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ANALYSIS OF THE PROTEOMIC PROFILE AND COMPOSITION IN PROSTATE CANCER PATIENTS

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ANALYSIS OF THE PROTEOMIC PROFILE AND COMPOSITION IN PROSTATE CANCER PATIENTS

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ABBREVIATIONS

APOA4 – apolipoprotein a-IV

AR – (human) androgen receptor

BPH – benign prostatic hyperplasia

CRC – colorectal cancer

FLNA – filamin A

FXI – coagulation factor XI

FXII – coagulation factor XII

HAI-1 – hepatocyte growth factor activator inhibitor type 1

HEG1 – protein HEG homolog 1

HDAC1 – histone deacetylase 1

HRG – histidine rich glycoprotein

LC-MS/MS – liquid chromatography-mass spectrometry

MMP-2 – matrix metalloproteinase 2

MMP-9 – matrix metalloproteinase 9

MSNs – mesoporous silica nanoparticles

NCI – National Cancer Institute

NF-kB – nucclear factor-kappa B

NSpC – normalized spectral counts

PAI-1 – plasminogen activator inhibitor-1

PCA – prostate cancer

PSA – prostate-specific antigen

siRNA – small interfering RNA

TGA – thermogravimetric analysis

uPA – urokinase -type plasminogen activator

uPAR – uPA-receptor

VEGF – vascular endothelial growth factor

ABSTRACT

Prostate cancer is a significant public health concern among American men. The current screening method for prostate cancer relies on elevated levels of prostate-specific antigen (PSA), which can be unreliable. Alternatively, a protein "fingerprint" identifies a characteristic set of proteins indicative of disease. Previous research in this laboratory group has identified a baseline protein fingerprint for prostate cancer. Here, an analysis is performed to identify how the protein fingerprint changes in composition throughout disease progression through inspection of the proteomic composition of human serum and review of the current scientific literature to investigate why these proteins might be dysregulated with respect to the metabolic activity of malignant cells. It was found that different disease states (metastasis, positive biopsy, negative biopsy, and healthy control) had distinctly different sets of upregulated and downregulated proteins. These dysregulated proteins had varying levels of relevance to physiological mechanisms involved in the progression of prostate cancer, with some proteins having a clearly defined role in cancer development while other proteins had unclear roles and appeared to have primarily diagnostic value at this time, elucidating areas for potential further research. Together these results provide a better understanding of the metabolic progression and consequences of prostate cancer, potentially contributing to earlier, more accurate diagnosis in the future.

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I. INTRODUCTION & BACKGROUND LITERATURE

Cancer is a well-known and widespread public health issue, with prostate cancer at the top of the list of concern for American men. The American Cancer Society cancer statistics for 2019 state that prostate, lung and bronchus, and colorectal cancers accounted for 42% of all cases in men, with prostate cancer alone accounting for nearly 1 in 5 new diagnoses.[1] The statistics for prostate cancer can be tricky, however, as incident rates have reflected some unusual trends in the past, with rates spiking dramatically in the late 1980s and early 1990s and more recent trends from 2011-2015 reflecting accelerated declines of approximately 7% per year for prostate cancer (which is significant when compared to 3% per year for cancers of the lung and colorectum).[1] These unusual trends have been attributed to the fact that diagnosis relies on elevated levels of prostate-specific antigen (PSA), a protein produced by the prostate gland, as the primary criterion. PSA, an androgen-regulated serine protease, is produced by prostate epithelial cells and functions to cleave semenogelins in the seminal coagulum as part of semen production.[2] However, inflammation, aging, and other problems unrelated to cancer can also elevate PSA levels, making it an unreliable biomarker. This caused a surge in the detection of asymptomatic disease as a result of widespread PSA testing among previously unscreened men in the 1980s followed by a sharp drop in prostate cancer incidence, not as a result of significant change in disease prevalence, but rather decreased PSA testing from 2008 to 2013 in the wake of US Preventive Services Task Force recommendations against the routine use of the test because of growing concerns about overdiagnosis and overtreatment.[3]

Multiple studies have determined PSA to be an unreliable biomarker for prostate cancer as it lacks specificity for the prostate cancer disease state, limiting its use in diagnosis and

potential for early detection of disease, a critical factor in the treatment of cancers and improving prognosis.[4, 5] The current standard for PSA in the detection of prostate cancer is unreliable given that ~20 % of men with PSA levels <4 ng/ml have prostate cancer and that many men with higher levels do not have prostate cancer where 4 ng/ml is defined as the upper limit of normal.[6] Despite this data, PSA is still the most used biomarker and the prevalence of prostate cancer in combination with the inaccuracy of diagnosis via PSA has prompted the search for novel, accurate and more reliable biomarkers for the disease.

The National Cancer Institute (NCI) defines 'biomarker' as a biological molecule found in blood, other body fluids, or tissues that can be objectively measured and evaluated as a sign of a normal/abnormal biological process and a pathogenic condition or disease.[6] Studies have identified many potential biomarkers of prostate cancer through various techniques, including via protein biochip surface enhanced laser desorption/ionization mass spectrometry coupled with an artificial intelligence learning algorithm to differentiate prostate cancer from noncancer cohorts,[4] iTRAQ 3D liquid chromatography with mass spectrometric detection (LC-MS),[7] metabolomics analysis using magnetic resonance spectroscopy, mass spectrometry and gas chromatography,[8] and protein adsorption onto mesoporous silica nanoparticles coupled with LC-MS to create a proteomic profile,[9] among other methods. In other words, a wide range of potential biomarkers for prostate cancer have been discovered with promising potential to replace the unreliable PSA test, including amino acids, lipids and metabolites involved in energy metabolism,[8] hundreds of proteins, and some non-FDA approved biomarkers including gene markers, RNA, miRNAs, and mitochondrial DNA.[6] Additional research is needed to determine which biomarkers are the most significant, effective, and accurate in diagnosis. Here, a "protein fingerprint" of the proteomic

composition in prostate cancer is proposed as a minimally invasive, inexpensive, and clinically accurate way to identify prostate cancer.

This work is relevant to public health because prostate cancer is highly prevalent, can detrimentally affect quality of life, and can be fatal, yet despite these significant negative aspects it is commonly misdiagnosed or diagnosed later than necessary due to the inaccuracy of the current screening methods. Diagnosis commonly results in false positive or negative results, causing both psychological stress and biological impacts to patient health in the case that no disease is present or by delaying diagnosis to a point that could worsen prognosis, prompting the need for a new, more reliable diagnostic method. This research is relevant because an understanding of the metabolic progression of prostate cancer could potentially aid in the development of a more accurate diagnostic method in the future.

II. PREVIOUS RESEARCH & STUDY AT HAND

In this topic area, research has been conducted to investigate the reliability of PSA as a biomarker and to identify other possible biomarkers for prostate cancer.[4-8] Although there is a solid body of research and review on the identification of alternative reliable biomarkers for prostate cancer, there has not been a significant amount of research on the metabolic effects of prostate cancer and how they influence the serum protein composition of these biomarkers, which is the focus of the study at hand.

