several hours to ensure commitment to DNA synthesis by progression factors. The dual requirement of both a growth factor and a progression factor prevents accidental triggering of proliferation as a barrier to neoplasm. They can also promote or block differentiation in turn or along with

proliferation. Consequently, oncogenes derived from growth factor genes cause inappropriate expression of growth factors that cause ongoing stimulation of cell growth and blockage of differentiation (Park, 1998).

Growth factor receptors are an additional product whose derived oncogenes provide cells with the ability to proliferate without the requirement of growth factors (Park, 1998). Under normal circumstances, binding of a growth factor to its receptor initiates a signal that activates certain proteins and transmits a signal to the cell nucleus. The end result is a change in gene expression that ushers the cell through the growth cycle (Loescher, Whitesell, 2003). Different subtypes of growth factor receptors include tyrosine protein kinases, which regulate signal pathway events that affect cell shape and growth through phosphorylation of the amino acid tyrosine, cytoplasmic adaptor proteins, which relay signals from the cell surface to the nucleus by allowing further phosphorylation, proteins with GTP-ase activity, and cytoplasmic serine-threonine protein kinases whose expression is normally limited to germ cells (Park, 1998).

The interaction and regulation of such different protein products is controlled through the interplay of multiple signal proteins through signal transduction pathways. The resulting signals pass within and between cells to coordinate cellular decisions during development (Kalderon, 2000). Transduction changes the conformation of the affected protein and activates its enzymatic product, while generating sites for the recruitment of proteins targeted for further phosphorylation to transmit signals. The ultimate result is the generation of complexes of signal-transducing molecules at sites within the cell where they will then act on the carried signal (Park, 1998). Recall again that the complexity of these pathways performs a service in the prevention of

carcinogenesis, carrying two implications for tumors: 1) the large number of components involved provides a variety of potential targets for oncogene activation and 2) because of the consequent redundancy and cross-regulation within pathways, the conclusion follows that human cancers rarely result from aberrant activation of a single proto-oncogene (Loescher, Whitesell, 2003).

Inheritance of Cancer

Five to ten percent of cancers result from hereditary causes, usually from an autosomal dominant mechanism (gene is present on a non-sex chromosome and only one defective gene is required to cause neoplasm) with a 50% risk to offspring. Those passed through an autosomal recessive mechanism (non-sex chromosome, two defective genes required for neoplasm) cause a 25% risk to offspring (Hunt, 2005). On the genetic level, cancers often consist of chromosomal abnormalities such as gain, loss, or rearrangement of chromosomes, which are heritable. Heritable fragile sites (those sensitive to gaps/breaks) on chromosomes are also associated with cancers. They can result in translocation with damaged material, deletion, or amplification.

However, although many cancers seem to be the result of inheritance, they are not a case of one single-gene inheritance and are not, as many believe, completely inevitable. Rather, it is the result of a multifactorial inheritance involving interaction of genes with the environment. Inheritance of certain genes simply increases one's susceptibility and lowers the threshold of environmental hits required to produce a neoplastic phenotype. Susceptibility in cancers with a genetic component is increased in the following cases: a person with three or more close relatives affected by one or more types of cancer, cases in which at least two generations are affected, and in those involving young people

(Kelly, 1983). The majority of hereditary cancers are caused by mutations of tumor suppressor genes in germline cells (Hunt, 2005).

Chemical Carcinogenesis

Chemical interference is another method of producing a neoplastic phenotype. Most chemicals require metabolic activation to become cytotoxic, mutagenic, or carcinogenic. The primary enzyme group involved in this process and conversely, in the process of detoxification is the microsomal monooxygenases or mixed function oxidases. They usually produce less harmful products but chemical interference can cause the production of more malicious, carcinogenic resultants. This interference can occur within intracellular regulation and biosynthesis leading to an increase or decrease of enzymatic activity, or by direct interference with the catalytic process. Another method of interference is inactivation or destruction of a key factor cytochrome in the detoxification process (Wiebel, 1980). Activation of such chemicals involves addition or alteration of the organic functional groups contained in the molecules and differs between each type of chemical carcinogen (Selkirk, 1980). Chemical carcinogens are typically electrophilic compounds which easily bind to DNA, causing bulky adducts which interfere with correct DNA replication and hence mutation.

After activation, chemicals can cause point mutations that subsequently activate proto-oncogenes and cause neoplasm (Park, 1998). However, there are a finite number of interactions at critical target sites that must occur to lead to tumorigenesis. In most cases, this is the result of a continuous bombardment of subthreshold doses of the chemical. The types of chemicals most known to cause cancer include polycyclic aromatic hydrocarbons such as coal tar, aromatic amines such as chemical dyes, nitrosamines and

nitrosamides such as alkyl urea, and aflatoxin, which is a mold that grows on food. Most of these predominantly cause liver tumors since chemicals are removed from circulation by the liver and metabolized, sometimes resulting in carcinogenic activation of the chemicals (Selkirk, 1980). Polycyclic compounds are capable of delocalization of charge and thus the maintenance of a very active electrophilic status.

Radiation Carcinogenesis

Genetic insult from exposure to radiation can also initiate carcinogenesis. There are two major types of radiation: ionizing and non-ionizing. Ionizing radiation is that which has the capacity to accelerate electrons in matter (Tomatis, 1990). The movement of electrons in macromolecules of the body resulting from the ionizing effects of this type of radiation causes a change in the DNA structure and subsequent expression and function of the products encoded therein. This mechanism of alteration to the cellular DNA is most likely responsible for primary cellular effects and consequently carcinogenesis.(Ullrich, 1980).

Exposure to ionizing radiation includes x-rays, gamma radiation from radioactive nuclides in the air, water, food, and minerals near the Earth's surface, cosmic rays, and nuclear reactors. Cancer as a result of ionizing radiation was first seen in radiologists who developed hand carcinomas and in industrial workers who developed leukemias 20-30 years after exposure to radiation. The effects of ionizing radiation were also investigated in survivors of the atomic bombs in Hiroshima and Nagasaki. An increased incidence of tumors was seen, accompanied by an increased risk to persons exposed in utero. Ionizing radiation has been seen to cause cancer in any organ in which cancer occurs naturally, though organs differ in rates of intrinsic susceptibility (Tomatis, 1990).

The only form of non-ionizing radiation that has been evidenced to have carcinogenic properties is ultraviolet (UV) radiation, which has been connected to skin cancer. The two were first linked in 1896 and the connection was directly demonstrated in 1928 by exposing mice to a UV light. Wavelengths between 280 and 320 nanometers have shown to have the most carcinogenic capacity. However, a single dose is not sufficient to cause tumors, which only appear at the edges of severely damaged tissue (Ullrich, 1980). This suggests that many insults are required before tumorigenesis occurs. Although the mechanism of carcinogenesis by UV radiation is largely unknown, it has been shown that absorption of UV radiation results in electron excitation which may cause carcinogenesis in macromolecules. The formation of pyrimidine dimers, cross-binding of bases in a strand of DNA left unrepaired by a damaged repair mechanism, has been the most studied means of carcinogenesis by UV radiation.

Viral Carcinogenesis

The cellular changes that characterize cancers may also be acquired as a combination of environmental mutagens and infectious agents such as viruses. There are six known oncogenic viruses that have been associated with almost 20% of all human cancers. They are hepatitis B, hepatitis C, human papillomavirus, the Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus, and human T-cell leukemia virus 1. Most oncogenic viruses cause carcinogenesis either by activating pre-existing oncogenes through insertion of a provirus into cellular chromosomes, encoding regulatory proteins to affect cell growth and death, or carrying an oncogene in their genome which has cellular homologues (Zheng, Ou, 2010). Some directly transform cells and are required

from that point forward to perpetuate the cancer, while others are only necessary during the initial stages of transformation (Tomatis, 1990).

