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Development of novel biomarkers in cancer: Detection of circulating miR-141 as a potential prognostic marker for prostate cancer

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DEVELOPMENT OF NOVEL BIOMARKERS IN CANCER: DETECTION OF
CIRCULATING MIR-141 AS A POTENTIAL PROGNOSTIC MARKER FOR
PROSTATE CANCER

by

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A thesis submitted in partial fulfillment of
the requirements for the

**Master of Science in Biochemistry
Department of Chemistry
College of Sciences**

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ABSTRACT

Development of Novel Biomarkers in Cancer: Detection of Circulating miR-141 as a Potential Prognostic Marker for Prostate Cancer

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Prostate cancer (CAP) is the most common epithelial malignancy and the second leading cause of cancer deaths in American men. The identification of predictive and prognostic biomarkers in CAP patients is critical for improving clinical outcomes. Although the measurement of prostate-specific antigen (PSA) and radiographic studies are clinically approved to predict response to therapy, these tests can oftentimes prove to be inadequate in certain patients. Thus, it is important to discover new biomarkers to improve chances of survivability. We and others have shown that longitudinal measurements of circulating tumor cells (CTC) and lactate dehydrogenase (LDH) may aid in predicting response to therapy. More recently, levels of microRNA (miRNA) have been implicated in disease processes such as cancer. Specifically, the expression of human miRNA miR-141 has been found to be elevated in the plasma of CAP patients. In our study, we have measured the levels of miR-141 in 21 CAP patients and compared it with other clinical markers (CTC, LDH, and PSA). We longitudinally examined these markers alone and in combination in relationship to the patient's clinical course and response to therapy. Our aim was to determine if miR-141

has the potential to be a putative marker for the prognosis of a patient's response to therapy.

For this retrospective study, plasma from 21 CAP patients were collected at different time points corresponding to treatment regimen or follow-up appointments. Levels of miR-141 in plasma were measured using quantitative RT-PCR and compared to temporal changes in miR-141, CTC, LDH, and PSA levels. Using PSA as the standard marker in monitoring CAP, correlation coefficients were determined for each biomarker's capability in predicting clinical outcomes. Our results indicate that there is a strong correlation between a patient's clinical characteristics and the plasma levels of miR-141. With further testing, we suggest that miR-141 has the potential to be a marker for the prognosis of CAP. We find that miR-141 is largely concordant with the other conventional markers and establish that miR-141 is a relevant biomarker worthy of further investigation.

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ABBREVIATIONS

BMI1	B Lymphoma Mo-MLV Insertion Region 1
CAP	Prostate Cancer
CRPC	Castrate-Resistant Prostate Cancer
CTC	Circulating Tumor Cells
DEPC	Diethylpyrocarbonate
DRE	Digital Rectum Exam
ERSPC	European Randomized Study of Screening for Prostate Cancer
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
IRB	Institutional Review Board
LDH	Lactate Dehydrogenase
miR-141	microRNA human miR-141
miRNA	microRNA
PIN	Prostatic Intraepithelial Neoplasia
PLCO	Prostate, Lung, Colorectal and Ovarian Cancer
PSA	Prostate Specific Antigen
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
SALL4	Sal-like Protein 4

CHAPTER 1

INTRODUCTION

1.1 Overview of prostate cancer

In 2006, there was a reported 2.4 million deaths in America with cancer and cardiovascular diseases as the two leading causes of these deaths. More specifically, cancer was responsible for 559,000 deaths alone (1). In 2009, there will be 1.4 million newly diagnosed cancers and approximately 562,000 deaths attributed to cancer. It is estimated that in 2010, cancer deaths will surpass heart disease in deaths (2).

For men, the three most commonly diagnosed cancers are prostate, lung and gastrointestinal (GI) (3). In terms of cancer deaths in men, prostate cancer (CAP) is one of the leading causes and second only to lung cancer. Approximately one in six American men will be diagnosed with CAP in their lifetime. In 2009, there will be an estimated 192,000 new cases whereas another one in six, approximately 27,000, will die from the disease.

Little is known as to what causes CAP. However, its increased prevalence has garnered more interest in discovering the biochemical and physiological functions associated with the disease. Although the pathophysiology is still not clearly elucidated, CAP is known to originate in glandular tissue of the prostate. A widely considered model has been proposed involving a population of precursor cells known as high-grade prostatic intraepithelial neoplasia (PIN). These precursor cells are normally isolated within the prostate gland, but can eventually become malignant and form tumors (4). Many genes or gene

products have been associated with the development and progression of CAP. A mutation in oncogenes such as c-myc and Ras results in a constitutive activation or gain-of-function which results in accelerated growth or facilitates abnormal cell division (5-14). On the other hand, mutations in tumor and metastasis suppressor genes such as PTEN, p53 and CD44 results in loss-of-functions (15-22). A proposed multistep process of prostate carcinogenesis involves a progressive accumulation of these genetic alterations that facilitate the transformation from normal prostate tissue to PIN and eventually to full blown CAP.

For CAP patients that have tumors isolated within the capsule of the prostate gland, the disease can be cured with therapy such as a prostatectomy or radiation therapy. However, as the disease progresses and becomes metastatic, curing it requires more surveillance and therapy. Metastatic CAP is heterogeneously comprised of two types of malignant cells: androgen-dependent and androgen-independent (23, 24). Androgens are steroid hormones that aid in the development and maintenance of male characteristics such as testes formation and spermatogenesis (25). In androgen-dependent cells, higher levels of androgens can repress the transcription of death-signaling genes necessary for apoptosis (26, 27). Therapies that are targeted to suppress or block the production of androgen (androgen ablation therapy) are aimed to allow the expression of these death-signaling genes (28, 29). In contrast, androgen-independent cells are resistant to any levels of androgens. These subsets of cells are considered castrate-resistant and are therefore insensitive to any type

of androgen ablation therapies (30, 31). The lethality of advanced CAP can mainly be attributed to this castrate-resistant form of the disease (32).

Although significant advances have been made in understanding the molecular biology of CAP, this disease continues to be a major health problem. Knowing the risk factors and understanding the screening and therapies available can help in reducing the burden of this disease.

1.2 Risk factors associated with prostate cancer

There are several common risk factors associated with CAP: age, ethnicity, familial disposition, and diet. Of these factors, age appears to be very important (33). CAP is usually rare for men under the age of 40, but the incidence dramatically increases with age progression, with the most dramatic prevalence of CAP occurring between the ages of 61-80 years (34, 35).

African-American men are more at risk of developing CAP. The average incidence for African-American men in their 70s is approximately 1,600 per every 100,000. This is approximately 1.5x the incidence for Caucasians and 2x the rate in Asian-Americans (36-38). It has also been reported that age of onset of CAP in African-Americans is earlier than most other ethnicities. Other studies have also discovered that at the time of diagnosis, diagnostics markers, such as prostate-specific antigen (PSA) and biopsy scores (Gleason score), and progression of disease were elevated or more advanced in African-Americans than in any other ethnic group (39, 40). There is no solid understanding for the

disparate figures regarding the risks in African-Americans; however poor access to healthcare and education have been suggested (41).

Studies have shown that men who have first-degree relatives diagnosed with CAP have an increased risk for CAP. For example, a man with at least one other immediate family member diagnosed with CAP has twice the risk of being diagnosed themselves. This incidence increases approximately 5- and 10-fold when two or more family members are affected (42-47). Another study has also linked prognosis of survival as a potential genetic predisposition whereas a son's survival can be directly correlated to his father's outcome (48).

Clearly, age and race seem to strong risk factors when ascertaining one's likelihood of developing CAP. Although it may seem apparent that one can closely predict who will be diagnosed with CAP based on age or genetic predispositions, clinical tests must be performed to properly diagnose CAP. Unfortunately, the clinical presentation of this disease may be subtle and present asymptotically. It has been said that most men will die with CAP as opposed to dying from it. An autopsy study analyzing serial sections of the prostate found that one-third of men 80 years or younger had CAP whereas in men greater than 80, two-thirds were positive for CAP (49).

1.3 Methods of screening

As with most cancers, early detection is essential for survival. In fact, the five-year survival rate for men diagnosed with cancer presenting localized or regional spreading is close to 100 percent. If the disease has progressed to a

more malignant, invasive form, there is a meager 31.9 percent survival rate (33). Men who clinically present often have difficulty urinating or have a feeling of discomfort in their pelvic region. Unfortunately, CAP can be present asymptotically and the need of sensitive and specific screening is needed.

Many tests exist for the screening of CAP. Digital rectum exams (DRE) and determination of prostate specific antigen (PSA) levels are some of the more common methods of diagnosis. Least common tests include prostate biopsies and transrectal ultrasonography; however these are not used for primary screening due to either cost, availability or their potentially low sensitivity and specificity in detecting CAP.

PSA is a protein produced by prostate epithelial cell and has a half-life of approximately 2 days (50). Total serum PSA has been found to be increased in men with CAP and thus has been detected with elevated levels in the serum of CAP patients. The increase in detectable PSA in the serum is due to a disruption of the tissue barriers between the prostate gland lumen and the capillary. Unfortunately, PSA levels can also be increased in the men exhibiting enlarged prostates, also known as benign prostatic hyperplasia (BPH). Prostatitis, digital rectal exams, ejaculation, and prostate biopsies can also lead to an increase of PSA in serum (51-56).

To assess an abnormal PSA level in the serum, a cutoff of 4.0ng/mL has been established. At this level, studies have determined the PSA sensitivity and specificity is at an estimated 70 to 80 percent and 60 to 70 percent, respectively (57). Furthermore, the positive predictive value (a measure of the ability to

accurately identify CAP in men with elevated PSA levels) of PSA levels between 4.0 -10ng/mL was determined to be at 30 percent (58-60). This predictive value increases to approximately 64 percent if PSA levels are determined to be greater than 10ng/mL (61).

To improve on the accuracy of PSA's diagnostic capabilities, it has been proposed to use multiple PSA parameters instead of a single PSA measurement. A few of these parameters include measuring free, unbound PSA, the change of PSA levels over time (PSA velocity), and PSA levels per unit volume of prostate (PSA density) (62). In some instances, men with PSA levels below 4.0ng/mL are found to have CAP; therefore some physicians have suggested lowering the cutoff to 2.5ng/mL (63-66). The effort to improve the diagnostic power of PSA has yielded multiple potential modifications; however there has been no consensus as to which of these modifications can be used to improve diagnosis or clinical outcomes. A major focus in CAP research is determining new prognostic serum markers with increased sensitivity and specificity.

Anatomical examinations such as the digital rectal exam can detect abnormalities of the prostate. These abnormalities include asymmetry, nodules, or hardening (induration of the prostate). A DRE can detect CAP in the lateral and posterior aspects of the prostate glands; however, 85 percent of cancers occur on the periphery and can only be detected by examination with a finger (67). Most cases of CAP that are diagnosed solely through a DRE present a clinically advanced stage of the disease (68). In fact, multiple studies have shown the specificity of the DRE to be at 59 percent, with a sensitivity of 94

percent and an overall positive predictive value of 28 percent (69). The low specificity and late disease onset of detection with DRE make it a test best used in conjunction with other tests such as PSA screening. Studies have shown that combining the PSA and DRE has greatly improved the rate of CAP detection (70).