Previous research has been done by other researchers in the Landry laboratory to characterize the proteomic profile of prostate cancer, allowing identification of 51 potential protein biomarkers for prostate cancer and the development of a protein fingerprint.[9] The protein

composition of the human serum of prostate cancer patients was determined by exposing human blood serum to mesoporous silica nanoparticles (MSNs), resulting in the adsorption of a specific subset of the proteins present in the serum onto the MSNs. Important to note are the effects of different nanoparticle properties on the types of proteins that adsorb, thus affecting the protein fingerprint.[10] Thiol-functionalized MSNs were chosen specifically for their ability to capture the greatest amount of protein relative to other types of nanoparticles, and the porosity of these particles allowed the capture a greater fraction of low-molecular weight proteins prevalent in serum that are relevant in disease states.[10] Following isolation of the MSNs and extraction and digestion of the adsorbed proteins, thermogravimetric analysis (TGA) was used to determine the total mass of protein present in a sample and LC-MS/MS was used to determine the identities of the proteins present.[9] The normalized spectral counts from mass spectrometry were multiplied by the total mass of protein from TGA to determine the individual protein masses. These data were then analyzed using the Elastic Net penalized multinomial regression model[9] and compared to controls (the serum protein composition of cancer-free patients), to identify statistically significant up- and down-regulated proteins. Z-scores, which represent the number of standard deviations from the mean, were calculated for each protein where the mean value for a given protein was taken across all patients in the study, including controls.[9] A characteristic protein fingerprint for the disease was developed based on the collective Z-scores for each represented protein (Figure 1).

This research identified 51 proteins that were found to be significantly up or down regulated in a particular patient group relative to the others, and it was also found that the types of proteins present strongly related to disease development.[9] Different proteins were seen to have distinctly different levels of significance in the plasma of patients at different stages of

prostate cancer. The statistical significance of these 51 proteins, which were identified as either significantly up- or down-regulated, was determined using the Elastic Net penalized multinomial regression model. Some proteins in individual patients demonstrated Z-scores of over 4 (meaning 4 standard deviations from the mean), indicating significant dysregulation of that protein with respect to other patients in the study, as 99.9% of a population is captured within 4 standard deviations from the mean (Figure 1).[9] The astounding standard deviations seen in some of these markers indicate a high level of significance, making them worthwhile candidates for the investigation of the metabolic consequences of the disease that could produce such significant levels of these protein products.

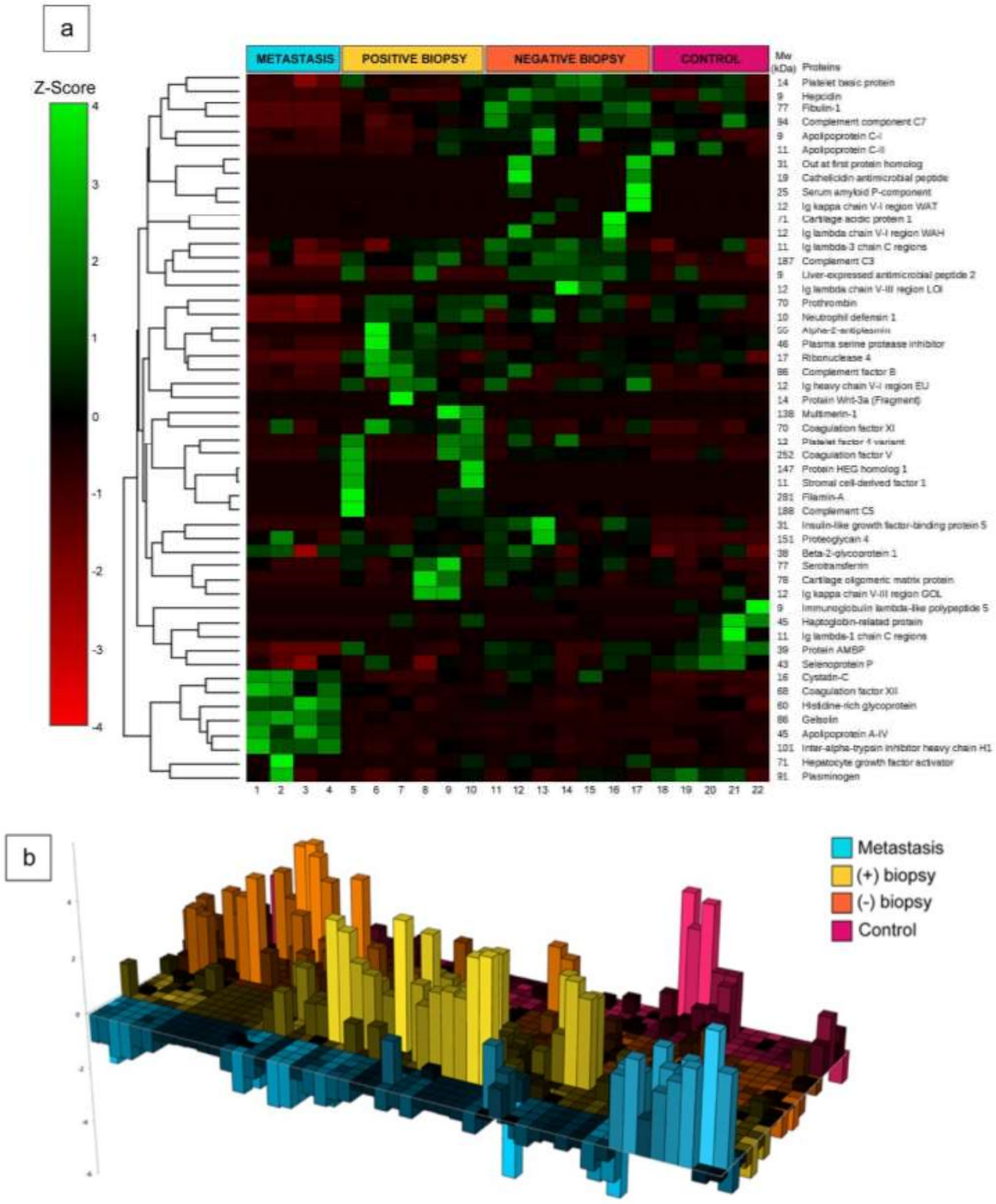

Figure 1a. Heat map representing Z-score values obtained by elastic net penalized multinomial regression model of 51 proteins identified in the serum of prostate cancer patients.[9] Proteins and their corresponding molecular weights (kDa) are listed on the right-hand side of the figure. Patient number is along the x-axis. Significance of each protein at different disease stages (metastasis, positive biopsy, and negative biopsy) as well is in a control patient is indicated through color coding of the Z-score, with green indicating up-regulated proteins (Z-score >1) and red indicating down-regulated proteins (Z-score < -1). Black indicates no statistically significant dysregulation. b. 3D representation of the heat map illustrating groups of dysregulated proteins based on Z-score. Figures used with permission.[9]

The study at hand aims to elaborate on this past research done by the Landry Group that identified a baseline protein fingerprint for prostate cancer and will investigate (1) how the protein fingerprint changes in composition throughout the progression of prostate cancer through analysis of the proteomic composition of human serum of prostate cancer patients and why such changes occur from a metabolic perspective and (2) if these protein fingerprints can be used to distinguish between cancerous disease states. In cancer, the levels of metabolic products (such as various proteins) of malignant cells can become abnormal, and much of this thesis will comprise of thorough review of the current scientific literature to determine the significance of proteins present at abnormal levels and why these proteins might be dysregulated with respect to the metabolic activity of the malignant cells, providing a better understanding of the metabolic progression and consequences of the disease which can potentially be used to develop a more accurate diagnostic method in the future.