Viral transformation is aided by the viruses' ability to interfere in cellular communication through signal transduction. This interference is accomplished through viral proteins which mimic cellular signal ligands, signaling receptors, and intracellular signaling adaptors. This mimicry activates cell surface receptors and fools the cell into entering continuous proliferation while blocking cell death, thus deregulating the cell cycle to cause the neoplastic phenotype.

Chronic infections of hepatitis B and C viruses have been linked to hepatocellular carcinoma and liver disease while human papillomavirus is associated with cervical cancer and skin or genital warts. Epstein-Barr virus has been studied in great detail in association with malignancies such as gastric and nasopharyngeal carcinoma, Burkitt's lymphoma, immunoblastic lymphoma, and T-cell lymphoma. Kaposi's sarcoma-associated herpesvirus is associated with its namesake, Kaposi's sarcoma, which is a multifocal angioproliferative disorder that most often presents as a cutaneous lesion. Finally, human T-cell leukemia virus-1 is most often associated with adult T-cell leukemia, a malignancy with poor prognosis (Zheng, Ou, 2010).

Human immunodeficiency virus (HIV) lowers the body's natural defenses by weakening the immune system and thus increases the risk of several types of cancer. Additionally, people with HIV often become infected with other viruses that also increase the risk of cancer, such as Epstein Barr Virus (EBV) and Human Papillomavirus (HPV). Those with HIV are at several thousand times higher risk of diagnosis with Kaposi's sarcoma, and 70 times higher risk of diagnosis with non-Hodgkins lymphoma, compared

to those without HIV. Women with HIV are 5 times more likely to be diagnosed with cervical cancer as compared to women without HIV. Other cancers with increased risk to those with HIV include anal, liver, and lung cancer, and Hodgkins lymphoma. However, the recent introduction of highly active antiretroviral therapy has decreased the incidence of Kaposi's sarcoma and non-Hodgkins lymphoma among people with HIV. This therapy acts by lowering the amount of HIV circulating in the bloodstream (National Cancer Institute, 2011).

Cell Proliferation and Tumorigenesis

The products of proto-oncogenes have direct effects in the control of gene expression in normally proliferative cells as transcription factors. They perform this function until a loss of the negative regulatory regions, producing uncontrolled proliferation, or a loss of positive regulatory regions, resulting in a lack of expression of genes whose products are required for differentiation. These two events are both key to the neoplastic phenotype. Proto-oncogene products are also required for a cell to transition from the resting state to the beginning of the normal cell proliferation cycle and at certain points during the cycle (Park, 1998).

The cell cycle consists of five phases: G1, preparation for DNA synthesis; S, DNA synthesis; G2, preparation for mitosis; M, cell division; and G0, the resting state. A number of mitogenic signals regulate these interconnected phases in a system that is cross-linked and double-checked (Nakamura, 1997). Normal cells have a limited propagation potential that averages 60-70 cell divisions. After this point, cells become senescent unless (as in carcinogenesis) loss of tumor suppressor genes confers additional replicative capacities. In this case, the cells eventually enter a state of crisis including

massive apoptosis and joining of chromosomes. Normally, propagation is limited by a sequence at the ends of chromosomes called a telomere which is shortened with each cell division. At a certain point when the telomere is gone, the cell can no longer divide because it begins to lose DNA from the ends of the chromosomes instead. However, an enzyme called telomerase can replace the telomeres and is expressed in low levels in healthy cells. Reactivation of this enzyme is seen frequently in malignantly transformed cells. The telomere may also be maintained by an alternative recombination-based mechanism. These two mechanisms are the main ones by which cancer cells attain an infinite multiplication potential (Zheng, Ou, 2010).

Genetic instability is one of the main characteristics of tumorigenesis and the resulting cancer, and is caused by gene mutations, microsatellite instability, or chromosomal instability. Accumulation of many of these genetic alterations in the genome is required for the multistep process of tumorigenesis and the continued progression of tumors. Healthy cells have a variety of protection mechanisms for genomic integrity such as highly accurate DNA polymerases to reduce error during replication, mechanisms employed to monitor DNA damage and restore damaged information (such as base excision repair), and mitotic checkpoints during the cell cycle to analyze abnormalities produced during mitosis or chromosomal segregation (Zheng, Ou, 2010).

Tumor Progression and Metastasis

Approximately 30% of new cancer patients with solid tumors have evident metastases. Metastases are the main cause of cancer deaths and the definition of malignant cancers. A “benign” tumor is one which is localized and amenable to local

surgical removal and resulting survival of the patient. Tumors become “malignant” when they invade and destroy adjacent organs and/or spread to distant sites. Malignancy is also characterized by a spread from one organ to another that is not directly adjacent to it. Dissemination of a tumor greatly decreases the possibility of a cure, if not prevents it entirely (Bani, Gaivazzi, 2000).

There are several mechanisms of metastatic spread of tumor cells which differ between types of cancers. Cells may invade a nearby natural body cavity, use the lymph fluid as an enhancement of motility, or use a hematogenous means. Carcinomas typically spread lymphatically, while sarcomas spread hematogenously. Due to numerous interconnections of all systems, most cancers use both of these most common routes. For example, a group of tumor cells may travel through the lymph nodes to reach the vascular compartments. However, all metastases progress through the same series of steps which may occur at the same time or in a series. First, a group of cells detaches from the solid tumor mass, invades the surrounding healthy tissue, and intravasates to the vascular channels. The cells must then survive as a clump or reduce to a single cell in the circulation before stopping in the capillary bed of a new site. Next the cell or cells extravasate through the vessel wall and infiltrate the surrounding tissue compartment before growing into a new solid tumor. During the whole process, the tumor cells have to evade constant immunologic surveillance, fail to respond to growth control factors, and promote angiogenesis (Bani, Giavazzi, 2000).

Malignant tumor progression is marked by the accumulation of genetic alterations which leads to permanent phenotypic changes, and is perpetuated more efficiently through metastasis (Bani, Giavazzi, 2000).

Angiogenesis

"Angiogenesis is the formation of new blood vessels from the existing vascular bed." (Piulats, Mitjans, 2000, p.271) In healthy (non-cancerous) body environments, angiogenesis is found in wound healing and in endometrium vascularization during the menstrual cycle, but it is also a key component of cancers (Piulats, Mitjans, 2000). A tumor may only grow so large until nutrients and blood can no longer reach the innermost cells by diffusion. New blood vessels must be produced to carry them to the central cells of the tumor mass.

According to Piulats and Mitjans (2000), neovascularization begins when the tumor grows larger than one millimeter cubed and is carried out by the extracellular matrix and endothelial cells when the tumor cells secrete angiogenic factors to attract endothelial cells. Similar angiogenic factors such as angiostatin and thrombospondin regulate the response in healthy environments. The first endothelial cells at the site become activated and produce paracrine growth factors for the tumor that increase tumor growth and angiogenic potential. This cross-communication between endothelial cells and tumor cells is one of the hallmarks of angiogenesis. Another hallmark is a precarious equilibrium between inducers and inhibitors of neovascularization, which is affected towards the inducement end of the spectrum by the effect of tumor cells to promote inducers such as transforming growth factor beta and platelet-derived endothelial growth factor (Piulats, Mitjans, 2000).

The three defined steps of angiogenesis are induction-initiation, proliferation-invasion, and maturation-remodeling. The initiatory step of angiogenesis, induction, is characterized by the release of inducers such as growth factors or cytokines both from

tumor cells and other cells recruited to the area. Proliferation-invasion involves a promotion of blood vessel growth in the direction of the tumor mass. Changes in cell adhesion are made that allow interaction of endothelial cells with the surrounding environment to promote angiogenesis and cell survival. Finally, maturation-remodeling consists of stopped proliferation and the beginning of differentiation of vessel walls and lumen (inner vascular space) formation followed by blood circulation to the tumor mass (Piulats, Mitjans, 2000).