For patients with high PSA levels and an abnormal DRE, a prostate biopsy can be performed to confirm a CAP diagnosis. In certain instances, CAP can be diagnosed solely through a prostate biopsy. A biopsy is a procedure where a small piece of suspected tissue is extracted from a patient using a needle. The resultant tissue is called a biopsy core. The cores are assessed to determine if cells of the tissue are normal or cancerous. Specifically, prostate biopsies are performed rectally through the perineum, with the use of a biopsy gun to extract cores of tissues (71). Approximately six needle cores are extracted from the base, midzone and apical areas of the prostate gland; however studies suggest the assessment of ten needle cores can increase the detection rate (72-75).

For CAP patients, biopsies of tumors are performed to assess the progression of the disease. Tumor biopsies are performed in the same manner as exploratory biopsies and are given numerical grades based on the differentiation and structure of the cells present in the tumor. Cells with normal structure and differentiation are given a grade of one, whereas cells with the least amount of structure and differentiation are scored a five. The grades ascertained from the biopsy cores are called the Gleason grade (76). In most instances, multiple cell types are exhibited in the core (i.e. a core exhibits cells with a grade

of 3 and 4). In situations where multiple grades exist, the core is given a combined score of the two highest-graded cell types exhibited in the core (i.e. a score of 7 where cell types of grade 3 and 4 exist). These combined Gleason scores allow physicians to determine the grade or severity of cancer present: combined scores of two through four represent a low-grade cancer; scores five through seven are considered moderate-grade; scores eight through ten are considered high-grade cancers (77, 78).

Another characteristic of a tumor biopsy that can increase clinical information is the extent of the biopsy that is occupied by the tumor. Whereas a Gleason score is a measure of cell differentiation of a tumor, this estimated tumor volume is determined by the amount of biopsy cores that are positive for tumor involvement and the percentage of the tumor involvement within each positive core. Together with the level of cell differentiation of a tumor (Gleason score), the extent of biopsy cores that are positive for tumors, and the percentage the tumor that is occupied within each positive core, a biopsy can provide more clinically significant information (79, 80).

1.4 Risks associated with screening

The value in screening for CAP lies in a test's ability to reduce the morbidity and mortality of this disease. Currently, only two large randomized trials have sought to determine the effectiveness of current screening methods – the European Randomized Study of Screening for Prostate Cancer (ERSPC) and the United States Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening

Trial. In both studies, men were randomly selected to have interval PSA screenings; however in the PLCO study, a DRE supplemented the PSA screening (81, 82). The tests were used to determine which patients should receive biopsies and ultimately receive proper treatment. Collectively, the two studies determined that the absolute mortality benefit from screening was relatively low. Meaning, these studies suggest that the current methods and frequency of screening for CAP may not help to prevent death versus those who do not regularly screen for CAP.

The low efficacy of the current screening methods may lead to potential harm from screening. Prostate biopsies, for instances, can lead to high anxiety, pain, and in rare cases, serious complications that can lead to hospitalization (83, 84). Even after a patient undergoes a biopsy, there is potential for a negative biopsy result which can lead to increased anxiety due to the high false-negative rates (85). Furthermore, there is an issue of an over diagnosis, whereas the detection of conditions through screening may not actually prove to be clinically significant. An over diagnosis can subject patients to unnecessary subsequent testing and rigorous treatment increasing the chances of harming the individual.

Although early screening for CAP with PSA and DRE can increase overall survivability, patients must be informed of the inherent risks involved with screening. Questions regarding quality of life be must be answered to weigh out the benefits of increased screening and treatment. The absolute risk reduction associated with screening is relatively low; therefore men who fall under certain

risk factors (i.e. race and family history) or those who are willing to accept the risks involved would more likely benefit from screening.

1.5 Treatment of prostate cancer

There are multiple options to treat CAP. Physicians most often strategize their approaches for treatment based on PSA values, age, and overall health of the patient. A more notable approach to delineating treatment options is based on the stage and grade of the disease. Treatment options for patients with clinically localized CAP are approached differently than those who have a more advanced progression of the disease (86).

Treatment option for men with localized CAP include: an active surveillance approach, radical prostatectomy and radioactive therapy. The active surveillance approach is an accepted option for patients exhibiting a form of the CAP that has a relatively low risk of progression or for patients with a high risk of side effects from certain treatments (87). This approach involves the postponement of immediate therapy, with treatment administered only if the patient is at increased risk or exhibits symptoms of disease progression (88). This surveillance method can drastically reduce the potential for over-treatment or unnecessary risk of harm to the patient.

As cancers progress to a more advanced stage, the disease is more likely to become metastatic and affect other tissues or organs. One of the more common organs involved in the metastatic form of CAP is the bone (89). For men with an advanced or recurring form of CAP, a more aggressive therapy must be

administered. Aside from a prostatectomy and localized radiation therapy, systemic (whole body) therapy would be a follow up option. Normal and cancerous prostate cells growth are stimulated by androgens; therefore, a systemic therapy approach to metastatic CAP would be androgen deprivation therapy (ADT). ADT can be achieved through surgical castration, chemical castration or a combination of both. Unfortunately, a form of CAP exists that is androgen-independent which would render ADT useless. This castrate-resistant prostate cancer (CRPC) is most often treated with chemotherapy (90). However, a new form of treatment involving the use of immunotherapy has recently been introduced to help treat CRPC (91, 92). Provenge (Sipuleucel-T) is a form of therapeutic cancer vaccine that utilizes a subset of a patient's leukocytes to present an immune-activating antigen. The immune response activates T-cells and is clinically shown to reduce the risk of death by 22% compared to placebo (93).

1.6 New and potential biomarkers

Biological markers, or biomarkers, refer to a measurable biological molecule that can reference or indicate any signs of normal or abnormal biological processes. Biomarkers can be used to diagnose and indicate the clinical stage of disease (94). Using biomarkers for diagnosing diseases has traditionally been focused on detecting enzymes and proteins circulating in the blood, other bodily fluids or tissues. PSA, a serum biomarker, is actively secreted by the prostate gland into semen and, in lower instances, other bodily fluids. Low levels are

released into the bloodstream and are a normal occurrence; however injury, inflammation, enlargement, or other traumatic events affecting the prostate can increase the amount of PSA being released into the bloodstream (95, 96).

Aside from diagnosing a disease, biomarkers can be powerful tools capable of predicting disease outcomes, predicting therapy response or identifying patients susceptible to severe side effects. The use of markers to evaluate disease progression is known as a surrogate marker (97). Surrogate markers are biomarkers that can substitute for a clinical endpoint (94). A clinical endpoint is a reference to the distinct overall well-being of a patient undergoing treatment. A few of these parameters include how a patient feels, how they are functioning, or ultimately if they have survived (94). Using such markers to predict outcome behavior has given rise to the notion of personalized medicine – a course of treatment tailored to each patient’s specific tolerance or the potential increase in drug efficacy based on the genetic makeup or biochemical expressions specific to each individual.

The current state of biomarkers has expanded to include the detection of intact, circulating cells in the bloodstream. A growing number of studies have focused on detecting circulating tumor cells (CTC) in blood as another potential biomarker for disease (98, 99). The CellSearch™ Circulating Tumor Cell test, developed by Veridex, LCC, is currently used to monitor breast and colorectal cancers and, in February of 2008, was also approved for its ability to assess CAP (100, 101). Using this test, prognosis for metastatic breast and CAP is defined by the number of tumor cells detected in 7.5mL of blood.

CTCs are prepared from whole blood in a two-step process: First, epithelial cell adhesion molecule (EpCAM) positive cells are selected using iron-conjugated EpCAM antibodies. Enrichment of EpCAM⁺ cells is achieved using a pull-down method utilizing magnets. Second, CD45 (leukocyte cell marker), cytokeratin (marker for keratin-containing intermediate filaments) and nucleic acids are stained via allophycocyanin (APC)-labeled antibody, phycoerythrin (PE)-labeled antibody and 4',6-diamidino-2-phenylindole (DAPI), respectively. The captured CTCs are imaged and scored based on the following criteria: cytokeratin⁺, CD45⁻, and DAPI⁺. Figure 1 summarizes the process to enrich and analyze for CTCs.

Detection of whole, intact cells characterized by CTCs is a clear indication of metastatic disease and has proven to be a strong indicator of disease progression. Studies have determined that a CTC count of five or greater as an independently predictive parameter of a decreased progression-free survival and overall survival (OS). More specifically, the detection of CTCs has shown to correlate well with the progression of metastatic castration-resistant CAP (102). Aside with being used as prognostic disease marker, CTCs may be useful in validating other biomarker candidates (103, 104).

The detection of circulating markers found freely in human bodily fluids is not limited to proteins or whole cells. More recently, studies have shown the potential of detecting circulating nucleic acids as a marker for diseases. Detection of abnormalities in DNA has long been used to diagnose disease; however, there are increasing studies focusing on the detection of RNA in

determining disease states. A species of RNA, microRNA (miRNA), has shown potential to be a marker for diseases. These miRNAs are shown, amongst other functions, to regulate gene expression.

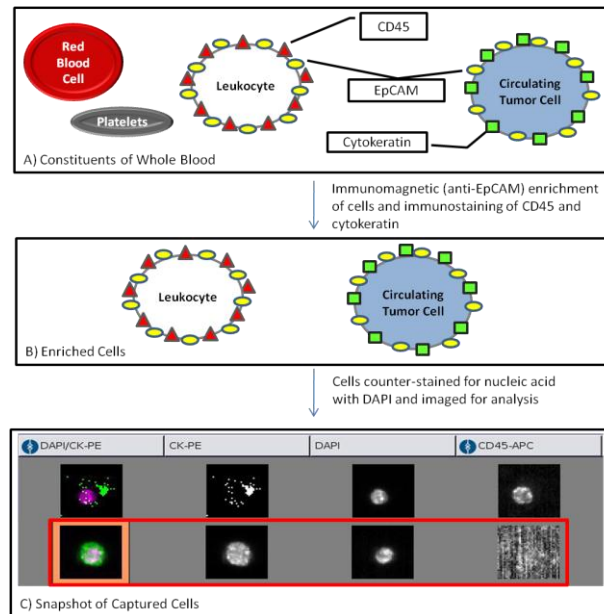


Figure 1: A schematic representation of CTC capture and analysis. Constituents of whole blood for patients with CAP are summarized in panel A. The whole blood is processed in a ferrofluid containing iron-coupled anti-EpCAM antibody to immunomagnetically enrich for EpCAM⁺ cells and remaining cells are stained with fluorescent antibodies against CD45 and cytokeratin (Panel B). Nucleic acid is stained and the captured cells are imaged. CTCs are defined as cytokeratin⁺ and CD45⁻ (panel c; red box), whereas leukocytes are CD45⁺ and cytokeratin⁻. Cell photographs in panel c are courtesy of Dr. Louis M. Fink and Kristine Scarbrough.