In the study at hand, the dysregulated proteins were screened for relevance according to several different factors, including cancer specificity (relevance to prostate cancer vs. other cancers), prostate cancer disease stage (metastasis, positive biopsy, negative biopsy, and control), cellular location, serum concentration by mass, and Z-score. One of the most significant issues in identifying relevant biomarkers for specific cancers is that there are many peptides and peptide fragments that are commonly dysregulated in various cancers due to the general health of cancer patients. An investigation of the specific metabolic mechanism causing a given protein to become dysregulated can provide insight into which potential biomarkers are worth pursuing as characteristic of prostate cancer vs other cancers (and could serve as potential novel therapeutic targets for prostate cancer).

First, Z-score was considered as proteins that are more significantly dysregulated are likely to serve as more reliable biomarkers and indicators of disease pathophysiology than proteins that are only slightly dysregulated with respect to non-disease state concentrations. The 51 proteins were sorted to identify the top and bottom 10% in the positive biopsy and metastasis groups. The most significantly dysregulated proteins, either upregulated or downregulated, were determined by identifying the top and bottom 10% in each group.

Cellular location is important, because to develop a proteomic profile for prostate cancer based on the plasma serum of prostate cancer patients, the relevant biomarkers must be present in the extracellular fluid as intracellular proteins would not show up in proteomic analysis of plasma serum. Therefore, in an investigation of these 51 dysregulated proteins, identification of any intracellular proteins that became detectable in extracellular fluid was significant as it might relate to the pathophysiology of prostate cancer.

Serum concentration for each protein was calculated using the product of the average values of the normalized spectral counts (NSpC) and thermogravimetric analysis (TGA) for all proteins found in protein corona analysis of prostate cancer patient serum samples. This factor is important to consider because it relates to the sensitivity of detecting biomarkers; dysregulated proteins present at low concentrations are harder to detect and therefore less reliable than proteins present at higher concentrations in plasma serum.

Currently, there is only one widely used biomarker to screen for prostate cancer (PSA), which has been shown to be unreliable as it may be dysregulated in other non-cancerous health conditions and result in inaccurate diagnoses, negative outcomes for patients, and unnecessary clinical expenses. Clearly there is a need to identify novel, more accurate biomarkers for prostate cancer. Other researchers have investigated families of biomarkers for evidence of disease,

mainly focusing on the presence or absence of prostate cancer in relation to these new biomarkers and not the stage of disease present (such as metastasis versus positive biopsy, for example). Evidence shows that families of bloodborne biomarkers change based on disease state. Therefore, it is expected that each disease state in prostate cancer patients will display a characteristic set of proteins that are unique from those in other disease states. This analysis investigates the question, can the disease state of prostate cancer be diagnosed based on families of biomarkers, i.e., a protein fingerprint? If that is indeed the case, what are the physiological functions of these biomarkers, and how do these biological mechanisms at work differ between the cancerous states of metastasis and positive biopsy?

III. LITERATURE REVIEW & RESULTS

In this study, the proteins that were significantly dysregulated specifically in the two cancerous disease states for prostate cancer (metastasis and (+) biopsy) were examined in detail to identify the biological mechanisms altering the proteomic composition in these patients and how they differ between these two cancerous states, providing a new way of understanding the metabolic state of prostate cancer patients and the how the disease progresses. Clinically, it is important to distinguish between disease states as it is relevant to determining the course of patient treatment, prognosis, and outcome. The top and bottom 10% of dysregulated proteins in four patient groups (metastasis, (+) biopsy, (-) biopsy, and healthy controls) were identified (Table 1), compared for similarity, and investigated for the role of the dysregulated proteins in mechanisms contributing to disease states. For additional data of all of the average Z-scores for each of the 51 proteins found in each of the four patient groups, see Appendix A.

Table 1. Top and bottom 10% of dysregulated proteins in metastasis, positive biopsy patient, negative biopsy, and control groups identified using elastic net penalized multinomial regression statistical model. The Z-scores listed represent average the Z-score for that protein across all patients in the indicated group.

Metastasis		**(+) Biopsy**	
Top 10%	Z-score	Top 10%	Z-score
Gelsolin	2.04550	Coagulation factor XI	0.93114
Apolipoprotein A-IV	1.95302	Plasma serine protease inhibitor**	0.89584
Inter-alpha-trypsin inhibitor heavy chain H1**	1.95216	Filamin-A	0.86908
Histidine-rich glycoprotein**	1.88158	Protein HEG homolog 1	0.84111
Coagulation factor XII	1.79890	Stromal cell-derived factor 1	0.83894
Bottom 10%	Z-score	Bottom 10%	Z-score
Prothrombin**	-1.60584	Hepcidin	-0.55623
Neutrophil defensin 1**	-1.50962	Cystatin-C	-0.51891
Fibulin-1	-1.33129	Complement component C7	-0.46808
Platelet basic protein	-1.32997	Plasminogen	-0.46232
Protein AMBP	-1.32051	Apolipoprotein A-IV	-0.42674

(-) Biopsy		**Control**	
Top 10%	Z-score	Top 10%	Z-score
Hepcidin	1.10146	Selenoprotein P	1.09008
Fibulin-1	0.957852	Protein AMBP	1.06105
Ig lambda-3 chain C regions	0.940796	Haptoglobin-related protein	1.03336
Complement component C7	0.856696	Ig lambda-1 chain C regions	0.935346
Apolipoprotein C-1	0.850408	Immunoglobulin lambda-like polypeptide 5	0.916202
Bottom 10%	Z-score	Bottom 10%	Z-score
Gelsolin	-0.487595	Proteoglycan 4	-0.892322
Apolipoprotein A-IV	-0.458882	Complement C3	-0.790098
Haptoglobin-related protein	-0.431284	Cystatin-C	-0.770817
Inter-alpha-trypsin inhibitor heavy chain H1	-0.403415	Beta-2-glycoprotein 1	-0.768302
Histidine-rich glycoprotein	-0.401478	Ig heavy chain V-I region EU	-0.735661

IVa. Metastasis – Top 10% (upregulated proteins)

Gelsolin: Gelsolin was the most significantly upregulated protein in the metastasis patient group, with an average Z-score of 2.05. Gelsolin exists in two forms, a cytosolic and secreted form, that are transcribed from the same gene [11]. Secreted gelsolin has not been extensively studied in the prostate cancer disease state, but researchers found that in disease states, gelsolin, a

normally harmless protein, can inhibit immunosurveillance and promote cancer progression.[12] This study found that gelsolin was highly expressed in prostate cancer cells and contributed to cancer progression through several different mechanisms. Researchers found that *in vitro*, secreted gelsolin inactivated $CD4^+$ T cells by binding to the surface glycoprotein CD37, induced apoptosis of activated $CD8^+$ T lymphocytes by binding to Fas ligand, and bound to sortilin (a membrane glycoprotein) to form a complex with another protein that enhanced endocytosis and intracellular transport of essential lipids needed to facilitate tumor growth.[12] These researchers also found that gelsolin is overexpressed in patients with prostate cancer and not in benign hypertrophic prostate epithelia (PSA is commonly elevated in both cases) and was negatively correlated with survival. Additionally, pathology studies demonstrated that overexpression of gelsolin in prostate cancer patients was correlated with significantly higher occurrence of metastasis.