Angiogenesis indicates a particularly advanced stage of disease. Therefore, knowledge about the process retains both prognostic and therapeutic value.

Apoptosis

Apoptosis is programmed cell death that usually occurs as a result of a cellular process involving both biochemical and morphological changes. It is often a response to extensively damaged DNA and allows for removal of old, dead, or nonfunctional cells (Loescher, Whitesell, 2003). Apoptosis is unique because it is the result of an inherent, regulated pathway that is a part of all cells of the body and not the result of trauma or external factors as in necrotic cell death. It also does not result in the release of cytokines or any inflammatory response (Rudin, Thompson, 1998). Characteristic morphologic marks of apoptosis include condensation of nuclear material, cytoplasmic condensation resulting in cellular contraction and loss of adhesion, and an irregular cell surface with protrusions that round up and break away (Archer, Trott, Dowsett, 2000). This process is highly regulated and a key part of inhibition of carcinogenesis (Rudin, Thompson, 1998).

Conversely, escape of apoptosis is also a key component of the progression of a neoplastic phenotype. For example, the cell cycle may become deregulated resulting in

uncontrolled proliferation but unless apoptosis is controlled, offending cells may be destroyed before progression can occur. Apoptosis is initiated by both intra- and extracellular signals including DNA damage (intracellular) and tumor necrosis factor-alpha (extracellular). Signal transduction occurs throughout the cell, culminating at the mitochondria where cytochrome c is released. This product activates a group of enzymes called caspases that initiate a cascade to perpetuate cell death signals to different cellular components, causing their disintegration. Mutations in genes involved in either the signaling or the consequent cascade can result in escape from apoptosis (Zheng, Ou, 2010).

Tumor Markers

Tumor markers are abnormal molecules or processes whose presence indicates a change in cellular environment to malignancy. They have great prognostic value because they allow medical personnel to differentiate a large population of cancerous individuals into subpopulations based on stages of increasing malignancy and differing in prognosis. They can also be used to predict which therapies are likely to have the best effect on a patient. Use of such markers can prevent the unnecessary treatment (e.g. potentially toxic exposure to drugs) of non-cancerous individuals exhibiting similar symptoms. Markers can include changes at the genetic level (mutations), the transcriptional level (over/underexpression), the translational level (increased/decreased level of product), or the functional level (presence of neovascularization). They can be assessed using various clinical methods and assays. The American Society of Clinical Oncology tests and approves various tumor markers through a rigorous method and consequently

recommends very few for analysis based on their sensitivities and specificities (Hayes, 2000).

An important tumor marker is TP53, derived from the p53 gene proteins. The p53 gene is integral to many cell processes and is highly conserved. Mutations thereof and subsequent production of TP53 are associated with almost all tumor types, especially colorectal carcinoma, and carcinomas of the breast, lung, esophagus, stomach, liver, and bladder. TP53 is an important marker predictive of poor prognosis, increased risk of relapse and cancer death risk, though this prognostic use is controversial. RAS is another group of highly conserved genes involved with cellular proliferation and homologous to several viral oncogenes. Mutations of RAS produce continuous signals leading to malignant transformation and are associated with cancers such as adenocarcinoma of the pancreas, colon cancer, and lung cancer. MYC is a marker whose cellular version has been isolated from neuroblastoma and small-cell lung cancer. It encodes for nuclear DNA-binding proteins involved in the regulation of transcription and is involved in control of proliferation, transformation, differentiation, and inducement of apoptosis. Other notable genetic markers include HER-2, RET, BCL2, BCL1-PRAD1-CCND1, REL, and BCL-6 (Birindelli, Aiello, Lavarino, et. al, 2000).

Tumor markers also include those derived from chromosomal or DNA-level instability. Microsatellites, or simple sequence repeats, can be detected by increased incidence. MSI are errors that occur in microsatellite sequences during replication and produce expanded or shortened sequences that cause cellular confusion issues. Loss of heterozygosity reflects mutations that cause a loss of function in tumor suppressor genes.

Loss of heterozygosity also has value as an early diagnosis marker and later as a prognostic and therapeutic response marker (Birindelli, Aiello, Lavarino, et. al, 2000).

Prostate Cancer

Epidemiology

Prostate cancer has been determined to be the most frequent cancer in men, representing about 30% of all male cancer cases, excluding non-melanoma skin cancers (IARC, 2010). In 2004, there were an estimated 230,110 diagnoses and 29,900 deaths due to prostate cancer in the United States. Risk factors include increased age, family history or genetics, African-American ethnicity, hormone levels, and increased serum level of prostate specific antigen (PSA). Ninety-five percent of total cases occur in men ages 45 and older with a dramatic increase in incidence at the age of 55 (Stratton, S., Ahmann, F., 2005). Less than 1% of prostate cancer cases occur in males less than 40 years of age and the peak frequency of 1 in 7 occurs in men in their 80s and 90s (Isaacs, Bova, 1998).

Prostate cancer has proven to be one cancer which has an appreciable genetic component. Five to ten percent of prostate cancers are thought to be attributable to the inheritance of certain alleles that increase susceptibility (Isaacs, Bova, 1998). Genetic study has even gone so far as to link the appearance of prostate cancer with specific anomalies on chromosomes eight and ten (Stratton, Ahmann, 2005).

Ethnicity is also a risk factor for prostate cancer. African American men have the highest incidence (32% more than American white males) and mortality rate. The increased mortality rate may also be due to the fact that African-American males are 25% less likely to undergo regular prostate screenings. (Stratton, S., Ahmann, F., 2005). Prostate cancer has shown to be uncommon in Asian males and alternatively high in

Scandinavian countries, such as Finland (Isaacs, Bova, 1998). There has recently been a rise in the incidence of prostate cancer in Japan, thought to be the result of the appearance of a more Westernized diet and lifestyle in the East in conjunction with increased exposure to environmental contaminants. Because of the increase in incidence with age, there is a higher incidence in developed countries with a longer life expectancy. Prostate cancer accounts for about 4% of male cancer in developing countries, as opposed to 15% in developed countries. The epidemiology suggests a combination of environmental and genetic causes (Stratton, S., Ahmann, F., 2005).

Signs and Symptoms

The signs and symptoms of prostate cancer are somewhat vague and indistinct and as a consequence, cases of prostate cancer are often asymptomatic and unexpectedly diagnosed as the result of a regular screening test (Bostwick, MacLennan, Larson, 1996). Signs and symptoms can include weak or interrupted urine flow, inability to urinate, difficulty starting or stopping urine flow, need to urinate frequently, especially at night, blood in the urine, pain or burning on urination, and continuing pain in the lower back, pelvis, or upper thighs (Cook, 1996). Other nonspecific symptoms can include a loss of appetite or weight loss (Bostwick, MacLennan, Larson, 1996).

Diagnosis and Staging

Diagnosis of prostate cancer is performed using a full medical history and a complete physical, followed by certain specific prostate tests. A rectal exam is often performed, in which a physician inserts a gloved finger into the rectum to palpate the prostate for hard or lumpy regions that could prove to be tumorous. A prostate specific antigen (PSA) test is also performed on a sample of the patient’s serum. Both prostate

cancer and benign prostatic hyperplasia (a non-cancerous condition associated with excess prostate growth) are accompanied by an increase in serum PSA. A test for prostatic acid phosphatase (PAP) is also sometimes performed; as PAP rises are seen in many prostate cancer patients, especially if the condition includes metastases of the tumor. Other tests include transrectal ultrasonography, in which a probe inserted in the rectum emits sound waves to produce a sonogram of the prostate; an IV pyelogram, which produces x-rays of the organs of the urinary tract to search for tumors causing pressure to the area; and urine tests for hormone levels and blood. A biopsy is often performed later in the diagnosis to confirm the cancerous nature of a suspected tumor mass (Cook, 1996).