Mature miRNAs, the functional form of miRNA, are described as a 22 nucleotide (nt) species of non-coding RNA. The biogenesis of mature miRNA is preceded by two intermediates: a pri-miRNA and pre-miRNA (Figure 2). Pri-miRNA is the initial transcription product of the miRNA-bearing gene and is characterized by a stem-loop structure. The opposing end of the stem loop is

cleaved by the Drosha RNase III endonuclease and yields the pre-miRNA. The pre-miRNA is exported into the cytoplasm and the loop end of the pre-miRNA is cleaved by the Dicer endonuclease leaving a short, 22nt, double-stranded RNA species. This double-stranded RNA is separated into the single-stranded mature miRNA by a helicase. The regulation of gene expression by miRNA is achieved through either translational repression or a site-directed cleavage of the mRNA (105, 106).

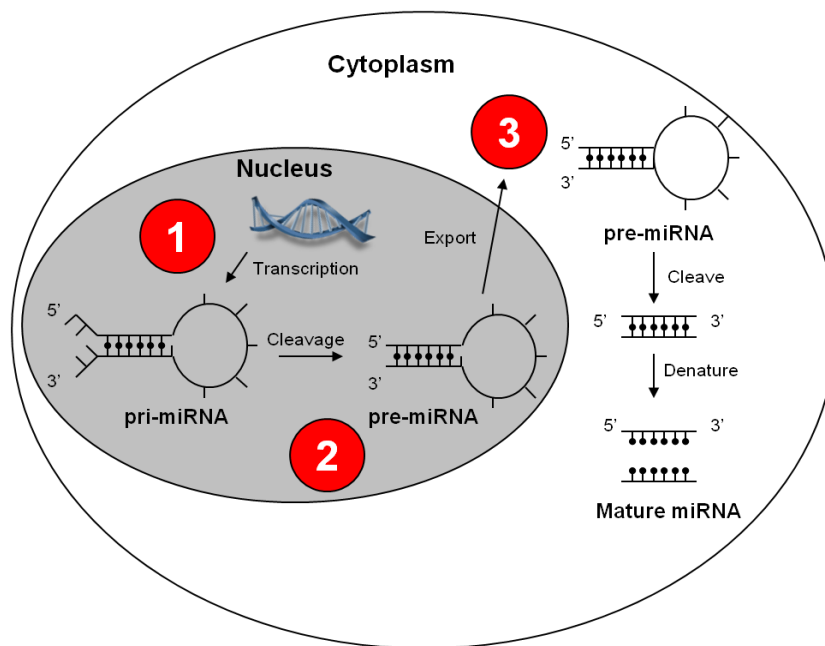


Figure 2: The biogenesis of microRNA. 1) The gene containing the miRNA is transcribed. The transcription product is called pri-miRNA and has a stem-loop structure. 2) The pri-miRNA is cleaved at the tail and is now called the pre-miRNA. 3) The pre-miRNA is exported into the cytoplasm and undergoes more modification (cleavage and denaturation) to yield the mature miRNA. Image adapted from Ambion website (107).

Since their initial discovery in 1993, the number of miRNA has expanded rapidly and the Sanger miRBase sequence database now contains over 900

characterized human miRNA (108). One of the first characterized miRNA, lin-4, was found in the roundworm *Caenorhabditis elegans* (*C. elegans*) and was determined to regulate the expression of *lin-14* gene (109). Today, it is well documented that miRNA play a major role in gene regulation and in certain disease processes (110, 111). For example, miRNA expression is known to be regulated in cancers such as chronic lymphocytic leukemia, and in lung and thyroid cancers (112-114).

Accurate determination of treatment efficacy is important to increase the overall survivability of CAP. It is therefore beneficial to expand on the current repertoire of prognostic serum biomarkers. Initial efforts to identify a putative marker can be expensive and must be validated to prove its prognostic capabilities. Fortunately, current validated markers can be used to compare and aid in the validation of the predictive characteristics of a putative prognostic marker.

1.7 Purpose of the study

Discovering novel biomarkers for diagnosing or prognosing diseases such as cancer was a major focus of my study. I have attempted to assess a variety of potential biomarkers that would allow for accurate monitoring of disease progression in breast and prostate cancers. My preliminary focus was aimed at developing both a protein-based and gene expression assay (through the detection of messenger RNA) for the simultaneous detection of SALL4 and BMI1 proteins in prostate and breast cancer. Furthermore, access to patient serum

samples has allowed me to assess the potential for a biomarker assay, currently marketed as a cancer biomarker panel that was developed with an emphasis towards ovarian cancer, to detect the progression of breast cancer.

A major hurdle of clinical and translational studies can be the availability and integrity of patient samples. The progression of my biomarker studies was complicated by such challenges. For instance, a change in samples procurement policies has impeded our access to more patient samples and an issue of sample degradation in banked samples complicated certain aspects of my study. We were fortunate, however, to have enough samples to focus attention to the detection of circulating miRNA in CAP. The focus of my thesis will be this on this miRNA study in prostate cancer; however I present a preliminary assessment of a cancer biomarker panel and its potential utility in detecting breast cancer can be found in appendix figure A1. Furthermore, our preliminary results of the gene expression assay for the detection of SALL4 and BMI1 in breast cancer can be found in appendix figure A2. A table that summarizes all experiments or attempts to detect novel biomarkers in prostate and breast cancer can be found in appendix table A1.

CAP is one of the leading causes of cancer deaths in American men. Physical and anatomical examinations such as the digital rectal and prostate biopsies are performed to detect and diagnose CAP. Furthermore, detection of biomarkers has also been used to diagnose and monitor CAP. Examples of these biomarkers include the prostate specific antigen (PSA) and lactate dehydrogenase (LDH). Currently, more emphasis has been placed in

discovering new diagnostic and monitoring tools for the early detection and accurate prognostication of this disease. Accurately monitoring the disease during a treatment regimen will allow physicians to determine the efficacy of the current treatment and can allow for punctual changes in treatment regimen. Just as PSA can aid in the diagnosis of CAP, PSA is also used as a predictive marker for monitoring disease.

Although tests such as the prostate biopsies and detection of PSA have been used confidently for many years, each test has known specificity and sensitivity drawbacks. It is for those reasons that physicians often use tests in conjunction with one-another, rather than individually, to diagnose or monitor the progression of CAP. Furthermore, tests such as the prostate biopsy are highly invasive and can lead to painful side effects. Digital rectal exams, although not invasive, can cause pain and stress to patients. An ideal test would have increased specificity and sensitivity than those of current tests and would ideally be less invasive and painful to the patients. Biomarkers are thought to be ideal in that these are normally detected from a patient's blood - meaning it is non-invasive and pain would only be measured from a patient's tolerance to needle-stick procedures.

A recent study by Mitchell *et al.* discovered that human miR-141 was stable and detectable in plasma of CAP patients (115). miR-141 is encoded on chromosome 12 and is a part of the miR-200 family known to regulate the transition from epithelial to mesenchymal tissue. This morphological change in cells reduces intercellular contacts and is a characteristic of metastasis (116-118). Nevertheless, the exact role of miR-141 in CAP is currently unclear. In this

study, we quantitate the circulating levels of miR-141 in plasma of castration-resistant and hormone-sensitive metastatic CAP patients undergoing treatment and retrospectively compared it to levels of PSA, CTC and LDH - conventional biomarkers used to monitor CAP. Temporal changes in copy number of circulating miR-141 were compared to the other markers and it was determined if miR-141 concomitantly correlated with values of the conventional markers determined at the same time points.

The purpose of this study is to demonstrate the possible benefits of using miR-141 as a biomarker through its correlation with treatment response. We hypothesize that values of miR-141 detected in patient plasma are concordant with values of PSA and clinical assessments and can prove to be a marker for the prognosis of CAP.

CHAPTER 2

METHODS

2.1 Chemicals, reagents and equipment

Trizol LS Reagent was obtained from Invitrogen (Carlsbad, CA). Diethylpyrocarbonate (DEPC)-treated water was obtained from EMD Chemicals (Gibbstown, NJ). 99.5% A.C.S. grade, 200 proof ethanol was obtained from Acros Organics (Morris Plains, NJ). PCR reaction tubes were obtained from VWR (West Chester, PA). Molecular biology certified chloroform and isopropanol was obtained from IBI Scientific (Peosta, IA). Ribooligonucleotides for the generation of standard curves were obtained from Integrated DNA Technologies (Coralville, Iowa). 7500 Fast Real Time PCR Thermocycler, reverse transcription kits for the cDNA amplification of *Caenorhabditis elegans* (*c. elegans*) miRNA cel-miR39 and *Homo sapien* miRNA hsa-miR-141, TaqMan MicroRNA Assays, 96-well Thermocycling Plate, and MicroAmp Optical Adhesive PCR sealing film were obtained from Applied Biosystems (Foster City, CA). miRVana miRNA extraction kit and THE RNA Storage solution were obtained from Ambion (Austin, TX). Sterilization of equipment and work surfaces from RNase and DNA contamination was performed with RNase Away and DNA Away obtained from Molecular BioProducts (San Diego, CA).

2.2 Biological sample collection

All patients and control subjects were consented by staff of the Nevada Cancer Institute and all biological samples pertaining to this study were stored on site in the institute's biorepository. Control samples were collected from consenting volunteers that, to the best of their knowledge, were known to be cancer-free. Biological samples of control subjects were kept anonymous and did not have any clinical tests performed on them (i.e. PSA or CTC testing). CAP patients used in this study were patients of Nevada Cancer Institute. In most instances, biological samples were collected in conjunction with their treatment or follow up visits.

For this retrospective study, CAP samples were chosen by one physician to reflect a range of clinical biomarker changes (rising or declining PSA and CTC values) against which miR-141 could be compared. These samples were chosen prior to the determination of miR-141 values. All samples were collected and processed under approved Institutional Review Board (IRB) protocols. These samples were chosen independent of the patient's clinical assessment, where one's clinical assessment is the diagnosis and prognosis of the patient based on medical history, performance and clinical lab tests. Each patient's clinical assessment was determined by one consulting oncologist and was based on overall chart review and reflects clinical and/or radiographic disease progression. Each assessment was made independently and in a blind fashion in respects to all biomarker values.

Blood was collected in K2-EDTA tubes from CAP patients with ages ranging from 60-77 at the time of first blood draw. Each tube was centrifuged at 3,300 rpm for 10 minutes to separate the peripheral blood platelet pool. Plasma was aspirated from the tubes, aliquotted into cryogenic tubes and stored at -80°C until analysis. A separate sample of blood was used to run PSA, LDH and CTC analysis on these CAP patients. The PSA was run on serum samples using the Bayer-Centaur methodology, CTC analysis was done according to Veridex's standard operating protocol, and LDH was determined using the Olympus LD procedure. All approved clinical testing were performed by licensed clinical laboratory staff. Each CAP patient also had subsequent blood draws at different time points coinciding with their schedule of therapy, thus allowing a longitudinal analysis for each biomarker. All control subjects were closely age-matched and were subjected to one blood draw only; therefore control samples do not have longitudinal analyses.

2.3 Determination of miR-141 expression

MicroRNA was extracted from plasma using the *mirVana* PARIS kit protocol (Ambion) (15). A modification of the extraction protocol was performed by including a second organic extraction with phenol:chloroform (Ambion) of total RNA prior to final purification of miRNA.