Apolipoprotein A-IV and inter-alpha-trypsin inhibitor heavy chain H1: Apolipoprotein A-IV (APOA4) and inter-alpha-trypsin inhibitor heavy chain H1 both had average Z-scores of 1.95 in the metastasis group. These proteins have not been studied for their specific mechanism in the prostate cancer disease state. One study investigating potential biomarkers for different cancers found that both proteins may belong to a larger group of proteins that are characteristic of the cancer state in general. A serum degradome analysis of cancer patients and healthy controls identified 1373 unique peptides, 40% of the identified peptides originated from five of the most prominently degraded proteins; fibrinogen alpha chain (FGA), APOA4, complement C3 (C3), apolipoprotein A-I (APOA1) and alpha-1-antitrypsin (SERPINA1).[13] The results on colorectal cancer (CRC) and prostate cancer peptidome presented by these researchers suggest that the vast majority of discriminating peptides are not specific to a particular cancer type, rather

their abundance results from general state of health of cancer patients. Interestingly, the researchers did suggest one protein fragments out of the hundreds of investigated proteins that could both differentiate cancer patients from healthy controls as well as colorectal cancer from prostate cancer; this was peptide ENADSLQASLRPHADEL derived from APOA4, which was significantly upregulated in prostate cancer patients, and only slightly upregulated in CRC patients and not at all in controls, and therefore could potentially be regarded as marker specific to prostate cancer.[13]

Histidine-rich glycoprotein (HRG): Histidine-rich glycoprotein was upregulated in metastasis with an average Z-score of 1.88. HRG is an abundant plasma protein with a variety of functions. Of relevance are its roles in immunity, angiogenesis, and thrombosis, in which HRG has been found to have both pro- and anti-angiogenic effects, interactions with platelets and other coagulation factors, and modulation of antitumor immune responses, all processes relevant to tumor progression in the development of cancer. HRG localizes in the stroma of some tumors upon release from platelets and may block interaction of anti-angiogenic proteins such as thrombospondins.[14] HRG is thought to interact with thrombospondin and promote angiogenesis by blocking binding of thrombospondin-1 to the thrombospondin receptor CD36, which is expressed on a variety of cell types and upon binding of the thrombospondin-1 ligand counteracts angiogenic signals mediated through growth factor receptors.[15] In the setting of breast cancer, HRG was found to mask the anti-angiogenic epitope of and co-localize with thrombsopondin-1 in the tumor matrix.[14] By contrast, HRG has also been found to have some anti-angiogenic activity. *Hrg*$^{-/-}$ mice with fibrosarcoma had tumor vessels with increased size, increased production of vascular endothelia growth factor (VEGF) and placental growth factor (PlGF).[16] HRG inhibited both VEGF and PlGF-mediated angiogenesis *in-vitro*, as well as

inhibiting endothelial cell chemotaxis, through a mechanism involving the minimal active domain of the histidine/proline rich region of HRG.[17] Angiogenesis is essential for tumor growth and metastasis, and an "angiogenic-switch" is proposed to occur when pro-angiogenic factors out-weigh anti-angiogenic factors and promotes tumor progression, and whether or not HRG is pro- or anti-angiogenic may be highly contextual depending on the tumor environment.[18] In addition, HRG has been demonstrated to be a potent tumor suppressor by regulating platelet signaling activity.[19] In the absence of HRG, platelet activation becomes enhanced, promoting tumor metastasis as platelet activation and coagulation play an important role by a proposed mechanism of shielding circulating tumor cells from detection by the immune system.[20] Interestingly, a study measuring the levels of plasma HRG in ovarian cancer patients found the same trend as in the study with the prostate cancer patients examined here; HRG was initially downregulated in earlier disease states, but upregulated in advanced disease states. In the study presented here, HRG was downregulated in the (-) biopsy, (+) biopsy groups, and then upregulated in metastasis, and in the ovarian cancer study HRG was downregulated in stage I/II of ovarian cancer relative to healthy controls and upregulated in stage III ovarian cancer.[21] Altogether, these results make HRG an interesting and seemingly worthwhile biomarker to continue to research in the context of prostate cancer.

Coagulation factor XII: Coagulation factor XII was upregulated in the metastasis group with an average Z-score of 1.80, which is not surprising given the role of thrombosis in cancers. Increased risk of thrombosis is common among cancers, especially once the cancer has progressed to metastasis. As cells proliferate and tumors grow in size, pieces are prone to break off and enter the systemic circulation as emboli. As such, it is likely for peptides involved in thrombosis to be dysregulated in many cancers. However, one study has identified a mechanism

specifically in prostate cancer cells contributing to the upregulation of FXII. The researchers identify a novel and unexpected role of the polyP/FXII-driven intrinsic pathway of coagulation in prostate cancer-associated thrombosis. Coagulation analyses of patient plasma models in genetically altered mice show that prostate cancer cells and prostasomes expose long-chain polyP on their surface[22]. The polymer activates FXII, triggers clotting in prostate cancer patient plasma, and causes thrombosis in mice [22]. These data identify a new coagulation mechanism that contributes to prostate cancer-driven thrombosis, and the mechanism causing upregulation of this protein in metastatic disease.

IVb. Metastasis – Bottom 10% (downregulated proteins)

Prothrombin: Prothrombin was the most significantly downregulated protein in the metastasis group, with an average Z-score of -1.61. This is interesting because it has been found that thrombin generation is increased in men with advanced prostate cancer.[23] A study investigating the role of prothrombin in prostate cancer found that thrombin induced the expression and secretion of interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF), two cytokines which promote prostate cancer growth and angiogenesis, suggesting a mechanism by which thrombin may contribute to the progression of prostate cancer.[24] These data seem to suggest that prothrombin should be upregulated in prostate cancer disease states, and prothrombin is in fact slightly upregulated in the (+) biopsy group, prompting further investigation of why prothrombin is downregulated in the metastasis group.

Neutrophil defensin 1: In the study of prostate cancer patients presented here, neutrophil defensin 1 was downregulated in the metastasis group with an average Z-score of -1.51.

However, it has been proposed that human neutrophil proteins 1, 2, and 3 are expressed in several tumor types, and human neutrophil peptides (α-defensin-1, -2, and -3) were found to be present in significantly elevated concentrations in the plasma from colorectal cancer patients[25] and in patients with metastatic bladder cancer[26]. More research is needed to investigate the mechanism resulting in the downregulation of neutrophil defensin 1 specifically in the context of metastatic prostate cancer.