After diagnosis, patients are classified by their tumor's grade and stage. Grading is based on the differentiation state of the tumor cells and is determined by the appearance of extracted cells viewed under a microscope. The tumor is then graded by the Gleason system with a number from one to five, with one being well-differentiated and five being undifferentiated. Staging concerns the extent of the cancer in the patient, how large it is, whether it has spread beyond the prostate, and if so, how far. Staging is complex and combines multiple testing parameters such as rectal exam results, PSA level, estimated tumor volume, and other tests. The tumor is then staged based on the tumor, node, metastases (TNM) system. The scale starts with T0 which goes to T4, then moves up to N0 to N3, then to M0 and M1. The T portion of the scale describes the extent of the primary tumor, which is the only mass present at that point. The N portion describes the appearance and state of pelvic area lymph nodes (often the first site of metastasis). Finally, the M portion of the scale indicates the presence or absence of

distant metastases. The numbers increase with the relative seriousness of each portion of the scale and the number 0 by a letter indicates that there is no evidence of that parameter (Bostwick, MacLennan, Larson, 1996).

Treatment

The choice of therapy generally depends on the patient's age and the stage of the tumor, as there are several methods to choose from. Surgery is an ideal choice if the cancer has not spread from the primary tumor. A radical prostatectomy consists of the removal of the prostate and some of the surrounding tissue and is often preceded by a dissection of the pelvic lymph nodes to ensure they contain no cancer. Side effects include impotence and leakage of urine from the bladder. A transurethral resection is also an option to cut the cancer from the prostate and is often used to relieve symptoms. Cryosurgery has become an option in recent times to kill the cancer by freezing it (Cook, 1996).

Radiation therapy is a local treatment option in which high energy rays damage the DNA of the cancer cells and prevent them from dividing. The patient's normal cells are also affected, but have a greater capacity to recover from the effects. Radiation also works best early on when the cancer is still confined. It is also used in conjunction with hormone therapy or after surgery to destroy remaining cancerous tissue. Radiation therapy is accomplished with external devices or internally implanted pellets (Bostwick, MacLennan, Larson, 1996).

Hormone therapy is a systemic treatment option to prevent the cancer cells from receiving the male hormones they require to grow, such as testosterone. It mainly controls the tumor and alleviates symptoms (Bostwick, MacLennan, Larson, 1996).

Removal of the testicles eliminates a source of testosterone, while administration of estrogen stops its production. However, the adrenal glands still produce small amounts of male hormones and side effects include growth of breast tissue, hot flashes, loss of sexual desire, impotence, nausea, and vomiting. This therapy cannot work indefinitely. Eventually, the tumor cells gain the ability to continue growth without the presence of testosterone (Cook, 1996).

The final treatment option is chemotherapy, or the administration of drugs to kill cancer cells. They may be taken in pill form or injected directly into the bloodstream or prostate. Thus far chemotherapy has had little significant effect on prostate cancer (Cook, 1996).

Markers of Prostate Cancer

Molecular markers of prostate cancer include oncogenes, tumor suppressors, proteins involved with inflammation, and serum proteins that usually increase in relation to prostate cancer. Androgens such as testosterone and 5α-dihydrotestosterone are involved in male sexual differentiation and are associated with prostate cancer. Androgen receptor polymorphisms such as trinucleotide repeats and single nucleotide polymorphisms are related to variations in prostate cancer risk. Nuclear factor κB is frequently overexpressed and activated in prostate carcinomas. Interleukin-6, cyclooxygenase-2, and B-cell lymphoma/leukemia-2 are also important indicators involved in processes surrounding the development of prostate cancer such as inflammation and apoptosis (Stratton, Ahmann, 2005).

Prostate specific antigen (PSA) is the primary marker screened for in potential cases of prostate cancer and is so far the most accurate indicator thereof. PSA is a serine

protease with strong substrate specificity and is normally secreted by the prostate in large amounts into the seminal fluid. The normal level of PSA in the blood stream is 4 ng/mL, though this fluctuates with age. The level dramatically increases with the development of prostate carcinoma and is a reliable determinant of the progression of the disease. It is often assayed after treatment to assess the function of the remaining prostate and possible presence of remaining cancer cells (Isaacs, Bova, 1998).

PSA is produced by the prostatic epithelium and functions in liquefaction of the seminal coagulum. Incidence of an elevated serum level increases with the stage of the tumor. Elevated amounts are found in both prostate cancer and benign prostatic conditions and falsely elevated amounts can be caused by a rectal examination. Additionally, not all prostate cancer cases are associated with levels above the normal range, with 20% of tumors accompanied by a normal level. However, in those with elevated content, the rate of increase can distinguish between patients with local or regional disease and those with advanced metastatic disease. A decrease after treatment indicates a patient with a good prognosis in remission and changing levels can indicate whether post-therapy irradiation is necessary (Horwich, Ross, 2000).

Levels of PSA between 4.0 and 10.0 ng/mL are associated with a diagnostically vague range of conditions, as levels caused by benign prostatic hyperplasia and prostate cancer mostly overlap. With a biopsy at this stage, 75% of men would prove to have no evidence of malignancy. PSA levels above 10.0 ng/mL indicate a 40-50% chance of prostate cancer and biopsies are frequently performed on men with serum levels above this point. The likelihood of an organ-confined malignancy is as low as 25% (Haese, Becker, Diamandis, et. al 2002).

Testosterone is a steroid hormone whose measurements are useful in evaluating the hypogonadal states. High levels of testosterone are associated with hypothalamic pituitary unit diseases, testicular tumors, congenital adrenal hyperplasia, and prostate cancer (Diagnostic Automation, Inc., 2001). Testosterone is the most important form of androgen and 95% is produced by the testicles. It is a requirement for prostate growth and male virilization. Because of the effect of testosterone on growing prostate cells, it also fuels the growth of prostate cancer cells and is required for the continued growth of the tumor mass (Bostwick, MacLennan, Larson, 1996). Consequently, the conclusion has been reached that an increased mass of prostate tissue, as caused by a tumor growth, would be accompanied by a rise in testosterone levels. Thus, it follows that an assay for the levels of testosterone in a patient suspected of having prostate cancer might indicate the presence of a tumor and possibly the tumor's size.

The first tumor marker used for prostate cancer was prostatic acid phosphatase (PAP). Acid phosphatases are a family of proteins found in many different tissues and whose isoenzymes have many different properties. PAP is mainly composed of two of these isoenzymes which are found in the prostate as well as the granulocytes and the pancreas. Consequently, levels of PAP may be elevated in a variety of conditions such as polycythemia rubra vera, granulocytic leukemia, Gaucher's disease, pancreatic cancer, as well as prostate cancer. PAP is produced by epithelial cells lining the prostatic acini and is found in high concentrations in prostatic fluid in healthy patients and in the serum of more than 75% of patients with metastatic prostate cancer. However, PAP tests have been shown to have relatively low sensitivity and specificity in the diagnosis of prostate cancer and were replaced by the PSA screening test (Horwich, Ross, 2000).

In recent times, PAP has mainly been used to monitor response to therapeutic approaches and determine patients' clinical status. Increasing levels correlate with increased progression of the disease and with metastasis. (Wu, 1997). PAP levels are also utilized in making therapeutic decisions such as predicting the efficacy of surgery on a particular patient. Some rare tumors may not produce PSA, in which case PAP is used as the primary tumor marker to assess their condition. PAP has also been reported to have predictive value in determining which patients are most likely to have recurrences of their cancer and when to expect long term failure. In these cases, PAP was reported to have more value than Gleason score or PSA level (Haese, Becker, Diamandis, et. al, 2002).