To normalize the reactions, we utilized an exogenous miRNA species not detected in human plasma, cel-miR-39 - an miRNA expressed exclusively in *Caenorhabditis elegans* (*c. elegans*). For most expression assays, normalization

is achieved through the detection of endogenous controls. Endogenous controls are most often associated with the detection of constitutively expressed proteins such as the housekeeping genes β -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH); however the normalization of studies utilizing miRNA has been complicated by the absence or inability to detect a true endogenous species of miRNA. Furthermore, since the mechanism of circulating miRNA in prostate cancer has not been deduced, nor has there been any true establishment of circulating endogenous miRNA, the nature of our study has further complicated the establishment of an endogenous control. Therefore, the use of an exogenous control was necessary. 500pg of synthesized cel-miR-39 ribo-oligonucleotide was spiked into each plasma sample following an initial denaturing step and served as our normalization, positive and loading miRNA control (IDT; cel-miR-39 sequence 5'-rUCACCGGGUGUAAAUCAGCUUG-3'). The resulting miRNA was eluted using THE RNA storage solution (Ambion) and either immediately subjected to a reverse transcription reaction or stored at -80°C.

In order to determine absolute quantitation of miRNA, standard curves were generated for both cel-miR-39 and miR-141 using synthesized ribo-oligonucleotides in separate reaction vessels (IDT; miR-141 sequence 5'-rUAACACUGUCUGGUAAGAUGG-3'). A 6-point standard curve was created starting with an RNA concentration of 100pg/ μ L with subsequent 10-fold dilutions with DEPC-treated water to a final concentration of 1.00fg/ μ L. Reverse transcription of miR-141 and cel-miR-39 from extracted samples and standard

curves was achieved using a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) with specific stem-loop primers for each miRNA to be assessed (Figure 3; Applied Biosystems). Reverse transcription reactions were incubated in a PTC-200 thermocycler (MJ Research) under the following conditions: 16°C for 30 minutes, 42°C for 30 min, 85°C for 5 min, and then held at 4°C (23).

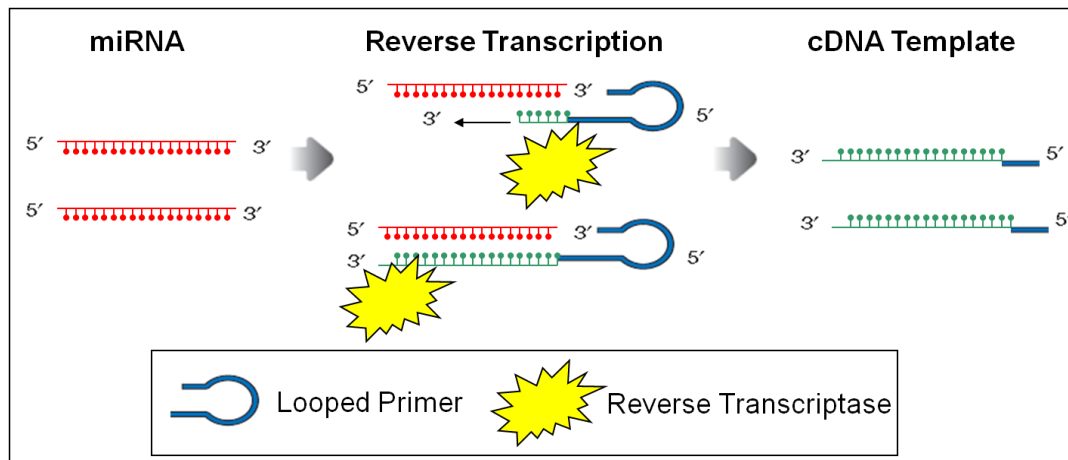


Figure 3: Principle of reverse transcription to produce cDNA template from miRNA using a looped primer. The unique stem-loop structure of the primer allowed for high specificity of binding of miRNA species only.

Quantitative RT-PCR for each sample and standard curve was performed in triplicate in a reaction mixture of TaqMan miRNA target-specific probe (Applied Biosystems) and 2x Universal PCR Master Mix (Applied Biosystems) in a final volume of 5uL. PCR reactions were performed in a 96-well thermocycling plate sealed with optically-clear sealing film and were run under the following conditions in 7500 Fast PCR Thermocycler (Applied Biosystems): non-fast conditions with an initial enzyme activation of 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minutes where data was collected at

the 60°C step. Although TaqMan signals were recorded at the last step of each cycle, the data were analyzed at the final cycle of the reaction (last step of cycle 40).

Our determination of miR-141 copy number for each sample was an endpoint analysis and the signal was interpreted into the amount of miR-141 determined on each sample based on a linear regression equation extrapolated from our standard curve. Figure 4 is a representative standard curve and extrapolated equation observed throughout the study. Normalization of the reaction was based on the cycle threshold values from the spiked endogenous control (cel-miR-39). Calculation of miR-141 copy number was as previously described (18).

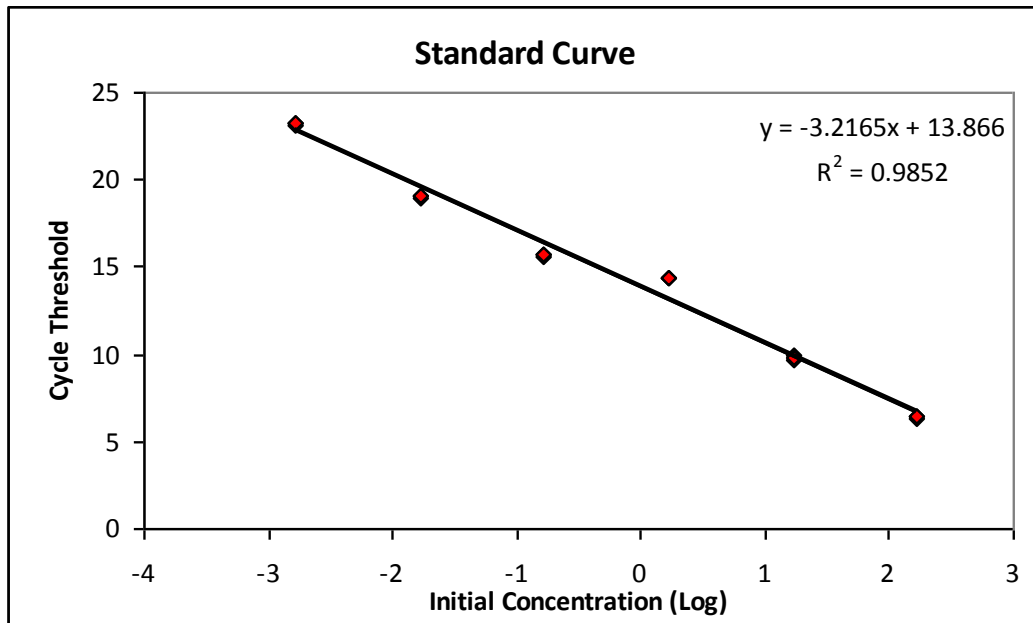


Figure 4: A representative standard curve to determine miR-141 concentration. Each standard curve represents the correlation of input RNA vs. expected. A linear regression equation was used to extrapolate the miR-141 concentration for each sample in the study. PCR efficiency can also be determined from the slope obtained in each reaction.

Aside from an extrapolated input miRNA concentration, we are also able to determine the efficiency of our qRT-PCR from our standard curves. In theory, a reaction efficiency of 100% should yield a PCR reaction where products are doubling after each subsequent cycle. Using the slope obtained from the standard curve, a measure of exponential amplification and PCR efficiency can be determined. A slope of -3.3 yields an exponential amplification of 2 (conferring a doubling of PCR product from previous cycles) and an efficiency of 100%. Throughout the study, we experienced an average slope of -3.42 that yielded an average exponential amplification of 1.96 and an average PCR efficiency of 96.1%.

2.4 Statistical methods

To assess the statistical relevance of the temporal changes of biomarker values, each data point was transformed to a \log_{10} value. The transformed data for each interval were used as data points on a regression plot yielding a slope corresponding to the temporal rate of biomarker change. Slopes were calculated for each biomarker and for each patient separately and were used as the outcome variables for the analyses described below. All analyses were conducted on two separate cohorts: cohort 1 included patients with exactly two blood samples and cohort 2 included patients with 3 or more blood samples. For all statistical analyses, we chose PSA as the predictor because it is considered the gold standard biomarker used to assess response to therapy of CAP.

1. Analysis of variance for each cohort.
 - a. Group 1 was defined as all patients with increasing PSA (positive PSA slope). Group 2 was defined as all patients with decreasing PSA (negative slope).
 - b. Analysis of variance was conducted for the 4 slopes (PSA, LDH, CTC's, and miRNA) as a function of Group as defined above. PSA slope ANOVA is biased because the PSA slopes defined the groups. Results for PSA are still provided for descriptive purposes.

2. Correlation analysis. Pearson correlation coefficients were calculated for the 4 slopes: PSA vs LDH, PSA vs CTC, PSA vs miR-141, LDH vs CTC, LDH vs miR-141, and CTC vs miR-141.

3. Binary Classification analysis
 - a. Binary variables were calculated for each of the 4 biomarker slopes. A "1" was assigned if the slope was positive. A "0" was assigned if the slope was negative.
 - b. Sensitivity and specificity were calculated for 3 pairs of biomarkers using PSA as the standard.
 - c. Sensitivity and specificity for other combinations were also calculated.

CHAPTER 3

RESULTS

The miR-141 values of the control samples are summarized in table 1 and observed value ranges are given in table 2. The clinical assessments and biomarker values for each patient in this study are summarized in table 3. For clarification of clinical assessments, patients deemed “regressing” is a patient that is clinically performing better, whereas a “progressing” patient has clinically gotten worse. The values of the clinical markers were not used to assess each patient as this would confound the analysis; rather the clinical assessments were based on the following weight-bearing percentages in determining the assessment: radiographic progression accounted for 50% of the assessment, performance status had a 30% weight, progression of pain at 15%, the need to change therapies was weighed at 5%, and other systemic symptoms such as anorexia or fatigue had <1% weight.

To assess the ability of miR-141 to prognosticate CAP, we observed two characteristics in regards to changes in miR-141 compared to the other clinical markers: 1) we sought to determine if temporal changes of miR-141 values would be concordant with the changes in other markers and its potential to correlate with clinical assessments and 2) the statistical relevance in which miR-141 can accurately and precisely correlate and classify a patient against the standard markers. When analyzing the results, biomarker data is separated into two cohorts: 1) patients providing only two data points (one interval) and 2) patients that had three data points to compare (two intervals). Each data point

corresponds to separate dates of sample draws and biomarker determination. The time lapsed between each data point differed for each patient and any matching intervals were strictly coincidental.

3.1 Raw temporal changes of miR-141 values

Figure 5 demonstrates the raw fold-change in each biomarker between the interval for each patient in cohort 1 (n=8 patients). Figure 6 demonstrates the same fold changes in the two intervals for patients in cohort 2 (n=13 patients). The significance of the raw biomarker change is defined by the ability of miR-141 value to change in concordance with the other markers in either a positive or negative manner. In doing so, we can estimate the potential for miR-141 to predict clinical progression.