Fibulin-1: Fibulin was downregulated in the metastasis group with an average Z-score of -1.33. Fibulin is downregulated by the inflammatory cytokines CXCL1/GROα through epigenetic regulation. CXCL1/GROα is associated with increased AKT activation and IkB kinase α phosphorylation, resulting in activation of nuclear factor-kappa B (NF-kB), which interacts with histone deacetylase 1 (HDAC1) to form a gene-silencing complex that decreases acetylation of histone H3 and H4 on the NF-kB-binding site on the fibulin-1 promotor,[27] thus repressing the expression of fibulin-1. Kuo *et al.* provide the first evidence that CXCL1/GROα specifically decreases fibulin-1 expression in prostate cancer cells and suggest that inhibition of the CXCL1/GROα-mediated AKT/NF-kB signaling pathway may be a potential therapeutic target for prostate cancer. [27] Another study identified fibulin-1 as a human bone marrow stromal cell-derived factor that induces apoptosis of prostate cancer cells.[28] Fibulin-1 is a clear example of how the downregulation of a peptide can permit the continued survival and proliferation of malignant cancer cells.

Platelet basic protein and protein AMBP: Platelet basic protein and protein AMBP were found to be downregulated in the metastasis patient group, with average Z-score of -1.33 and -1.32, respectively. A clear role these proteins in the progression of prostate cancer, or indeed in any type of cancer, has not yet been identified, and further research is needed. At this time, these

biomarkers may primarily fulfill a diagnostic role in the protein fingerprint for metastatic prostate cancer as statistically significant biomarkers.

IVc. (+) Biopsy – Top 10% (upregulated proteins)

Coagulation factor XI: Coagulation factor XI (FXI) was the most upregulated protein in the (+) biopsy group, with an average Z-score of 0.93, which is logical given the previously discussed role of thrombosis in cancers. FXI is the substrate of FXII leading to fibrin formation. As seen previously, FXII was significantly upregulated in metastasis as a result of the exposure of the activator polymer long-chain polyP on the surface of the plasma membrane of prostate cancer cells. In addition, polyP accelerates other downstream mechanisms for pro coagulation including FXI feedback activation, leading to upregulation of FXI protein.[22] Interestingly, FXII was upregulated in metastasis but slightly downregulated in (+) biopsy, and FXI was essentially not dysregulated at all in metastasis but was significantly upregulated in (+) biopsy. This could possibly be the result of greater consumption of FXI substrate by FXII in metastasis, but further research is needed to investigate the relationship between these two coagulation factors in the context of prostate cancer.

Plasma serine protease inhibitor: Plasma serine protease inhibitor was found to be significantly upregulated in (+) biopsy with an average Z-score of 0.90, and the current literature shows unclear data about the role of this type of peptide in prostate cancer. These peptides are expressed by most epithelial cells. Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a type I transmembrane serine protease inhibitor that is the cognate inhibitor of the serine protease matriptase and was first identified in a human gastric cancer cell line.[29] Recent studies

have suggested the importance of the matriptase/HAI-1 balance in the progression of various cancers.[30] In one study examining this relationship, it was found that HAI-1 expression levels were significantly higher in all proliferative prostate diseases (including benign prostate hyperplasia as well as localized and metastasized cancers).[31] Additionally, studies investigating the suppression of HAI-1 using HAI-1 deficient prostate cancer cell lines found that these cancer cells displayed decreased invasiveness and slower growth compared to controls, suggesting that loss of HAI-1 can lead to more aggressive phenotypes.[32] Yet, other studies have found that in prostate cancer tissues and cell lines, expression of the serine protease matriptase is significantly increased and correlates with a corresponding decrease in the expression of the serine protease inhibitor HAI-1.[33] Suggested explanations for these inconsistencies between studies are small patient numbers, different patient populations and different assays used in the detection of the peptides. It is also possible that dysregulation of HAI-1 is a marker of abnormal prostate epithelial cell proliferation independent of cancer phenotype.

Filamin-A: Filamin A (FLNA) was found to be upregulated in the (+) biopsy group with an average Z-score of 0.87. FLNA is a well-characterized actin protein involved in cross-linking and cell scaffolding and has functions in cell motility and adhesion. Multiple studies have found FLNA to be overexpressed in prostate cancer,[34, 35] as is seen here. An investigation of the clinical significance of filamin A (FLNA) in prostate cancer found that FLNA may have a dual role in cancer progression by either promoting or suppressing tumor progression depending on the localization of the protein (either to the cytoplasm or within the nucleus) and its regulation of the human androgen receptor (hAR).[36] The majority of FLNA is localized to the cytoplasm as a full-length peptide, where it interacts with the hAR[36] and influences the hepatocyte growth factor (HG)/c-MET axis,[35] ultimately resulting in the promotion of tumor growth by inducing cell

invasion and migration. On the other hand, FLNA is highly susceptible to proteolysis, and the cleavage product is localized to the nucleus rather than the cytoplasm where it may act to suppress tumor growth and metastasis by interactions with transcription factors resulting in apoptosis and cell death.[34] In metastatic tissue, FLNA is primarily localized to the cytoplasm, while in localized prostate cancers, neoplasia, and normal tissues, FLNA is mostly nuclear. FLNA expression was found to be significantly decreased in prostate cancer tissues relative to normal tissues and that overexpression of FLNA in prostate cancer cell lines resulted in lower cell survival fraction, decreased migration and invasion, and lower expression of the matrix metallopeptidase 9 (MMP-9),[37] a peptide belonging to a class of enzymes involved in degradation of the cellular matrix in physiological processes such as cell migration (relevant to metastasis) and remodeling during angiogenesis (relevant to tumor progression). Further research is needed to determine how serum concentrations of FLNA correlate to the intracellular concentrations described here. Overall, these results suggest that FLNA correlates with prostate cancer disease stage and serves as a negative regulator of prostate cancer, making it a useful biomarker in the context of a protein fingerprint that could be indicative of disease state.

Protein HEG homolog 1 (HEG1): HEG1 1 was seen to upregulated in the (+) biopsy group with an average Z-score of 0.84. This peptide has not been studied for its role in the context of prostate cancer, but studies of malignant mesothelioma found that HEG1 is involved in cell proliferation of malignant cells through a mechanism involving microRNA-23b.[38] In the study of malignant mesothelioma cells, Fujii *et. al.* demonstrated that HEG1 contains epidermal growth factor-like domains that influence cancers through aberrant signaling during cell adhesion and protection from invasion of tumor cells, thus supporting the survival and proliferation of malignant cells. This study found that cell proliferation significantly decreased

following transfection with microRNA-23b inhibitor and/or HEG1 siRNA, and that suppression of microRNA-23b and/or HEG1 resulted in apoptosis through the induction of LC3-II, a protein involved in autophagy.[38] Together these results demonstrate that HEG1 and HEG1 mediated microRNA-23b signaling contribute to cell proliferation via evasion of apoptosis and autophagy in malignant cells. Further research is needed to identify if this is the same mechanism in prostate cancer cells.