Prostate Cancer Assays

Assays for markers of prostate cancer are accomplished by incubating a patient serum sample with various reagents and colorimetric indicators in microwell plates with antibodies to a particular marker embedded in its walls. The marker attaches to the antibody and unbound antigen is washed away. The intensity of the color produced is dependent upon the amount of marker-antibody complex and is indicative of the concentration of the tumor marker. The intensity is quantitated by its absorbance using spectrophotometric methods.

For assays to be used in the diagnosis of patients, both high specificity and sensitivity are desired. An antibody should be chosen which has a high affinity for binding to the marker to be tested to increase the sensitivity, and exclusive binding to the specific marker to increase the specificity. The use of monoclonal antibodies also increases specificity by exclusively binding to one epitope of a tumor marker, as opposed to polyclonal antibodies which bind to any of a number of epitopes which may be shared

by several different tumor markers. The most popular test format is a sandwich format enzyme-linked immunosorbent assay (ELISA), in which a specific antibody is absorbed in the solid phase on the walls of a microwell plate. A sample of serum is added and the antigen (the tumor marker) is allowed to bind during an incubation period in the presence of a reagent. The solution is washed and any unbound antibody is washed away. The solid phase contains the tumor marker "sandwiched" between the solid phase capture antibody and the added indicator antibody. An enzyme substrate is added which produces a colorimetric reaction whose intensity is directly proportional to the concentration of the tumor marker in the original sample. The other popular test method involves the principle of competitive binding, in which antigen (tumor marker) in the sample competes with a determinate amount of radioactively labeled antigen for binding with the antibody. The complexed antigen is separated from the free antigen and the amount of radioactivity is used to determine the concentration of antigen in the original sample (Wu, 1997).

PSA is typically measured by radioimmunoassay. Sensitivity and specificity may be increased by refining the assay's parameters to include PSA density, velocity, and relativity to age, fractionation, and measurement of cells in the circulation expressing PSA mRNA. (Horwich, Ross, 2000). PAP is measured using electroimmunoassay using monoclonal antibodies (Wu, J., 1997). Testosterone is measured using electroimmunoassay based on the principle of competitive binding (Diagnostic Automation, Inc., 2001).

A normal reference interval (NRI) is determined by sampling a large range of the population and plotting the results on a graph of frequency versus concentration. The mean of the set of values plus and minus two standard deviations is usually accepted as

the range of healthy patient values as this range covers 95% of healthy people. These ranges are used in making diagnoses to determine the disease states of patients and the extent of their conditions. They vary by analyte, gender, age, and test method. The normal range for PSA is less than 4 ng/mL and for PAP, less than 5 ng/mL. For men under 50, the range for testosterone is 2.9-13 ng/mL and for men over 50 it is 1.8-7.5 ng/mL.

This study will examine the efficacy of tests for PAP and testosterone in comparison with the current standard PSA in the diagnosis of prostate cancer. It is designed to determine the percent sensitivity and specificity of each test based on calculations using the number of false positives and negatives; and true positives and negatives of each test. The sensitivities and specificities will be compared to determine the most accurate test. Finally, predictive values will be determined on all three markers as a whole to evaluate their use together. This study is executed on the hypothesis that PAP was discarded too early in its use as a guide to the diagnosis of prostate cancer and that present test methods provide a more accurate quantitation of its concentration, making it a better indicator of the presence of prostate cancer.

Materials and Methods

Materials

The kits used in this project were acquired from Diagnostic Automation, Inc. (Calabasas, CA). All solutions utilized were prepared using diluents present in the kits. Tests were performed using immunoassays for prostatic acid phosphatase and testosterone. Statistical analyses were performed using SPSS version 18 statistical software. The samples were tested for prostate specific antigen at the hospitals of their origin. Permission for this study was granted by the University of Southern Mississippi Institutional Review Board under the protocol number 11080903 in accordance with Federal Drug Administration regulations, Department of Health and Human Services, and university guidelines to ensure adherence to stipulated criteria.

Patient serum samples were obtained from Memorial Hospital at Gulfport (Gulfport, MS) and Singing River Hospital (Pascagoula, MS) with only a patient number and the cancer diagnosis provided. Normal samples, obtained from Wilford Hall Medical Center (U.S. Air Force Base in San Antonio, TX) and Forrest General Hospital (Hattiesburg, MS), were also utilized from persons not suspected of having cancer to provide a basis of comparison. All procedures detailing the confidentiality of patient medical records were followed and no information regarding the identification of a specific patient was released by the hospitals involved. Aseptic techniques were used at all times with the samples. Samples were collected by hospital personnel at the respective hospitals, allowed to clot, and centrifuged before being frozen and packaged in plastic tubes for transport. Before testing, all samples were sorted into test tube racks and

allowed to reach room temperature by soaking in a low level water bath at approximately 25˚C.

Patient samples were classified by the hospital pathologists as either cancerous or cancer free (Table 1). This diagnosis was provided for comparison only. One hundred two normal control samples (from males in good health) were tested without bias in order to generate a normal (healthy) interval for reference.

Three test procedures were used in this experiment and consequently three sets of materials were required. The results of the assays performed in the laboratory were read with a Beckman Coulter AD 340 microplate reader. The washing of the micro-well solutions was done with a Stat Fax 2600 microplate washer. The assays performed at the provider hospitals were done with a Beckman Coulter Synchron LXI 725/Beckman Access process.

Table 1

Test Sample Classification

Number of Samples	Cancer Diagnosis
82	Cancerous
469	Cancer free

Total patients evaluated: 551

Prostatic Acid Phosphatase kit

The kits catalog #42272 and lot #12301054 used for this procedure came from Diagnostic Automation, Inc. Materials that were required and not provided with the kits include disposal tips, pipettors of 25 µL and 100 µL, a microwell reader, and deionized water for use as blanks.

The prostatic acid phosphatase (PAP) kit used is a quantitative solid phase enzyme linked immunosorbent assay with a detection range of 0-30 µg/mL. The test requires 50 µL of serum and performs to a specificity of 96% at a sensitivity of 1 µg/mL (as recorded by Diagnostic Automation, Inc.). The wells provided are coated with anti-PAP antibodies and the enzyme conjugate is a mixture of anti-PAP antibodies chemically conjugated to horseradish peroxidase. The antibodies in the conjugate have different affinities toward epitopes of PAP molecules. The conjugate binds to the sample mixture in an amount proportional to the amount of PAP in the sample. Washing the solutions with the wash buffer removes any unbound conjugate. After addition of the TMB solution, a colorimetric reaction occurs whose final color intensity is proportional to the bound enzyme conjugate and thus the concentration of PAP present (Diagnostic Automation, 2010).

In preparation for the assay, all reagents and samples were brought to room temperature (24±3˚C) and gently mixed. The kit components, reagents and samples were unpackaged and placed in the work space. The wash buffer was prepared by adding 10 mL washing buffer concentrate into 990 mL distilled water in a large flask. The mixture was capped and inverted several times before pouring into the wash solution bottle.

Blanks (deionized water), calibration solutions, and controls (calibration solution of 3 ng/mL was used as the control) were run in duplicate in the first 14 wells of each kit. The remaining wells contained serum samples or extra controls. A data sheet was kept to identify samples, calibrators, and controls with their respective well locations. New pipet tips were used for each dispensation. The procedure in figure 1 was followed.

Figure 1

Prostatic Acid Phosphatase Immunoassay Kit Procedure

1. Secure the wells in the holder.
2. Bring all reagents and samples to room temperature and mix gently.
3. Dispense 25 µL of references, controls, or serum samples into the appropriate wells.
4. Dispense 100 µL of enzyme conjugate into wells.
5. Incubate for 30 minutes at room temperature.
6. Remove incubation mixture.
7. Rinse the wells 5 times with washing buffer (300µL/well/each rinse).
8. Dispense 100 µL of TMB solution into each well.
9. Incubate for 15 minutes at room temperature.
10. Stop reaction by adding 50 µL of stop solution into each well.
11. Read O.D. at 450 nm with a microwell reader within 5 minutes.