Of the eight patients in cohort 1, 6/8 (75%) patients had all biomarkers changes in concordance with one another. Meaning, fold changes in miR-141, PSA and CTC either increased or decreased in the same direction during the time intervals. For patients 7 and 14 (25% of cohort population), only two of the three biomarkers were concordant with one another. Since PSA is used as a standard assessment tool in progression of this disease, an important observation can be made for patient 7: as PSA went down so did his miR-141 value with an overall clinical assessment of regression. Whereas in patient 14, a clinically progressing patient, miR-141 and CTC values both decreased, with a net increase in PSA. It is important to note, however, that markers changes observed in this patient may reflect the emergence of a second metastatic

neoplasm (pancreas cancer) during the course of treatment of the metastatic CAP. However an autopsy was not done to confirm the presence of two malignancies.

For patients in cohort 2, two intervals were observed. For five patients (patients 6, 5, 8, 17, and 21), all biomarkers were concordant with one another during both intervals (38% of cohort). Two patients (patients 1 and 12) had the biomarkers concordant in only the first interval (15% of cohort). Four of the patients (patients 9, 15, 18, and 20) had observed concordant biomarkers in the second interval only (30% of cohort). Finally, two patients (patients 16 and 19) did not observe any three marker concordance in either one of the two intervals (15%). Interestingly, of all interval data points that were observed (between both cohorts), only one patient had a miR-141 change not be in concordant with another biomarker – patient 16. During interval 1 for this patient, PSA and CTC changes were observed to be concordant with each other; specifically, miR-141 value decreased where PSA and CTC increased. Clinically, this patient had a slowly progressing disease during that interval and the PSA and CTC values reflected that. During this patient's second interval, the disease rapidly progressed, but PSA had a net decrease, where both miR-141 and CTC increased.

Table 4 illustrates the degree of concordance each biomarker had with each patient's clinical assessment. Keeping in mind that the true clinical assessment was ascertained independent of the biomarkers, this assessment of biomarker concordance is based on the biomarker interval change and the actual clinical

assessment. Conventional logic deems a clinically progressing disease should be associated with an increase of a biomarker and vice versa for a clinically regressing disease. For this assessment, each biomarker was given a “+” if the change in marker was concordant with the clinical assessment and given a “-“ if it was not; meaning if the clinical assessment was deemed progressive, each biomarker should illustrate net increase and scored with a “+”.

The first draw for each patient was labeled as baseline (designated “b”) and therefore a concordance score could not be assessed. A total of 34 intervals were assessed in the study (combined all patients). For PSA, 29/34 (85%) intervals were concordant with the clinical assessment whereas the other markers had 27/34 (79%), 25/34 (74%) and 24/34 (71%) for CTC, miR-141 and LDH clinical concordance respectively.

3.2 Statistical analysis

One-way variance of analysis (ANOVA) was performed to determine if there was a significant difference between the two groups (positive versus negative slope), in their respective marker of study. This analysis would give us an initial indication whether any of the markers could differentiate a regression or progression prognosis based on marker values alone. Tables 5 (cohort 1) and 6 (cohort 2) provide the p values of both cohorts for each group compared to the tested biomarkers. In cohort 1, we observed $p < 0.001$ for PSA values between the two groups. LDH, CTC had values of $p = 0.172$ and $p = 0.154$, respectively and

miR-141 had $p=0.007$. In cohort 2, the p -values were $p<0.001$, $p=0.277$, $p=0.007$ and $p=0.024$ for PSA, LDH, CTC, and miR-141, respectively.

To determine the predictive relationships that each biomarker has with one another, Pearson correlation coefficients were determined and summarized in tables 7 and 8 for cohort 1 and 2, respectively. As correlation coefficients gets closer to 1.00, the tighter the relationships two biomarkers have with one another and can, in theory, predict the same degree of change. Correlation coefficients for cohort 1 for miR-141 versus PSA was $R=0.94$ ($p<0.001$), miR-141 versus CTC was $R=0.65$ ($p=0.082$), and miR-141 versus LDH was $R=0.85$ ($p=0.008$). For cohort 2, miR-141 versus PSA was $R=0.63$ ($p=0.021$), miR-141 versus CTC was $R=0.79$ ($p=0.001$), and miR-141 versus LDH was $R=0.67$ ($p=0.013$).

A binary classification analysis, although similar to a correlation analysis, was performed to indicate the sensitivity and specificity of a marker in relation to a standard. For a correlation analysis, the outcome variables (slope) was plotted to determine the correlation, a binary classification study differs in that each slope was assigned binary values based on its regression: either positive or negative. A positive slope was assigned a value of "1" whereas a negative slope was assigned a value of "0." LDH, CTC and miR-141 were subjected as test outcomes for the classification analysis comparing it individually against PSA, LDH and CTC values as gold standards. Classification of biomarkers against miR-141 as a standard was not performed as it is not a conventional marker. Tables 9 and 10 summarize the classification analysis for cohort 1 and cohort 2, respectively.

Since a positive slope is a true indicator of a disease that is progressing (a marker values that has increased during time point), the percentage of people who have a positive slope for a tested marker correctly identified as having a positive slope of the standard is an indicator of a marker's sensitivity. The specificity of a marker is a measure of the proportion of the patients that tested with a decline of a test marker (negative slope) with a decline of the standard marker. An overall correct classification was determined by samples that correctly identified sensitivity and specificity against the whole cohort.

Testing miR-141 against PSA as the standard yielded a sensitivity of 75%, specificity of 100% and a correct classification of 87.5% in cohort 1. For cohort 2, the same analysis yielded a value of 75%, 80% and 76.9% for sensitivity, specificity and correct classification, respectively. Performing the same analysis on CTC with PSA as the standard measured a 75% of all classifications in cohort 1 and a 87.5% sensitivity, 100% specificity and 92.3% overall performance in cohort 2. In this classification analysis, miR-141 values outperformed the CTC test in its capability to predict a PSA classification for patients with 1 interval, whereas with multiple intervals, CTC performed better. Interestingly, LDH did not perform any better than other two markers in predicting a PSA response. Testing miR-141 to predict CTC had a sensitivity, specificity and total classification percentages of 75.0%, 100% and 87.5%, respectively for cohort 1 and 85.7%, 83.3% and 84.6%, respectively for cohort 2.

Table 1: miR-141 values of control samples

Control Sample	miR-141 Value*
1	1997
2	1514
3	263
4	159
5	54.0
6	67.6
7	115
8	82.9
9	3.63
10	1.63

* = Copy number/8.35 μ L

Table 2: Ranges of miR-141 values observed with controls

miR-141 Value Range*	# Samples in Range
1-10	2
11-100	3
101-300	3
301-1000	0
1001-3000	2

* = Copy number/8.35 μ L

Table 3: Patient biomarker values and clinical assessment

Patient	Draw #	CTC Value*	PSA Value†	LDH Value ‡	miR-141 Value§	Clinical Assessment
1	1	2	90.4	134	29.8	Interval 1: Progressing rapidly (t = 2 months) Interval 2: Regressing slowly (t= 2 months)
	2	40	314.1	1246	135.6	
	3	56	238.2	159	760	
2	1	3	4.5	194	595	Interval 1: Progressing slowly (t = 13 months)
	2	401	9.6	282	5165	
3	1	201	71.8	328	1679	Interval 1: Progressing rapidly during interval. Patient died 4 days after draw 2 with liver metastases (t = 3 months)
	2	356	173.5	2729	18851	
4	1	0	0.2	311	338	Interval 1: Progressing slowly (t = 11 months)
	2	9	2.6	219	1479	
5	1	0	85.8	143	142	Interval 1: Progressing slowly (t = 4 months) Interval 2: Stable on treatment (t = 21 days)
	2	4	152.4	157	227	
	3	9	173	168	389	
6	1	3	21.5	205	95.9	Interval 1: Progressing rapidly (t = 2.5 months) Interval 2: Progressing slowly (t= 2.5 months)
	2	101	93.0	253.0	1025	
	3	206	143.2	291.0	1370	
	**4	326	119.0	141.0	4650	
7	1	0	74.3	155	3337	Interval 1: Progressing slowly during interval (t = 4 months)
	2	7	9.2	121	589	
8	1	65	288.8	229	950	Interval 1: Net slow regression (t = 3 months) Interval 2: Regressing rapidly (t= 20 days)
	2	1	168.0	155.0	99.8	
	3	0	69.7	174.0	36.7	
9	1	72	57.5	212	475	Interval 1: Progressing slowly (t = 5 months) Interval 2: Regressing slowly (t= 7 months)
	2	12	118.0	262.0	697	
	3	1	15.6	N/A	182	
10	1	41	14.3	234	295	Interval 1: Regressing slowly during interval (t = 4 months)
	2	0	<0.1	159	24.3	
11	1	113	259.1	183	9454	Interval 1: Regressing slowly during interval (t = 2 months)
	2	0	73.1	156	2559	
12	1	145	2.3	161	1067	Interval 1: Progressing slowly (t = 2 months) Interval 2: Regressing slowly (t= 4 months)
	2	13	1	151	219	
	3	0	2.7	132	201	
13	1	1295	71.8	352	856	Interval 1: Regressing rapidly during interval (t = 6 months)
	2	1	0.2	278	23.4	
14	1	67	138.1	242	790	Interval 1: Progressing rapidly (t = 4.5 months)
	2	16	172.5	339	622	
15	1	3	6.1	225	256	Interval 1: Regressing slowly (t = 7 days) Interval 2: Net slow progression (t= 2.5 months)
	2	5	4.9	324	258	
	3	26	17.2	294	1615	
16	1	28	247	188	795	Interval 1: Progressing slowly (t = 5.5 months) Interval 2: Net slow progression (t= 2.5 months)
	2	30	1184	168	349	
	3	273	858	N/A	674	
17	1	0	4	194	119	Interval 1: Progressing slowly (t = 3.5 months) Interval 2: Rapid progression (t= 3 months)
	2	2	26.3	200	642	
	3	17	238	219	1737	
18	1	0	4.8	147	138	Interval 1: Progressing slowly (t = 7 months) Interval 2: Slow progression (t= 1 month)
	2	3	2.4	170	123	
	3	29	18.2	120	427	
19	1	95	44.7	95	63.0	Interval 1: Regressing slowly (t = 2 months) Interval 2: Slow regression (t= 2 months)
	2	2	6.1	87	64.1	
	3	0	3.6	74	94.34	
20	1	9	1.6	215	1152	Interval 1: Net slow progression (t = 4.5 months) Interval 2: Slow regression (t= 3 months)
	2	7	6.4	290	1323	
	3	0	0.3	192	310	
21	1	8	593.2	340	2053	Interval 1: Slow regression (t = 2 months) Interval 2: Net Slow regression (t= 10.5 months)
	2	2	51.4	265	1923	
	3	0	0.2	258	271	

