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Identification and Characterization of Serum Biomarkers Associated with Breast Cancer Progression

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Doctor of Philosophy

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ABSTRACT

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Despite the recognized advances in the treatment of breast cancer, it still accounts for 15% of all cancer-related deaths. 90% of breast cancer deaths are due to unpredicted metastasis. There is neither successful treatment for metastatic patients nor a specific test to predict or detect secondary lesions. Patients with primary tumor will be either over-treated with cytotoxic side effects or under-treated and risk recurrence. This necessitates the need for personalized treatment, which is hard to offer for such heterogeneous disease. Obstacles in treating breast cancer metastasis are mainly due to the gaps exist in the understanding of the molecular mechanism of metastasis. The linear model of metastasis is supported by several observations that reflect an early crosstalk between the primary and secondary tumor, which in turn makes the secondary microenvironment fertile for the growth of disseminated cells. This communication occurs through circulation and utilizes molecules which have not been identified to date. Identifying such molecules may help in detecting initial stages of tumor colonization and predict the target organ of metastasis.

Furthermore, these molecules may help to provide a personalized therapy that aims to tailor treatment according to the biology of the individual tumor. Advances in proteomics allows for more reproducible and sensitive biomarker discovery. Proteomic biomarkers are often more translatable to the clinic compared to biomarkers identified using other omics approaches. Further, protein biomarkers can be found in biological fluids making them a non-invasive way to treat or investigate cancer patients. We present in this manuscript our study of the use of a proteomic approach on blood serum samples of metastatic and non-metastatic patients using LC-MS/MS quantitative analysis machine to identify molecules that could be associated with different stages of breast cancer metastasis. We focused on the deferential expression of low molecular weight biomolecules known to reflect disease-specific signatures. We manually analyzed 2500 individual small biomolecules in each serum sample of total of 51 samples. Comparisons between different sample types (from stage I and III Breast Cancer patients in this case) allows for the detection of unique short peptide biomarkers present in one sample type. We built a multi-biomarker model with more sensitivity and specificity to identify the stage of the tumor and applied them on blinded set of samples to validate prediction power. We hope that our study will provide insights for future work on the collection, analysis, and understanding of role of molecules in metastatic breast cancer.

Keywords: breast cancer, metastasis, low-molecular weight, serum peptidome, biomarkers, multimarker model, cLC-MS/MS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction and Background

Cancer Metastasis

Cancer is considered the second leading cause of death in the united state after heart diseases. It is expected to overtake the heart disease death rate imminently. Approximately, 90% of human cancer deaths are due to metastasis [1]. The survival rate of almost all cancer types drops dramatically once the tumor disseminates and establish a secondary growth at distant organs. Therefore, metastasis is considered the most determinant prognostic factor in cancer [1, 2]. The mystery surrounding metastasis makes it hard to predict, diagnose and treat. Despite all the advancement in early cancer detection and treatment, metastasis remains a complicated puzzle with no foreseeable solution. The current clinical practice to diagnose and treat metastasis are mainly to prolong patients life as much as possible regardless of the quality of patients life [3].

Breast Cancer Metastasis

Maymuna is a 35-year old assistant professor of clinical anatomy at the College of Medicine at Sultan Qaboos University in Oman. She has a happy, social and optimistic personality. She is a mother of five kids; the youngest is 6 months old. Her life dramatically changed after she has been diagnosed with Breast Cancer. Luckily she was at stage II and the tumor was positive to estrogen receptor. She was treated according to the guideline, where she underwent surgery and received hormonal therapy with adjuvant chemotherapy. She got back to her normal life after a long period of hospital admission, pain and chemotherapy-induced side effects. The doctors assured her that she has a very low risk of relapse or metastasis because of her age, multi-parity, negative family history of tumor and her hormone sensitive tumor. Unfortunately, a year later, she started to have shortness of breath and high fever. She has been admitted in intensive care unit because of her rapidly deteriorating health. Within 10 days of these symptoms she passed away due to

lung and pleural metastasis leaving a husband and 5 kids with tearful memories. This is a true story of my friend, who, unfortunately, was not the first nor the last to suffer this fate. According to the American Cancer Society, one woman in the U.S. will lose her life to Breast Cancer every 13 minutes [4].