Testosterone kit

The kits catalog #RN-42074 and lot #RN-42010 used in this procedure came from Diagnostic Automation, Inc. Materials required for the assay and not provided with the kits include disposable tips and pipettors of 10 µL, 50 µL, 100 µL, and 1.0 mL, deionized water, and a microwell reader.

The testosterone kit used is an enzyme immunoassay intended to quantitatively determine the concentration of testosterone in human serum. Diagnostic Automation, Inc. recorded its sensitivity to 0.05 ng/mL. The assay requires 10 µL of serum. Samples are dispensed into anti-rabbit IgG-coated wells and incubated with testosterone-HRP conjugate and rabbit anti-testosterone. The testosterone-HRP (fixed, known amount) competes with the testosterone in the sample to bind to the testosterone antibody (with a fixed number of binding sites). Unbound testosterone is washed away. Consequently, the detectable amount of testosterone-HRP bound to the wells decreases as the amount of testosterone in the sample increases. The TMB reagent added to the solution produces a colorimetric reaction which is then stopped by the addition of the stop solution. The intensity of the color produced can be measured spectrophotometrically to determine the amount of enzyme bound to the wells, which has an inversely proportional relationship to the concentration of testosterone in the samples (Diagnostic Automation, Inc., 2001).

In preparation for the assay, all reagents and samples were brought to room temperature (24±3˚C) and gently mixed. The kit components, reagents and samples were unpackaged and placed in the work space.

References, controls, and serum samples were run in duplicate at the beginning of each procedure. A data sheet was recorded with the identification of samples, references, and controls and their well numbers. The procedure in figure 2 was followed.

Figure 2

Testosterone Immunoassay Kit Procedure

1. Secure the coated wells in the holder.
2. Dispense 10 µL of standards, specimens, and controls into appropriate wells.
3. Dispense 100 µL of testosterone-HRP conjugate reagent into each well.
4. Dispense 50 µL of rabbit anti-testosterone reagent into each well.
5. Thoroughly mix for 30 seconds.
6. Incubate at 37˚C for 90 minutes.
7. Rinse and flick the microwells 5 times with distilled or deionized water.
8. Dispense 100 µL of TMB reagent into each well.
9. Gently mix for 5 seconds.
10. Incubate at room temperature (18-25˚C) for 20 minutes.
11. Stop the reaction by adding 100 µL of stop solution to each well.
12. Gently mix 30 seconds until all the blue color turns to yellow.
13. Read absorbance at 450 nm with a microwell reader within 15 minutes.

Prostate-Specific Antigen test

This assay was performed in the hospital laboratories where the patient samples originated. The reagent kits came from Siemens Healthcare Diagnostics with the catalog name ADVIA Centaur Assay.

This PSA assay procedure has been labeled a "two-site sandwich immunoassay" (Siemens, 2009) because of its use of two antibodies that "sandwich" the antigen. Constant amounts of both antibodies are used. The first antibody (a polyclonal goat anti-PSA antibody) is labeled with acridium ester, while the second (a monoclonal mouse anti-PSA antibody) has been linked to paramagnetic particles. The combination of these antibodies with the antigen (PSA) leads to a chemiluminescent reaction that can be measured in relative light units (RLUs). The amount of RLUs expressed is in direct correlation with the amount of PSA present in the patient sample. This test requires 35µL of serum and is performed automatically by the ADVIA Centaur system.

In preparation for the assay, all reagents and samples were brought to room temperature (24±3˚C) and gently mixed. The kit components, reagents and samples were unpackaged and placed in the work space. The procedure in Figure 3 was followed.

Figure 3

Prostate Specific Antigen Procedure

1. Prepare the specimen container for each specimen, and place barcode labels on the specimen containers, as required.
2. Load each specimen container into a rack, ensuring that the barcode labels are clearly visible.
3. Place the racks in the entry queue.
4. Ensure that the assay reagents are loaded.
5. Start the entry queue if required.

Results

Over the course of the project, quality control samples were incorporated into the assays to determine within- and between-run precision (Table 2). For the PAP assays, the calibrators provided were used, and additionally the provided 3 ng/mL calibrator was used as a control. For the testosterone assays, the calibrators and controls provided (control 1=0.486-1.5 ng/mL, control 2=5.2-14.0 ng/mL) were utilized. The coefficient of variation (%CV) for PSA was low (2%), but those for PAP and testosterone (41.78% and 23.29%, 10.74%, respectively) varied a great deal. Serial dilutions of patient samples were used to determine the linearity of the assays (Table 3, Graphs 1-3). These results indicate good linearity, with all results being around 0.98. The minimum concentration each assay is able to detect (assay sensitivity) was determined by analyzing 20 replicates of the diluent and calculating the mean±2 standard deviation, which was established as the cut-off value (Table 4). Assay sensitivities ranged from 0.000-2.330.

The normal reference intervals (NRI) are given in Table 5. The NRIs were obtained by assaying sera from approximately 100 healthy adult males and calculating the mean±2SD. The intervals obtained were significantly increased over those given in the manufacturers' inserts for the PAP assay

In determining the normal (negative) and abnormal (positive) patient results, cut-off values from the manufacturers' inserts were used (Table 6). In this way, diagnostic sensitivities of 30.12% (PSA), 20.73% (PAP), and 0.00% (testosterone) were obtained. Sensitivities for combined markers are 30.12% (Testosterone and PSA), 43.37% (PAP and PSA), and 43.37% (testosterone, PAP, and PSA). Diagnostic sensitivity is the proportion of individuals with a disease who test positively with the test in question for

that disease. The higher the sensitivity, the more accurate the test is. Similarly, diagnostic specificity is the proportion of individuals without the disease who test negatively with the test in question. Diagnostic specificities of 91.29% (PSA), 80.38% (PAP), and 96.80% (testosterone) were obtained, which are all relatively good. Combined specificities were 89.15% (testosterone and PSA), 75.11% (PAP and PSA), and 72.77% (testosterone, PAP, and PSA). Other diagnostic parameters evaluated are predictive value (+), which is the fraction of positive tests that are true positives, predictive value (-), which is the fraction of negative tests that are true negatives, and diagnostic efficiency, which is the fraction of all test results that are either true positives or true negatives.

Table 2

Assay Precision: Comparison of PSA with PAP and Testosterone using Control Sera

Within-Run

Assay	N	X (ng/mL)	SD (ng/mL)	%CV
PSA	2	1.00	0.02	2.00
PAP	20	2.13	0.89	41.78
Testosterone level 1	20	4.25	0.99	23.29
Testosterone level 2	24	19.65	2.11	10.74

Between-Run

Assay	N	X (ng/mL)	SD (ng/mL)	%CV
PSA	40	1.00	0.02	2.20
PAP	22	3.51	2.05	58.40
Testosterone level 1	15	5.11	4.69	91.96
Testosterone level 2	Not done			

Table 3

Assay Linearity: Comparison of Linearity of PSA with PAP and Testosterone

Assay	R Squared
PSA	0.9996
PAP	0.9850
Testosterone	0.9830

Table 4

Assay Sensitivity: Comparison of Sensitivity of PSA with PAP and Testosterone

Assay	N	X (ng/mL)	SD (ng/mL)	Range (ng/mL)
PSA	20	0.00	0.004	0-0.008
PAP	19	0.32	0.830	0-1.980
Testosterone	20	1.21	0.560	0-2.330

Table 5

Normal Reference Intervals: Comparison of Healthy Adult Reference Intervals for Total PSA with PAP and Testosterone