Interval 1= time period between draw 1 and draw 2, Interval 2 = time period between draw 2 and draw 3
t = time, * = Number of CTC/7.5mL of blood, † = ng/mL, ‡ = IU/L, § = Copy number/8.35µL as measured by qRT-PCR
** = No raw or statistical analyses were performed with data from patient 6, draw 4

Table 4: Biomarker concordance with clinical assessments

Patient	Draw #	Clinical Assessment	Concordance			
			CTC	PSA	LDH	miR-141
1	1	Interval 1: Progressing rapidly (t = 2 months) Interval 2: Regressing slowly (t= 2 months)	b	b	b	b
	2		+	+	+	+
	3		-	+	+	-
2	1	Interval 1: Progressing slowly (t = 13 months)	b	b	b	b
	2		+	+	+	+
3	1	Interval 1: Progressing rapidly during interval. Patient died 4 days after draw 2 with liver metastases (t = 3 months)	b	b	b	b
	2		+	+	+	+
4	1	Interval 1: Progressing slowly (t = 11 months)	b	b	b	b
	2		+	+	-	+
5	1	Interval 1: Progressing slowly (t = 4 months) Interval 2: Stable on treatment (t = 21 days)	b	b	b	b
	2		+	+	+	+
	3		-	-	-	-
6	1	Interval 1: Progressing rapidly (t = 2.5 months) Interval 2: Progressing slowly (t= 2.5 months)	b	b	b	b
	2		+	+	+	+
	3		+	+	+	+
7	1	Interval 1: Regressing slowly during interval (t = 4 months)	b	b	b	b
	2		-	+	+	+
8	1	Interval 1: Net slow regression (t = 3 months) Interval 2: Regressing rapidly (t= 20 days)	b	b	b	b
	2		+	+	+	+
	3		+	+	-	+
9	1	Interval 1: Progressing slowly (t = 5 months) Interval 2: Regressing slowly (t= 7 months)	b	b	b	b
	2		-	+	+	+
	3		+	+	N/A	+
10	1	Interval 1: Regressing slowly during interval (t = 4 months)	b	b	b	b
	2		+	+	+	+
11	1	Interval 1: Regressing slowly during interval (t = 2 months)	b	b	b	b
	2		+	+	+	+
12	1	Interval 1: Progressing slowly (t = 2 months) Interval 2: Regressing slowly (t= 4 months)	b	b	b	b
	2		+	-	-	-
	3		+	-	+	+
13	1	Interval 1: Regressing rapidly during interval (t = 6 months)	b	b	b	b
	2		+	+	+	+
14	1	Interval 1: Progressing rapidly (t = 4.5 months)**	b	b	b	b
	2		-	+	+	-
15	1	Interval 1: Regressing slowly (t = 7 days) Interval 2: Net slow progression (t= 2.5 months)	b	b	b	b
	2		-	+	-	-
	3		+	+	-	+
16	1	Interval 1: Progressing slowly (t = 5.5 months) Interval 2: Net slow progression (t= 2.5 months)	b	b	b	b
	2		+	+	-	-
	3		+	-	N/A	+
17	1	Interval 1: Progressing slowly (t = 3.5 months) Interval 2: Rapid progression (t= 3 months)	b	b	b	b
	2		+	+	+	+
	3		+	+	+	+
18	1	Interval 1: Progressing slowly (t = 7 months) Interval 2: Slow progression (t= 1 month)	b	b	b	b
	2		+	-	+	-
	3		+	+	-	+
19	1	Interval 1: Regressing slowly (t = 2 months) Interval 2: Slow regression (t= 2 months)	b	b	b	b
	2		+	+	+	-
	3		+	+	+	-
20	1	Interval 1: Net slow progression (t = 4.5 months) Interval 2: Slow regression (t= 3 months)	b	b	b	b
	2		-	+	+	+
	3		+	+	+	+
21	1	Interval 1: Slow regression (t = 2 months) Interval 2: Net Slow regression (t= 10.5 months)	b	b	b	b
	2		+	+	+	+
	3		+	+	+	+
# of intervals			34	34	34	34
number concordant			27	29	24	25
% Correct			79%	85%	71%	74%

b = baseline, Interval 1 = time period between draw 1 and draw 2, Interval 2 = time period between draw 2 and draw 3

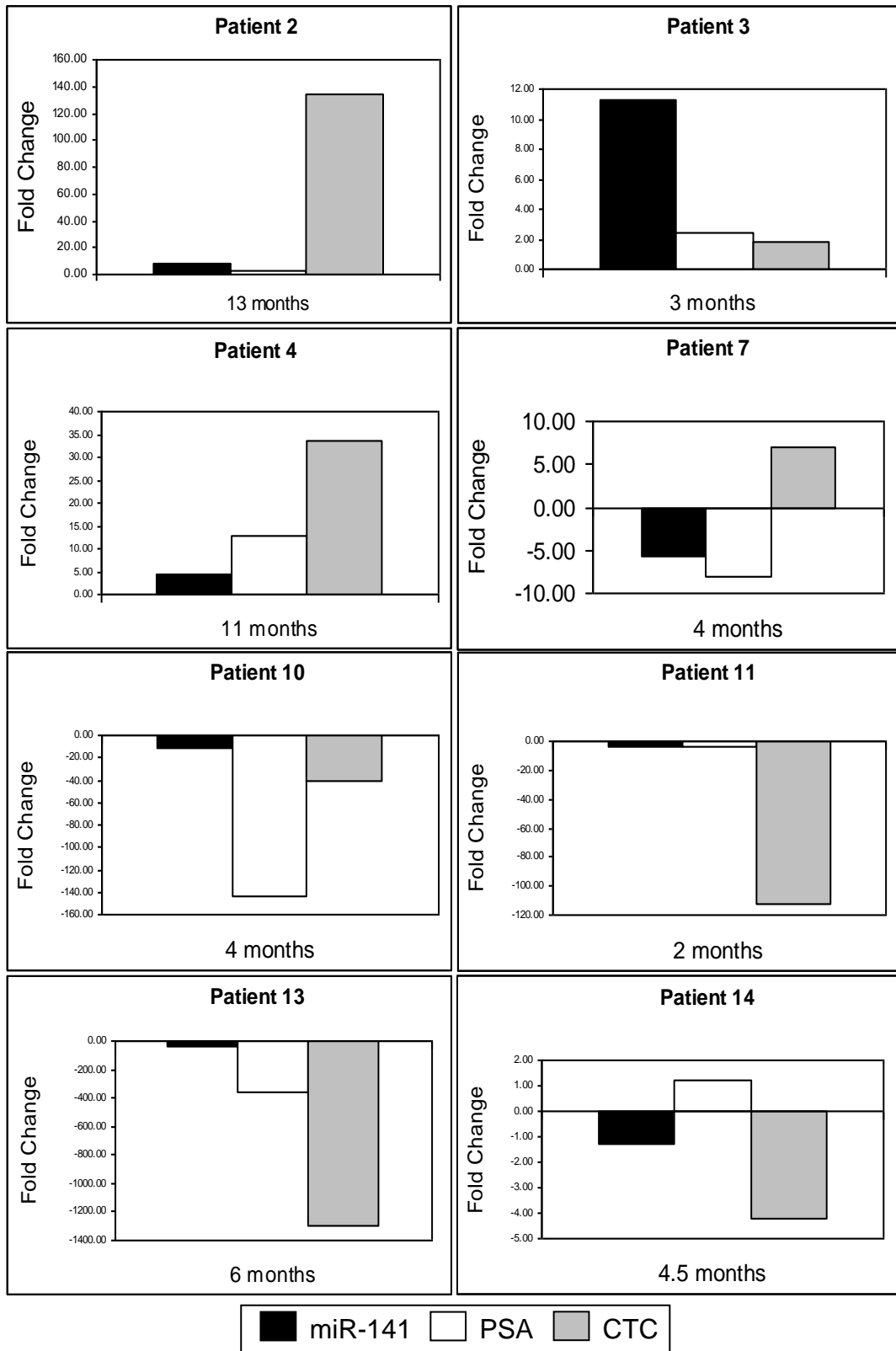


Figure 5: Fold changes in biomarker values for patients with 2 time points

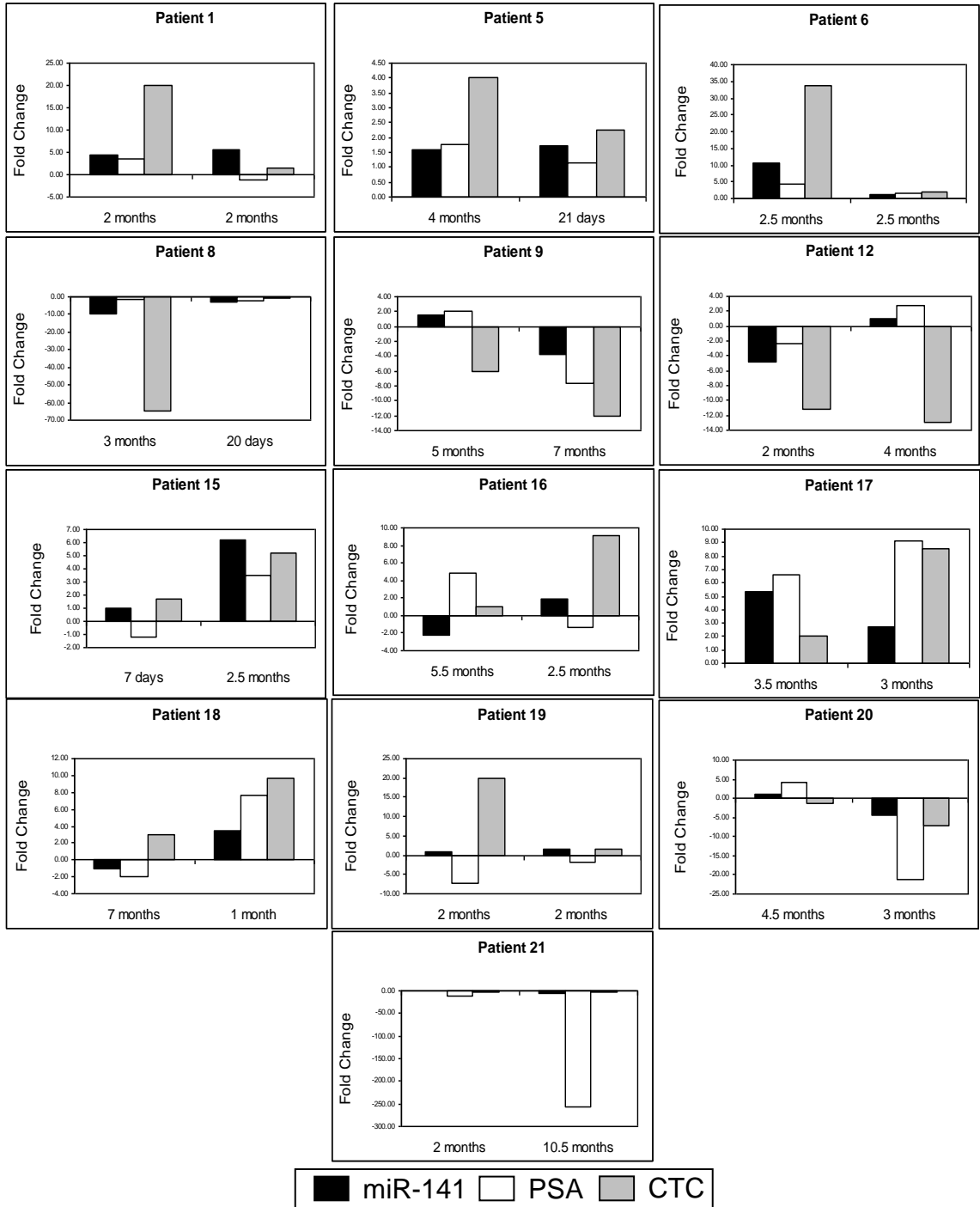


Figure 6: Fold changes in biomarker values for patients with 3 time points

Table 5: Analysis of variance for cohort 1 (patients with 1 interval)