Breast Cancer (BC) is the second leading cause of all cancer deaths among women worldwide. In the United States, about 40,000 women succumb to Breast Cancer annually. 90% of deaths from Breast Cancer occur in patients with advanced, metastatic disease. Five year survival rates of Breast Cancer patients drop from 99% for women treated in early stage cancer to 25% for women treated in later stages [5, 6]. Treatment costs increase as the disease progresses and furthermore the intent of treatment becomes more of controlling the disease as long as possible rather than cure [7].

Diagnosis of Breast Cancer Metastasis

The mystery of Breast Cancer metastasis is that it is unpredictable, can remain dormant for several decades, and is far less responsive to available cancer therapy [8]. Unfortunately, there is no accurate method to measure or monitor metastatic disease at very early stages of the metastatic spread. Monitoring of metastatic disease is performed by time consuming, costly, invasive and complicated radiological studies that suffer from low sensitivities and specificities [5, 8]. Once suspected, diagnosis of Breast Cancer for local or distant recurrence is accompanied by invasive, and painful biopsies of the affected organs [9]. As a result, assessment of whether Breast Cancer patients are at risk for recurrence due to early metastatic spread or whether treatments have been successful in eliminating residual metastatic disease remain inconclusive and inaccurate. The ability to detect metastatic disease is a critical driver in whether continued gains in Breast Cancer treatment can be achieved. Therefore, the identification of each individual tumor's metastatic potential is required to personalize treatment protocol for each patient [9, 10, 11, 12, 13, 14].

Treatment of Breast Cancer Metastasis

Transcriptomic studies divided primary Breast Cancer into five subtypes; Table 1.1 [15]. The most common and least invasive Breast Cancer subtype is the luminal type. This tumor type expresses estrogen receptors (ER) and usually responds to tamoxefin (an estrogen receptor inhibitor) and/or aromatase inhibitors with a good prognosis. The second subtype over-expresses human epidermal receptor (HER-2) and behaves more aggressively, but responds well to targeted therapy with trastuzumab, a monoclonal antibody against HER-2. The third and a more aggressive subtype is the basal subtype, which does not express any key hormonal receptors. Thus, Breast Cancers of this subtype are referred to as triple negative tumors for their lack of estrogen, progesterone and HER-2 receptors [16]. This tumor subtype does not respond to any targeted therapy and therefore needs a broad spectrum chemotherapy, which has unpleasant short and long term side effects that impair the patient's quality of life. Despite reliance on broad spectrum chemotherapy, triple negative breast cancer (TNBC) also has the worst prognosis among all Breast Cancer subtypes [17, 18].

It is very straightforward to identify subtypes by determining the hormone status of the patient's tumor and thus predict the patient's prognosis and response to treatment. However, in the real world, scenarios are completely different. Breast Cancer patients with identical tumor histopathology will respond differently to the same treatment. This can be explained by the molecular heterogeneity between the tumors that challenges development of personalized therapy for Breast Cancer [19, 20, 21].

Despite the use of tamoxefin and trastuzumab which are considered as targeted treatment, American society of Clinical Oncology (ASCO) latest recommendation is to use adjuvant chemotherapy as a precaution to eradicate escaped tumor cells, even if undetectable, and thus reduce risk of recurrence. This is because tumor aggressiveness cannot be easily assessed and there is currently no test that can evaluate patient's risk of local or distant recurrence. It has been estimated that almost 80% of primary BC patients

are receiving adjuvant therapy although 50% of those patient will never get progressed to an advance stage and they will benefit from local treatment alone [3]. Metastatic lesions, when identified, are usually not treated by surgical resection, but rather by a more systemic therapy. This is because the presence of a metastatic lesions often indicate a more systemic disease that needs to be attacked [21].