Tumor Marker	N	X (ng/mL)	SD (ng/mL)	Range (ng/mL)
PSA	80	0.98	0.96	0-2.90
PAP	101	7.79	14.99	0-37.77
Testosterone	102	4.44	3.40	0-11.24

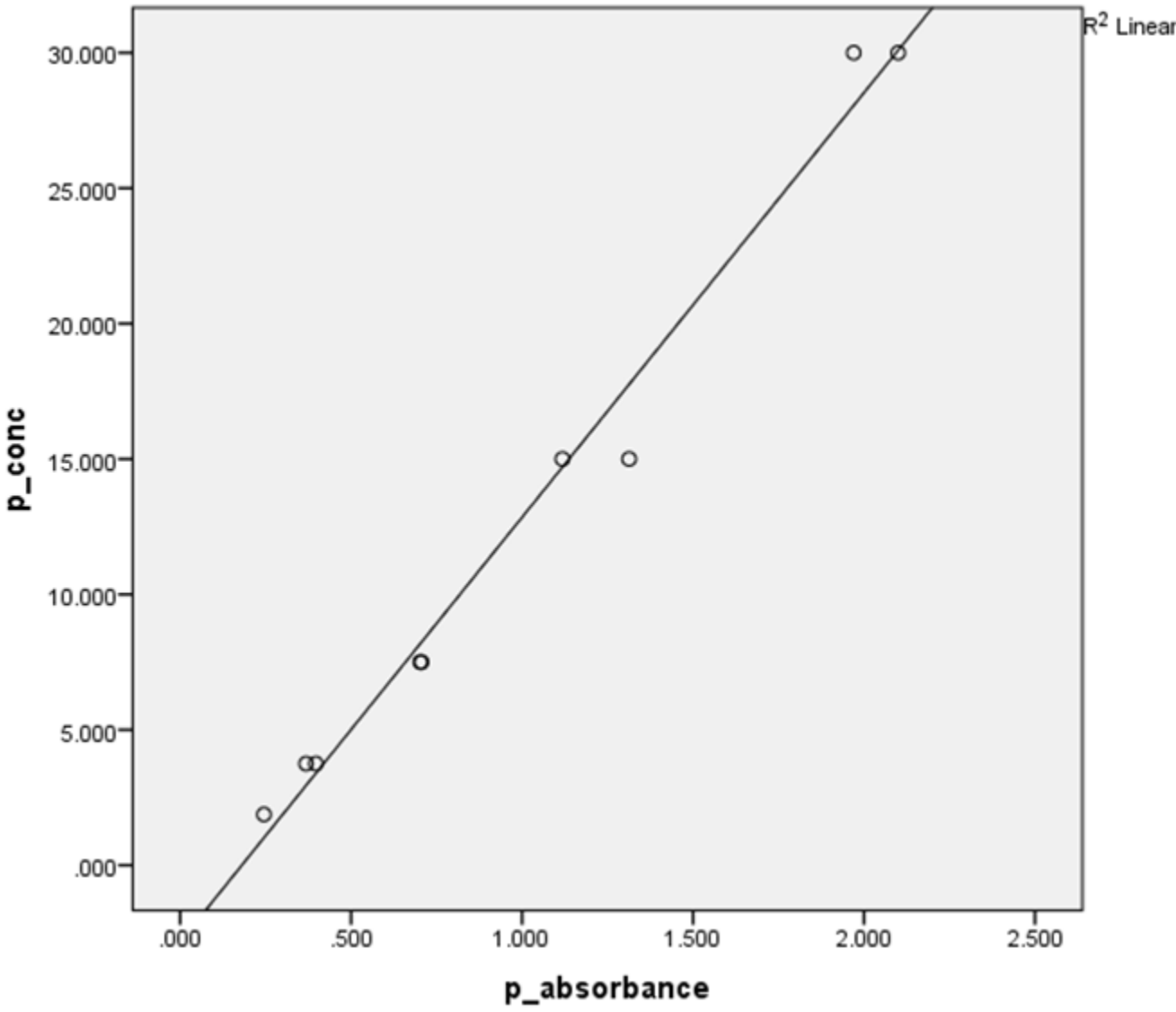
Table 6

Predictive Values: Comparison of Diagnostic Parameters of PSA, PAP, and Testosterone for Prostate Cancer in 551 Patients

Tumor Marker	Sensitivity (%)	Specificity (%)	PV + (%)	PV – (%)	Efficiency (%)	Cut-off (%)
PSA	30.12	91.29	39.06	87.58	81.73	4.00
PAP	20.73	80.38	15.60	85.29	71.51	5.00
Testosterone	0.00	96.80	0.00	84.70	82.40	10.00
Combination of Testosterone and PSA	30.12	89.15	32.89	87.84	80.29	N/A
Combination of PAP and PSA	43.37	75.11	23.53	88.25	70.34	N/A
Combination of Testosterone, PAP, and PSA	43.37	72.77	21.95	87.92	68.35	N/A