	Marker			
	PSA	LDH	CTC	miR-141
Group 1				
Mean	0.189	0.308	0.13	0.394
Std	0.178	0.531	0.479	0.59
N	4	4	4	4
Group 2				
Mean	-0.924	-0.105	-1.521	-0.869
Std	0.135	0.04	1.967	0.194
N	4	4	4	4
p-value	<0.001	0.172	0.154	0.007

Table 6: Analysis of variance analysis for cohort 2 (patients with 2 intervals)

	Marker			
	PSA	LDH	CTC	miR-141
Group 1				
Mean	0.322	0.006	0.462	0.411
Std	0.262	0.042	0.702	0.493
N	8	8	8	8
Group 2				
Mean	-0.436	-0.028	-0.853	-0.313
Std	0.314	0.066	0.687	0.47
N	8	8	8	8
p-value	<0.001	0.277	0.007	0.024

Table 7: Correlation analysis for cohort 1 (patients with 1 interval)

	Marker		
	vs. LDH	vs. CTC	vs. miR-141
PSA	0.68	0.66	0.94
	p=0.065	p=0.074	p<0.001
LDH		0.34	0.85
		p=0.412	p=0.008
CTC			0.65
			p=0.082

Table 8: Correlation analysis for cohort 2 (patients with 2 intervals)

	Marker		
	vs. LDH	vs. CTC	vs. miR-141
PSA	0.48	0.78	0.63
	p=0.097	p=0.002	P=0.021
LDH		0.63	0.67
		p=0.020	p=0.013
CTC			0.79
			p=0.001

Table 9: Classification analysis for cohort 1 (patients with 1 interval)

	Test Parameter					
	LDH		CTC		miR-141	
PSA (standard)	Decrease	Increase	Decrease	Increase	Decrease	Increase
Decrease	4	0	3	1	4	0
Increase	1	3	1	3	1	3
Sensitivity	75.00%		75.00%		75.00%	
Specificity	100.00%		75.00%		100.00%	
Correct classification	87.50%		75.00%		87.50%	
LDH (standard)			Decrease	Increase	Decrease	Increase
Decrease			3	2	4	1
Increase			1	2	1	2
Sensitivity			66.70%		66.70%	
Specificity			60.00%		80.00%	
Correct classification			62.50%		75.00%	
CTC (standard)					Decrease	Increase
Decrease					4	0
Increase					1	3
Sensitivity					75.00%	
Specificity					100.00%	
Correct classification					87.50%	

Table 10: Classification analysis for cohort 2 (patients with 2 intervals)

	Test Parameter					
	LDH		CTC		miR-141	
PSA (standard)	Decrease	Increase	Decrease	Increase	Decrease	Increase
Decrease	4	1	5	0	4	1
Increase	4	4	1	7	2	6
Sensitivity	50.00%		87.50%		75.00%	
Specificity	80.00%		100.00%		80.00%	
Correct classification	61.50%		92.30%		76.90%	
LDH (standard)			Decrease	Increase	Decrease	Increase
Decrease			5	3	5	3
Increase			1	4	1	4
Sensitivity			80.00%		80.00%	
Specificity			62.50%		62.50%	
Correct classification			69.20%		69.20%	
CTC (standard)					Decrease	Increase
Decrease					5	1
Increase					1	6
Sensitivity					85.70%	
Specificity					83.30%	
Correct classification					84.60%	

CHAPTER 4

DISCUSSION AND CONCLUSION

There is great demand for more specific and sensitive biomarkers for prognosticating disease progression and determining treatment efficacy in CAP patients. It is well known that PSA values can be affected by tumor differentiation state; where poorly differentiated tumors produce significantly less PSA compared to tumors with well differentiated cells. Significant differences in PSA production can lead to false-negatives leading a patient to believe that he is cancer-free. Despite its inherent shortfalls, the use of PSA levels as a diagnostic and prognostic marker has been the most commonly used test and seen as the gold standard in the detection and care of CAP.

There has been major focus in discovering new biomarkers that will facilitate in the monitoring of disease progression. Proper monitoring of disease progression throughout a treatment regimen is vital for increasing a patient's chance of survival. The monitoring of disease progression or treatment efficacy through the detection of biomarkers is ideal in that it requires a less invasive approach and limits potential harm and discomfort to the patient. As mentioned, PSA is the gold standard in monitoring CAP; however new approaches such as detecting circulating tumor cells have been developed and is currently being used in conjunction with PSA tests.

Discovering new biomarkers associated with disease states can be a daunting task. Initial efforts in discovering new markers require "fishing" for any changes or expression levels compared to normal or non-disease cohorts.

Determining these changes requires numerous gene expression, microarray analyses or other experimental procedures that detect unique disease-associated genetic signatures. These experiments can even be complicated in the potential lack of samples or a model system. Once a potential marker becomes identified, these candidate markers must prove to be sensitive and specific enough to be given approval for use in clinical testing.

Markers currently approved for clinical use can help in validating a potential marker's potential to indicate a possible disease state. Candidate markers can use approved biomarkers as standards with which to compare its potential predictive characteristics. More often, comparative studies would ideally be performed under a prospective study. For this type of study, the expression of a putative marker should be determined or tested concomitantly with the standard marker. Furthermore, marker values should be compared over time and determine if there is any correlation or concordance of changes comparable to the standard. Prospective studies can prove to be difficult to establish due to potential lack in patients exhibiting the specific disease or the lack of tests being offered by certain facilities.

In lieu of prospective studies, a retrospective study can be performed on samples collected and stored in biorepositories. Many facilities proactively collect extra biological samples from patients in the anticipation of certain studies. Unfortunately, retrospective studies can introduce bias in the sample selection or can risk the degradation of an analyte if the sample is not prepared or stored properly. Nevertheless, a positive retrospective study can lead to a

prospective study if evidence suggests a strong correlation between the putative and standard marker.

In this study, we took a retrospective approach to determine if detectable miRNA in CAP samples can accurately predict a patient's prognosis. An initial study (Mitchell *et al.*, 2008) determined that miR-141 was stable and detectable in CAP in the plasma of patients. We expanded on their study to compare miR-141 with other conventional markers clinically approved for monitoring CAP prognosis. Furthermore, these studies were performed temporally over either two or three draw points and determined if these changes over time correlated with the changes of the other conventional markers, and most importantly, if they there was concordance with the patient's clinical outcome.

Although we detected miR-141 in our control population, the control data proved to be inconclusive. Since control samples are healthy individuals, no follow-up samples were collected leaving us with only one data point per control; therefore we cannot determine any temporal changes. Furthermore, no other clinical values were ascertained limiting our ability to compare the miR-141 values against other markers. The individual data points also demonstrate an inability to establish any baseline values that can distinguish a healthy individual from a diseased individual.

We observed a range of miR-141 values from our control samples of 1.6 (control sample 10) to 1997 (control sample 1) copy numbers – a difference of over 1000 fold. However, analyzing the control miR-141 values broken down into ranges illustrate that 80% of the samples fell between ranges of 1- 300 copy

numbers, whereas the remaining fell in the range of 1000 or more. It would be easy for us to classify the spike in these two samples as outliers from the remainder of the group, but unfortunately, the control patients were kept anonymous and their health status was not clinically validated. Perhaps this illustrates miR-141's involvement in processes we are not quite aware of at the moment. An improvement for a study like this would be the ability to include validated health statuses on control samples. Nonetheless, we found significant findings in the changes of miR-141 for patients who have progressing or regressing CAP.

We first sought to determine if raw values of miR-141 could accurately predict a clinical response by determining any degree of concordance that miR-141 values had with the true clinical assessments. For all of our comparison studies, PSA was used as the standard. PSA had an 85% concordance with the clinical assessments whereas CTC and miR-141 had a 79% and 74% concordance, respectively. This analysis clearly illustrates that, although PSA is considered the benchmark marker, there is no test that can predict a clinical response with 100% accuracy for CAP. Furthermore, it illustrates that miR-141 has predictive qualities that can potentially discern a clinical assessment.

Our initial ANOVA study clearly indicates (as expected) that PSA is capable of differentiating the difference of a progressing or regressing CAP patient. For both cohorts, p-value for PSA of $p < 0.001$ is a strong indicator that the differences between the two groups were not by random chance alone. As we compared the miR-141 p-values, we observed fairly significant values ($p = 0.007$ and $p = 0.024$ for

cohort 1 and 2, respectively). Meaning, to some degree of confidence, we can differentiate groups of patients based on miR-141 values. Comparing these p-values to LDH and CTC for those in cohort 1, we confidently say that miR-141 outperforms LDH and CTC in its ability to properly classify CAP prognosis in patients with one interval. However, in patients with two interval (cohort 2), the performance of miR-141 was not as impressive and was outperformed by CTC ($p=0.007_{\text{CTC}}$ vs. $p=0.024_{\text{miR-141}}$). An important observation was made in that LDH did not outperform any markers in any of the two cohorts for this particular study

Our study of variance also illustrated a high coefficient of variation (CV) observed for each group and in each cohort with CVs ranging from 30% or greater. For a translational study such as this, it would be important to limit this CV to 10% or less. To achieve this, we would need to determine what would be the sufficient sample size for ensuring a CV of our ideal percentage. Using our observed means for each marker studied, a confidence interval of 99% and limiting the margin of error to 1%, it is estimated that we would have needed to study 663 patients achieve a CV of 10% or less.

We conducted a correlation study to compare the predictive relationship of each marker versus the standard (PSA). We observed a high correlation ($R=0.94$, $p<0.001$) of miR-141 for cohort 1; however, these values decreased in cohort 2 ($R=0.63$, $p=0.021$). Again, the performance of miR-141 in the correlation study for cohort 1 was significantly better than both CTC and LDH, but this cannot be said in cohort 2. In cohort 2, CTC ($R=0.78$, $p=0.002$) clearly outperformed the other two analytes. Also for cohort 2, we observed a

signification correlation between CTC and miR-141 values ($R=0.79$, $p=0.001$), thus allowing us to conclude that there could potentially be a synergistic quality between these two analytes in prognosticating CAP.