Stromal cell-derived factor 1 (SDF1): SDF1 was found to be upregulated in the (+) biopsy group, with an average Z-score of 0.84. There is evidence that overexpression of the SDF1/CXCR4 (chemokine receptor type 4) axis is involved in the promotion of prostate tumor growth, localization of prostate cancer cells to bone, and facilitation of interactions between malignant cells and the bone environment, possibly helping to facilitate the transition from (+) biopsy to metastasis. This is especially significant given that in prostate cancer, metastatic disease is one of the leading causes of cancer-related death, and the primary location of metastasis in prostate cancer is bone.[39] Studies have demonstrated that SDF1 signaling can be activated by chemokine receptor type 4 (CXCR4) in prostate cancer cells via the loss of the tumor suppressor phosphatase and tensin homolog (PTEN) and resultant activation of the PI3K/Akt pathway which regulates SDF1/CXCR4 signaling.[40] SDF1 is produced by vascular endothelial cells and increases expression of the β_3 subunit of α_v β_3 receptors in prostate cancer cells, increasing the adhesiveness and invasiveness of these cells, and simultaneously induces expression and blockade of the cell adhesion molecule CD164 in prostate cancer cells,[39] decreasing their ability to adhere to endothelial cells in bone marrow and enhancing metastatic behavior. Additionally, silencing of CXCR4 was found to lead to a significant decrease in VEGF secretion and MMP-9 (which was previously seen to be downregulated by FLNA in the

suppression of tumor progression), resulting in delay of tumor growth, while higher levels of CXCR4 activity were associated with mitotic dormancy that facilitated colonization of the bone marrow by tumor cells in prostate cancer.[39] These data suggest that SDF1 is an important protein contributing to tumor progression in the development of prostate cancer.

IVd. (+) Biopsy – Bottom 10% (downregulated proteins)

Hepcidin: Hepcidin was the most significantly downregulated protein in the (+) biopsy patient group, with an average Z-score of -0.56. This is interesting because multiple studies have demonstrated the opposite, the overexpression of hepcidin in both malignant and metastatic prostate cancer.[41-43] One study found that hepcidin-silenced prostate cancer cells had significantly lower iron levels and reduced expression of ferroportin (an iron transporter), and that hepcidin-silenced cells treated with exogenous iron had significantly greater proliferative capacity and a higher migration rate.[41] The researchers proposed that hepcidin is involved in the onset of prostate cancer through a mechanism involving downregulation of ferroportin expression and increased intracellular iron levels to enhance cell proliferation, migration, and evasion of apoptosis by cancer cells. Another study found that prostate epithelial cells synthesize their own hepcidin (through a unique intersection of pathways involving BMP4/7, IL6, Wnt, and the agonist of both BMP and Wnt, SOSTDC1), and that hepcidin synthesis and secretion are significantly increased in prostate cancer cells and tissues and promote prostate cancer growth and progression by the same mechanism described above, by decreasing cell surface ferroportin and increasing intracellular iron retention.[43] It is unclear why in the protein fingerprint generated here hepcidin was found to be downregulated in every patient in both the (+) biopsy and

metastasis groups. Further analysis with a greater number of patients is needed to confirm these findings.

Cystatin C: Cystatin C was downregulated in the (+) biopsy patient group, with an average Z-score of -0.52. Cystatin C is a secreted cysteine protease inhibitor and that processes including neutrophil chemotaxis, tissue inflammation, resistance to infections, and bone resorption (as such, this protein has been proposed as a biomarker for increased osteoblastic activity in prostate cancer patients who have progressed to the metastasis disease state). Cystatin C has previously been found to be downregulated in prostate cancer, and one group of researchers proposed that cystatin C prevents tumor progression by exerting influence on the MAPK/Erk (specifically by inducing Erk2 inhibitor, thus inhibiting MAPK/Erk2 activity) and androgen receptor pathways in the invasion of prostate cancer cells.[44] Researchers found that cystatin C expression was inversely correlated with matrix metalloproteinase 2 (MMP-22), which along with MMP-9 discussed above in the context of upregulation of filamin A in (+) biopsy, is a protein that contributes to tumor progression and metastasis. In vitro experiments showed that silencing of cystatin C using siRNA resulted in increased invasiveness of prostate cancer cells, while overexpression of cystatin C significantly reduced the invasiveness of malignant cells.[44] Researchers also found that tissue expression of androgen receptor (AR) is inversely correlated with cystatin C expression, and that overexpression of AR is a mechanism that further enhances tumor progression by overcoming the inhibiting effects of transforming growth factor beta 1 (TGFbeta1), which induces growth inhibition and apoptosis.[45] Cystatin C expression was significantly lower in malignant than benign tissues, and decreased cystatin C levels correlated with lower patient survival, suggesting the relevance of using cystatin C as a biomarker to discriminate between cancerous and non-cancerous prostate disease states.

Plasminogen: Plasminogen was found to be downregulated in the (+) biopsy group, with an average Z-score of -0.46. One study measuring the expression of urokinase-type plasminogen activator system members (including urokinase-type plasminogen activator (uPA), uPA-receptor (uPAR), and plasminogen activator inhibitor-1 (PAI-1)) in prostate cancer patients demonstrated that these proteins have important roles in prostate cancer tumor progression through regulation of a uPA/uPAR/PAI-1 axis via both autocrine and paracrine mechanisms.[46] Urokinase-type activator (uPA) is a serine protease that activates plasminogen by catalyzing the conversion of plasminogen to plasmin, which degrades various matrix proteins. The binding of uPA to the uPAR also results in signaling cascades leading to angiogenesis,[47] chemotaxis,[48] cell adhesion,[49] and cell proliferation.[50] Researchers found that uPA was overexpressed in prostate cancer cells, both in primary prostate cancer and metastasis, and silencing of uPA in prostate cancer cells *in vivo* using siRNA resulted in decreased uPA, suppressed uPAR, and resulted in significant reduction of prostate tumor growth.[46] Given that plasminogen is the substrate of uPA, it is logical that overexpression of uPA resulted in the downregulation of plasminogen seen in the (+) biopsy group examined here. Interestingly, the researchers found that PAI-1 was also overexpressed in prostate cancer cells, which they found was due to soluble factors that stimulated PAI-1 gene transcription in bone cells (subsequently, uPA silencing by PAI-1 then led to reduction of PAI-1 expression).[46] This suggests that plasminogen could briefly become less significantly downregulated, depending on the amount of circulating PAI-1. Altogether, these data suggest that prostate cancer-cell mediated dysregulation of proteins in the enzymatic cascade in which plasminogen is a component are the source of dysregulation of plasminogen.

Complement component C7: Complement component C7 was found to be downregulated in the (+) biopsy group, with an average Z-score of -0.46. Complement C7 has been identified by

multiple studies as a potential tumor suppressor.[51, 52] Researchers found that C7 expression was significantly lower in prostate cancer cells than in normal tissues, that lower expression was correlated with higher clinical T stage (referring to the size and extent of the main tumor), and that C7 was significantly correlated to nine cell signaling pathways (including cytokine/cytokine receptor interaction, focal adhesion, chemokine signaling, calcium signaling, JAK stat signaling, cell adhesion, axon guidance, leukocyte transendothelial migration, and vascular smooth muscle contraction pathways).[51] The researchers did not identify the mechanism causing downregulation of C7, but did point out genetic alteration was found in 26 of 136 (19%) prostate cancer patients, with the most common mutation being a missense mutation.[51] Further research is needed to identify the mechanism resulting in the downregulation of complement C7 in prostate cancer.