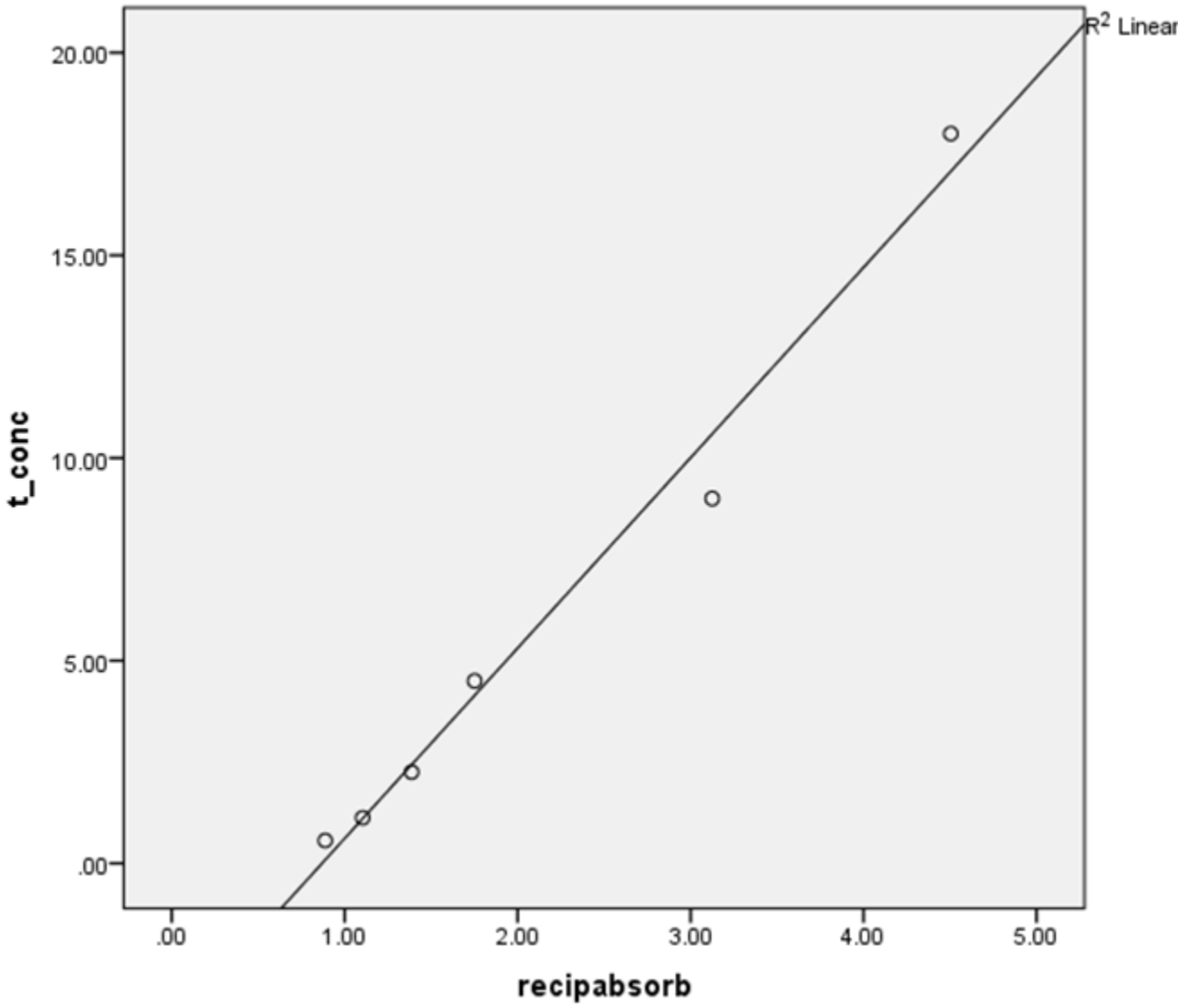
Graph 1

PAP Linearity



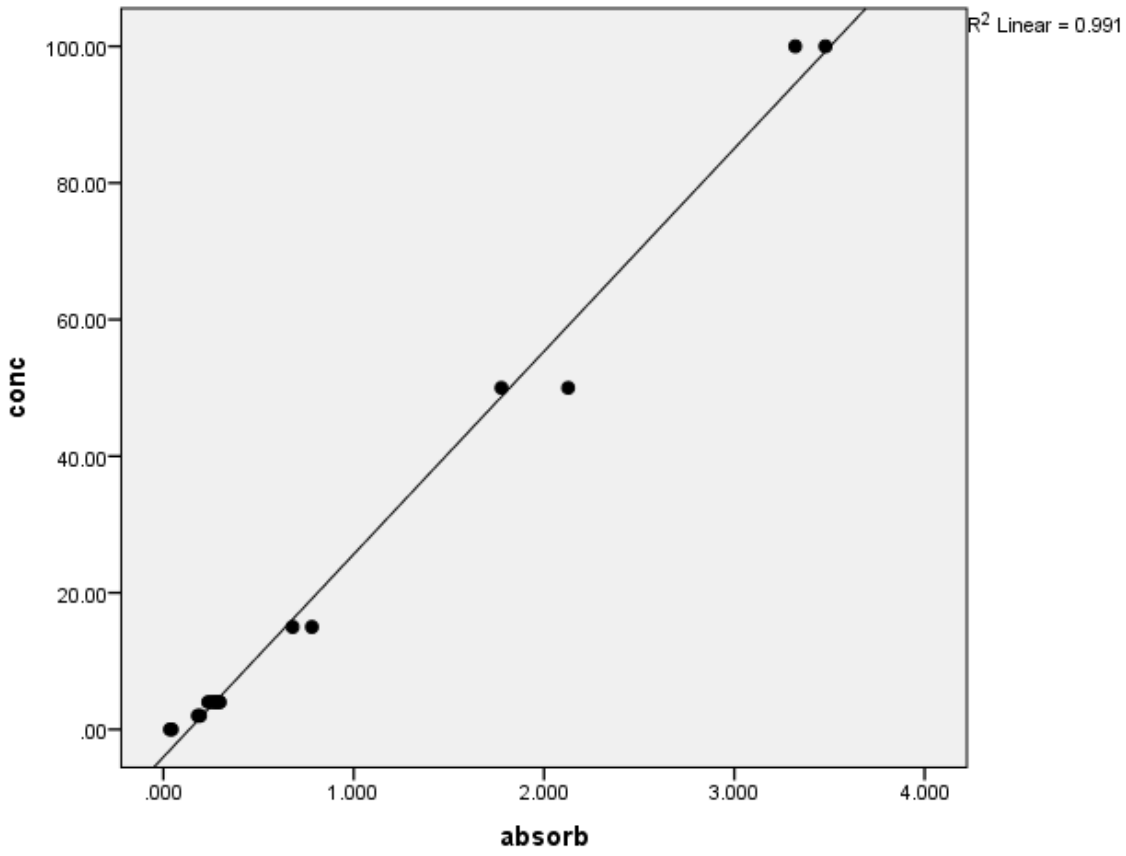
Graph 2

Testosterone linearity



Graph 3

PSA Linearity



Discussion

Analytical parameters for each of the three testing methods were adequate. As previously stated, the normal reference intervals calculated for PAP were considerably higher than the manufacturer's specifications. This was possibly due to a few falsely diagnosed subjects or an intrinsic defect with the testing procedure itself. None of the diagnostic sensitivities were optimal, but of the three examined, PSA remained the most accurate by that measure. The diagnostic specificities obtained were much better, with testosterone representing the most specific assay (96.80%). This result was in great contrast to the 0% sensitivity of testosterone. The "cutoff points" for testosterone used were those of the manufacturer (uncorrected). By adjusting the cutoff points one would obtain higher % sensitivity but lower % specificity. PAP specificity (80.38%) was below either of the other tests (PSA-91.29%; testosterone-96%). Predictive values (+ and -) were similarly comparable. One notable result was the 0% PV+ of testosterone and its 84.70% PV- value. Consequently, it could be theorized that testosterone has more value in ruling out prostate cancer than in confirming it. PAP stayed consistently second or third in the comparison of diagnostic parameters. Testosterone had the highest diagnostic efficiency (82.40%), followed closely by PSA (81.73%).

Concerning the combined marker results, three conclusions may be drawn from the data presented. First of all, it is apparent that adding testosterone evaluation to the current measurement of PSA does not improve any of the diagnostic capabilities. Secondly, measuring both PAP and PSA improves the diagnostic sensitivity alone over that of PSA by itself. Finally, combining all three markers in diagnostic evaluation also improves only the diagnostic sensitivity over that of PSA alone.

Lee, C., et. al stated "serum prostatic acid phosphatase has been reported as the most valuable enzyme marker for the diagnosis of prostate cancer." (1982). More recently however Haese, et. al (2002) wrote, based on further testing, that "most experts now agree that PAP analysis has no role in the diagnosis and monitoring of prostate cancer and that PSA is clearly the superior marker." These results confirm those of our tests. While PSA does not have the ideal hallmarks of a tumor marker (high sensitivity and specificity, PV+ and -, and efficiency), it is comparably the best available within the spectrum of this study. Neither of the other markers assayed showed as much consistent diagnostic accuracy as PSA. The initial statement by Lee, et. al (1982) that PAP is the most valuable marker was most likely made before the major discovery of the assay for PSA was widely known. Although PAP is still used in some cases to monitor cancer progression and detect tumors that do not produce a sizable increase in PSA concentration, it has largely been replaced by PSA due to evidence reported by Haese, et. al (2002) and others. These latter reports are in agreement with the findings of this study.

A strong point of this study is the small number of people directly involved in testing the samples. This keeps the amount of human error relatively standard among all the testing runs and makes the study more reliable. Also, there was always more than one person present during testing to as backup to prevent pipetting error. All the testing kits for each tumor marker were from the same company, standardizing the potential equipment error. Conversely, the age of some of the samples is a possible weakness due to potential sample degradation. Those samples from the Wilford Hall Medical Center were several months old, in contrast to the more recent samples from Singing River Hospital and Forrest General Hospital. To improve the accuracy of this study, a larger

number of samples should be tested from multiple geographic regions. The samples used should be as fresh as possible, and only thawed once, when tested.

Conclusion

From the data, it was concluded that the test for prostate specific antigen is the most accurate and efficient screen for prostate cancer. The hypothesis that prostatic acid phosphatase would be a better screening test was rejected. PAP is not more efficient than, or even as efficient as PSA in the diagnosis of prostate cancer. The sensitivity and specificity were both lower than that of PSA, although the results were relatively close. This finding is in line with other studies of the same nature.

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