As for any medical test, it is necessary to determine the predictive value and accuracy of test in order to be used for clinical settings. To conclude our statistical analysis of biomarker comparisons, we performed a binary classification analysis to determine the sensitivity (true positives) and specificity (true negatives) of a marker in relation to a gold standard. Performing this type of comparison would allow us to further conclude the capability that miR-141 can screen and confirm a CAP prognosis. This test indicated that miR-141 had strong classification characteristics compared to CTC and LDH. Although we only observed an 80% specificity (compared to PSA as gold standard) for cohort 2, we were able to observe 100% specificity for cohort 1. Furthermore, comparing the sensitivity and specificity of LDH and CTC against PSA, miR-141 performed better than LDH and comparable to CTC in this classification analysis. Ideally, any test should have sensitivity and specificity close to 100%, but no such perfect test exists for CAP.

Our study was designed to evaluate the performance of miR-141 in predicting clinical outcomes in CAP patients. Our goal was to determine if temporal changes of miR-141 correlated with the clinical outcomes of patients and if these changes concomitantly changed with other conventional biomarkers used in monitoring and prognosticating CAP. The study of raw temporal changes, however, did not allow us to determine a baseline miR-141 copy number value

that can accurately predict a clinical outcome (meaning we cannot determine an upper limit of normal). Determining a threshold or baseline miR-141 value would require a more extensive study involving more patients.

Our statistical analyses were performed on the slopes determined from plotting two interval points; these slopes are interpreted as the rate of biomarker change over time. The analysis compared miR-141 changes to the rates of change observed in the other clinical markers. The correlation and classification analyses indicate that miR-141 has the potential to be a new biomarker for the progression of CAP. In some instances, miR-141 outperformed CTCs but in all cases it performed better than LDH.

For this study, no inference can be made on the mechanisms for miR-141 detection in the bloodstream. However, our ANOVA analysis may point to a link with PSA and miR-141 secretion into the bloodstream. Perhaps there can also be a link with miR-141 values and CTCs detected wherein a CTC could possibly undergo apoptosis or lyse releasing miR-141; however, further characterization and mechanism of CTC release is needed to characterize any potential links. Nonetheless, this retrospective study provides evidence that miR-141 has potential to aid in prognosticating CAP progression or predict response to treatment; however additional studies need to be performed prospectively with a much larger sample population to confirm the utility of miR-141.

APPENDIX
SUPPLEMENTAL DATA

Table A1: Summary of preliminary data obtained our study

Disease	Platform	Analyte Type	Analyte	Status
Breast cancer	Luminex®	Protein	CA-125, IGF-II, Leptin, MIF, Osteopontin, Prolactin	Sample procurement issues hindered further results
	qRT-PCR	Gene Expression	BMI1 and SALL4	Preliminary results obtained
Prostate Cancer	Luminex®	Protein	BMI1 and SALL4	Sample procurement issue hindered preliminary results
	qRT-PCR	Gene Expression	BMI1 and SALL4	Degradation of analyte yielded no results
	qRT-PCR	microRNA	miR-141	Pilot study completed

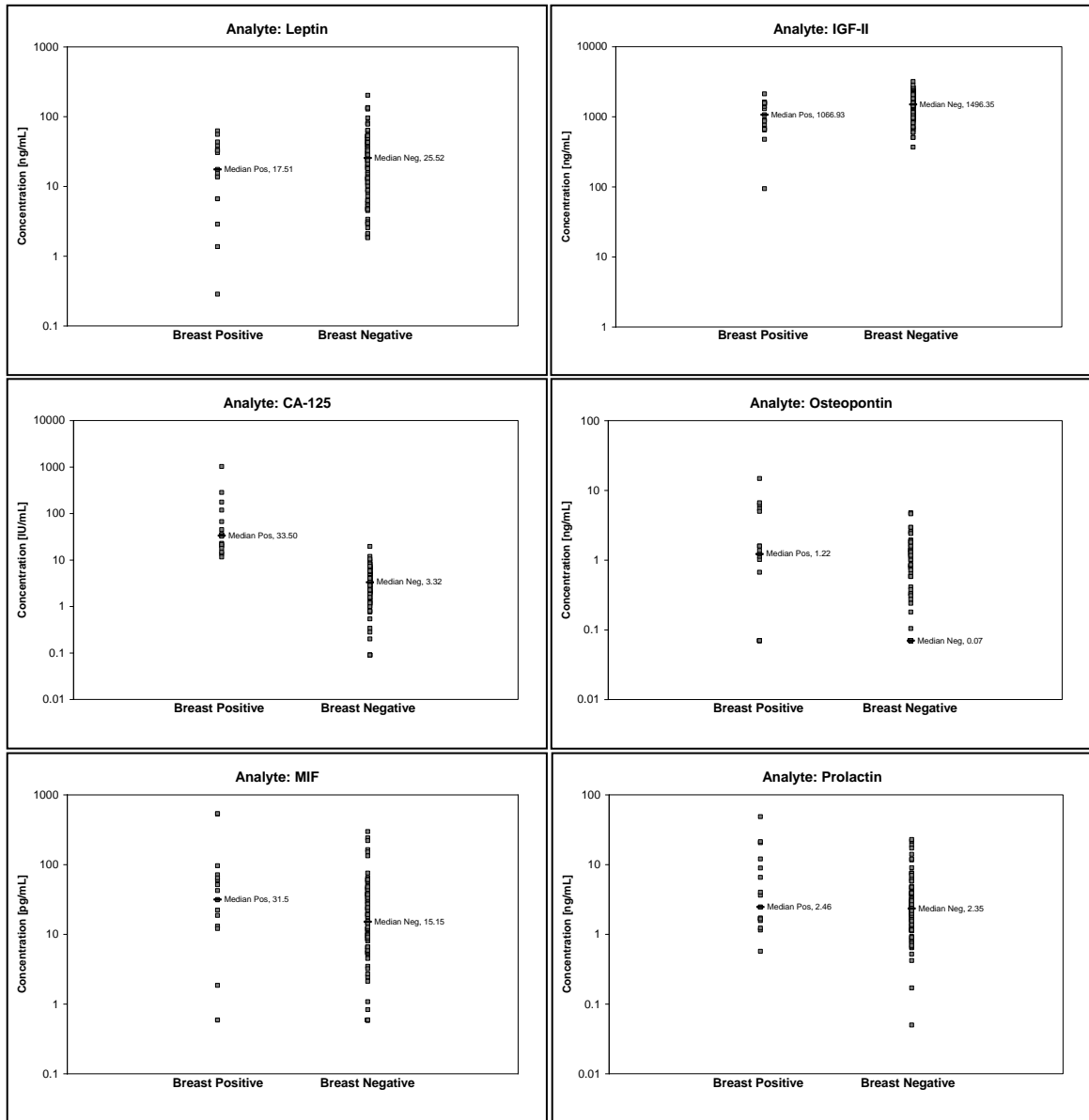


Figure A1: 6-analyte assay of breast cancer patients. Samples of serum normal women and breast cancer patients were subjected to a 6-analyte, Luminex®-based biomarker panel obtained from Millipore. Our objective was to determine the utility of this marketed biomarker panel in detecting and differentiating breast cancer patients from normal samples. Policies in samples procurement hindered attempts to follow-up with results and perform more studies.

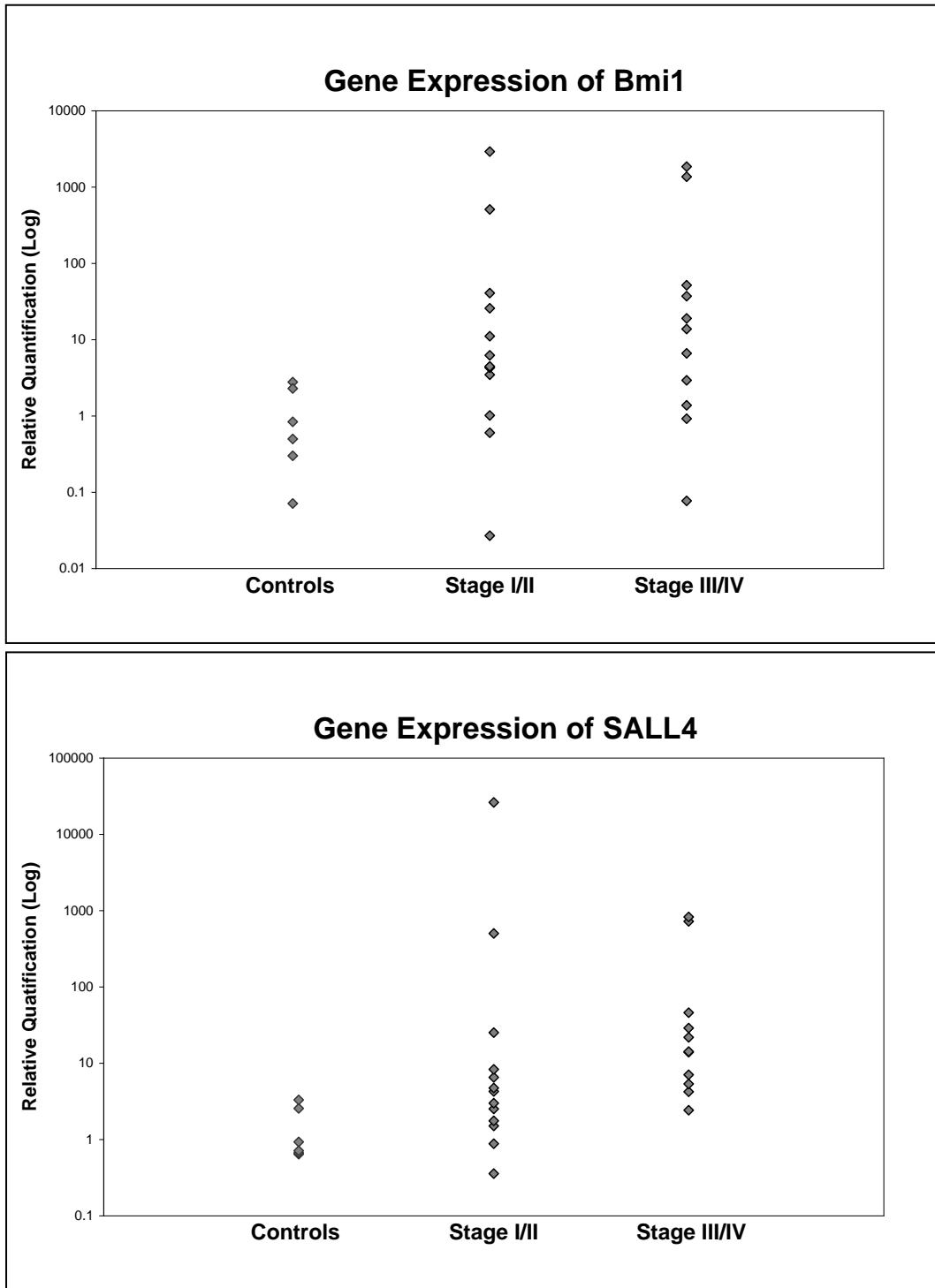


Figure A2: SALL4 and BMI1 gene expression assay on breast cancer patient serum. In-house developed multiplex TaqMan gene expression assay was performed to determine relative quantification of SALL4 and BMI1 in breast cancer patients with varying stages of disease progression. Policies in samples procurement hindered attempts to follow-up with results and perform more studies.

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