Apolipoprotein A-IV (APOA4): APOA4 was found to be downregulated in the (+) biopsy group, with an average Z-score of -0.43. Important to note, this was the only biomarker in common between the metastasis and (+) biopsy groups top/bottom 10% and was oppositely dysregulated between these two groups (average Z-score for APOA4 in metastasis was 1.95). APOA4 is a lipid-binding protein synthesized in the small intestine which is packaged into chylomicrons and is present on chylomicron remnants and high-density lipoproteins as well as in lipid-free form in systemic circulation.[53] APOA4 has many identified physiological roles; one that could potentially be relevant to prostate cancer is its role in platelet aggregation and thrombosis. ApoA-IV negatively regulates αIIbβ3-mediated platelet aggregation and thrombosis.[54] However, study of this pathway specifically in prostate cancer patients is needed to identify if APOA4 has any sort of relevance in this context. Given the diverse functions and locations of APOA4, it could simply be dysregulated in prostate cancer due to the dysregulation of various other biological pathways that interact with or influence this peptide.

IV. DISCUSSION

The questions central to this study were *can* different families of protein biomarkers be used to diagnose specific disease states, specifically metastatic versus malignant prostate cancer, and *why* are various proteins found to be dysregulated in these disease states?

The answers are complex; immediately of interest is that there are no proteins in common between the (+) biopsy and metastasis groups top and bottom 10% except apolipoprotein A-IV, which is upregulated in metastasis and downregulated in (+) biopsy. This suggests significant changes or differences in the metabolic processes occurring in the body during different disease states as the prostate cancer progresses and supports the development of unique protein fingerprints to accurately predict disease state and discriminate between metastasis and (+) biopsy. On the other hand, several biomarkers were commonly downregulated across the (+) biopsy, (-) biopsy, and control groups. Cystatin C was downregulated in both the (+) biopsy and control groups, and plasminogen and apolipoprotein A-IV were both downregulated in (+) biopsy and (-) biopsy groups. While this isn't relevant to the conversation about discriminating between metastatic and (+) biopsy disease states, this predicts potential difficulty in using these specific biomarkers to discriminate between the disease and non-disease states of (+) and (-) biopsy, such as is commonly needed to distinguish a malignant tumor from benign prostatic hyperplasia and suggests the need for further analysis to determine if there is a significant threshold level to identify disease state despite these being similarly dysregulated between disease and non-disease state groups.

Which of the investigated proteins would make good biomarkers for prostate cancer? Primarily, proteins that are statistically significantly dysregulated, proteins that have a role in a

mechanism relevant to prostate cancer (specificity), and that are present at a detectable level in plasma are great candidates. One thing to consider about the protein biomarkers investigated here is that these peptides specifically resulted from the unique method used to extract them (thiol-functionalized MSNs were used for their demonstrated ability to capture low-molecular weight proteins). Other methods investigating serum protein composition in prostate cancer patients might yield slightly different results, depending on the methods used to identify the proteins present. This leads us to the question, should we rely only on proteins that have a clearly defined role in prostate cancer, or can simply diagnostic markers (i.e. markers that do not have a clearly defined role in prostate cancer or are common to other cancers as well) be just as accurate? Further studies repeating the methods used here are needed to further validate the data by increasing the number of patients, and thus serum samples, and increasing the confidence in the results obtained in this analysis. Additionally, investigation of the serum levels of the 51 peptide biomarkers identified in the heat map for prostate cancer in patient groups with other cancer types would help to identify biomarkers that are potentially specific to prostate cancer.

How many biomarkers are needed to achieve high-accuracy and consistently reliable diagnosis? Although more data is needed to determine the ideal number of biomarkers to form the fingerprint that would be used to screen patients, the past unreliability of PSA demonstrates the need to rely on more than one biomarker to diagnose prostate cancer. A fingerprint relying on potentially dozens of biomarkers would provide a novel approach that could help to reduce the prevalence of inaccurate diagnoses (both false positives and incorrect negatives) by allowing patients to vary in serum levels of one or more biomarkers, a natural phenomenon in humans that all differ with respect to metabolism and possible comorbidities, while overall still demonstrating good correlation to the determined fingerprints. In the study analyzed here, over 400 proteins

were initially screened, of those 51 were determined to be significantly dysregulated,[9] and approximately 20 were analyzed here, suggesting that a relatively small subset might be indicative of disease and sufficient to create an accurate fingerprint.

Also,worth noting is that the Z-score values, while fairly significant for the most dysregulated proteins in the metastasis group (ranging from around 1.5 to 2 standard deviations away from the mean), were less significant for the most dysregulated proteins in the (+) biopsy group (ranging approximately 0.5 to <1 standard deviation from the mean). These statistics suggest that the protein fingerprint for metastasis would have more diagnostic power as a test for the metastatic disease state than the protein fingerprint for (+) biopsy. Yet, given the need to catch cancer early to improve patient outcomes, the goal would be to diagnose prostate cancer before it achieves metastasis. Therefore, relying on correlation to a greater number of proteins seems appropriate, especially in the (+) group where patients are apparently less likely to have significant dysregulation in the proteomic composition.

The major limitation to this study was the utilization of a small patient group. There was a total of 22 patients used in this study. While average values were used for Z-score across patient groups for each disease state, high outliers (Z-scores of >4 or <-4) had the potential to inflate Z-score values than if a larger patient group was used. The small number of patients might also be able to explain some of the discrepancies seen where the direction of dysregulation didn't correspond with the current scientific literature. In addition, this study compared prostate cancer patients to healthy controls and did not compare to other cancer patient groups. This makes it difficult to identify biomarkers unique to prostate cancer based off statistical data alone.

V. CONCLUSIONS & FUTURE DIRECTIONS

In conclusion, current literature clearly shows the need for a more reliable diagnostic test for prostate cancer than the current PSA test to increase diagnosis accuracy and improve prognosis through earlier identification of and higher specificity for prostate cancer. A protein "fingerprint" identifies a characteristic set of proteins indicative of disease. In this analysis, different proteins were seen to have distinctly different levels of significance in the plasma of patients at different stages of prostate cancer. This is promising evidence for the potential use of protein fingerprints specific to each disease state and the ability to diagnose a patient's stage of cancer (metastasis, positive biopsy) and differentiate from certain non-disease states such as benign prostatic hyperplasia (negative biopsy). Additionally, peptide biomarkers were investigated for the specific mechanisms resulting in their dysregulation in the context of prostate cancer, elucidating some of the physiological processes that are occurring in each disease state and contributing to a greater understanding of the way in which prostate cancer progresses.

Future research is needed with a larger patient group to reproduce and expand on the data presented here. A patient group of 55 participants has been identified at the University of Vermont Medical Center as highly genetically pre-disposed for developing prostate cancer and would serve as a good resource for this research. Groups such as this would provide an excellent opportunity for a longitudinal study, such as an observation cohort study, to identify trending biomarkers over time and distinguish "delta values," or how quickly each protein biomarker is changing in its level of significance of dysregulation over time. For example, repeatedly analyzing the biomarkers of interest in a group of (+) biopsy patients over a significant period

would clearly indicate how quickly these proteins are becoming dysregulated. Future analysis using the same methods as described in this analysis to compare protein fingerprints in non-cancerous disease states, such as negative biopsy and benign prostatic hyperplasia, is needed to help identify the biological mechanisms that differ for non-cancerous disease states as well. The development and comparing of protein fingerprints of different cancer types would also be useful to help distinguish biomarkers specific to prostate cancer versus other cancers, and which biomarkers are common to multiple cancer types. The development of "control fingerprints," in which the average Z-score of each protein is set to zero in the control patient group, would provide a different way to look at the dysregulation of the proteins in the fingerprint (in this study, the Z-scores were calculated relative to an average taken across all patients in the study. Therefore, proteins were also necessarily apparently up- and down-regulated in controls as well). Another interesting angle to investigate would be to include the PSA levels of these patients in this study and to compare the rate of dysregulation of the protein biomarkers to the amount of change in PSA levels. Additionally, further studies comparing the power of various families of biomarkers (such as peptides compared to miRNA) to accurately predict disease are needed to identify the most worthwhile biomarker candidates to pursue in the context of prostate cancer.

Ultimately, the analysis of the proteomic profile and composition in prostate cancer patients presented here demonstrates the relevance of certain proposed biomarkers to the physiological mechanisms in the cancerous disease states, demonstrates the specificity of protein fingerprints for different disease state (metastasis, (+) biopsy, and (-) biopsy), and reinforces the validity of pursuing families of peptide biomarkers found in human serum to generate a protein fingerprint characteristic of prostate cancer, with the goal of future application as a less-invasive diagnostic method providing the earlier detection and more accurate diagnosis of prostate cancer

and thus improving both patient prognosis and psychological well-being. The greater understanding of the biological mechanisms resulting in the dysregulation of the proteomic profile presented here also presents potential opportunities for the identification of novel therapeutic targets in prostate cancer.

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VII. SUPPLEMENTAL DATA

Appendix A. Average Z-score values for each protein within the specified patient group (metastasis, (+) biopsy, (-) biopsy, and control). The top and bottom 10% of proteins (i.e., the most statistically significantly up- and down-regulated proteins) are highlighted within each patient group. The top 10% are highlighted green while the bottom 10% are highlighted red.

PT GROUP AVG + GROUP SPECIFIC +/-10%

METASTASIS	(+) BIOPSY	(-) BIOPSY	CONTROL	PROTEINS
0.396982523	-0.462319313	-0.340403189	0.713761622	Plasminogen
0.805209491	-0.215840045	-0.017057411	-0.361279165	Hepatocyte growth factor activator
1.952159967	-0.404240234	-0.403414587	-0.511859271	Inter-alpha-trypsin inhibitor heavy chain H1
1.953023229	-0.426738842	-0.45888192	-0.407897285	Apolipoprotein A-IV
2.045496541	-0.389038758	-0.487595384	-0.486917186	Gelsolin
1.881577337	-0.385438293	-0.401478266	-0.480666346	Histidine-rich glycoprotein
1.798898278	-0.271553522	-0.344482967	-0.630978242	Coagulation factor XII
1.631240511	-0.518913067	0.063228514	-0.770816648	Cystatin-C
-0.993802527	-0.28964158	0.037520775	1.090082832	Selenoprotein P
-1.320506835	-0.12143411	0.1007687	1.061050219	Protein AMBP
-0.275101847	-0.275101847	-0.275101847	0.935346281	Ig lambda-1 chain C regions
-0.474018854	-0.041951864	-0.431284159	1.033355141	Haptoglobin-related protein
-0.304014084	-0.253045213	-0.263811424	0.916201515	Immunoglobulin lambda-like polypeptide 5
-0.406654571	0.72500378	-0.303333877	-0.12001345	Ig kappa chain V-III region GOL
-0.667813028	0.64416359	0.071916017	-0.33942831	Cartilage oligomeric matrix protein
-0.619785052	0.68558344	0.090784187	-0.453969949	Serotransferrin
0.185069859	0.256863521	0.222864509	-0.768302425	Beta-2-glycoprotein 1
0.210349176	-0.05335261	0.562904418	-0.892322394	Proteoglycan 4
-0.698657238	-0.115844262	0.847143297	-0.488061711	Insulin-like growth factor-binding protein 5
-0.379228924	0.757345068	-0.190143436	-0.339230133	Complement C5
-0.325903472	0.869075924	-0.325903472	-0.325903472	Filamin-A
-0.314602472	0.838939925	-0.314602472	-0.314602472	Stromal cell-derived factor 1
-0.315415646	0.84110839	-0.315415646	-0.315415646	Protein HEG homolog 1
-0.464665391	0.746459088	-0.002745954	-0.520174257	Coagulation factor V
-0.535207477	0.738509972	0.055115352	-0.535207477	Platelet factor 4 variant
-0.084878553	0.931144651	-0.266977476	-0.675702272	Coagulation factor XI
-0.307743559	0.820649491	-0.307743559	-0.307743559	Multimerin-1
-0.248416615	0.662444308	-0.248416615	-0.248416615	Protein Wnt-3a (Fragment)
-0.73566082	0.544644784	0.479011239	-0.73566082	Ig heavy chain V-I region EU
-0.786466044	0.807387435	0.138186235	-0.533152816	Complement factor B
-1.259394798	0.721089013	0.164455222	-0.088028289	Ribonuclease 4
-0.79793323	0.895841793	-0.334221677	0.031246781	Plasma serine protease inhibitor
-0.421916409	0.819259856	-0.248534197	-0.297630824	Alpha-2-antiplasmin
-1.5096161	0.729673406	0.284302118	-0.065938172	Neutrophil defensin 1
-1.605839416	0.509309657	0.259982588	0.309524321	Prothrombin
-0.29292294	-0.29292294	0.627692013	-0.29292294	Ig lambda chain V-III region LOI

-0.797441669	0.042636624	0.723320292	-0.425859022	Liver-expressed antimicrobial peptide 2
-1.179275601	0.510851872	0.800354232	-0.79009769	Complement C3
-0.721912407	-0.277825075	0.940796233	-0.40619471	Ig lambda-3 chain C regions
-0.314982789	-0.314982789	0.674963119	-0.314982789	Ig lambda chain V-I region WAH
-0.308596121	-0.286179957	0.642063546	-0.308596121	Cartilage acidic protein 1
-0.25593189	-0.25593189	0.548425478	-0.25593189	Ig kappa chain V-I region WAT
-0.27859434	-0.27859434	0.596987872	-0.27859434	Serum amyloid P-component
-0.307944315	-0.307944315	0.659880674	-0.307944315	Cathelicidin antimicrobial peptide
-0.316130282	-0.316130282	0.677422032	-0.316130282	Out at first protein homolog
-0.801503239	-0.234678017	0.158630107	0.700734061	Apolipoprotein C-II
-0.71125986	-0.307089601	0.850408431	-0.253056394	Apolipoprotein C-I
-1.27097777	-0.468084189	0.856696359	0.379108341	Complement component C7
-1.331293523	-0.258530256	0.957851926	0.034278429	Fibulin-1
-0.934966529	-0.556230091	1.101458398	-0.126592426	Hepcidin
-1.329966954	-0.020840033	0.785827599	-0.011177036	Platelet basic protein