Prognostic Indicators of Metastatic Breast Cancer

Every Breast Cancer patient will be evaluated comprehensively to determine her metastatic risk and whether she requires for only local or more aggressive systemic therapy. The prognostic factors that are currently used are mainly clinicopathological parameters, such as patient's age at diagnosis, lymph node status, grade of malignancy, and hormone receptor status by focusing on Estrogen receptor (ER), Progesterone receptor (PgR), and human epidermal growth factor receptor (HER-2). The grade of malignancy predicts metastasis with circular logic as it is generally determined by the extent of invasion and metastasis observed [22]. The only molecular prognostic biomarker used is hormonal receptor status. These three proteins biomarkers have been widely utilized in the clinic, both for prognosis and for determining treatment options. Although these biomarker provide partial insight on the risk of metastasis, they are generally considered to be insufficient and might result in mistreatment. This is because about 15% of patient within the lower risk group (i.e. ER and PgR +ve) will recur and die of metastasis and paradoxically about 15% of patient in the high risk group (i.e. Triple negative tumor) will have a favorable outcome. No studies have demonstrated clinical utility of other molecular biomarkers beyond the expression of these three receptors [18]. The complexity and massive molecular heterogeneity of primary and secondary Breast Cancers are major reasons for failure to find other potential biomarkers [20, 23]. There is a hope that recent advancement in "omics" studies would decipher the complexity of Breast Cancer and reveal genotypic and phenotypic signatures underlying tumor heterogeneity. The promise of this approach is

Table 1.1: Intrinsic Subtypes of Breast Cancer.
Surrogate definitions of intrinsic subtypes of Breast Cancer according to the 2015 St. Gallen Consensus Conference and the ESMO Clinical Practice Guidelines. Reprinted from [24].

Intrinsic Subtype	**Clinicopathologic Surrogate Definition**
Luminal A	ER-positive HER2-negative Ki67 low PgR hig Low risk molecular signature (if available)
Luminal B	ER-positive HER2-negativ either Ki67 high or PgR low low high-risk molecular signature (if available)
HER2 over-expression	HER2-positive ER and PgR absent
Basal-like	Triple-negative (ductal) ER and PgR absent HER2-negative

that the molecular profile of each tumor can be determined and then categorized into a larger array of subtypes, each with its own molecular biomarker, which when detected will identify the most successful treatment regimen.

History of Metastasis

The term metastasis is originally from the Greek and means "change of place" [25]. Cancer metastasis, as a medical term, was first recorded by a famous French physician, Recamier, in 1829 more than 150 years ago [26]. Metastasis refers to the establishment of a secondary growth in a body organ other than the site of the primary tumor and that is not directly connected to it [21]. Despite the clinical significance of metastasis, its genetic and biomolecular basis is not yet well understood [1]. In fact, tremendous efforts have been undertaken in order to understand the cell biology of metastasis. One of the earliest theories is "seed and soil" theory proposed by the British physician, Paget, in 1889. He stated, "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil". His theory simply equalizes the contribution of primary tumor cells (he referred to them as seeds) and the potential secondary sites microenvironment (he referred to them as soil) in the establishment of the metastatic growth [27] . This theory was dormant till 1980 when Hart and Fidler found that intravenously injected radio-labeled melanoma cells metastasized to orthotopically grafted lungs and ovaries but not to the grafted kidneys in a synergetic mice [28]. Their findings demonstrate that tumor cells only colonize specific organs that have "fertile soil" for the corresponding mature "seeds". Subsequent to their work, a plethora of theories and models have been proposed to explain the process of metastasis [1, 3, 14, 29, 30, 31, 32, 33, 34, 35, 36]

Molecular Mechanism Underlying Metastasis

Interestingly, almost all proposed models agree on the complexity of metastasis and that it is composed of sequential steps, where each step is considered crucial to establish metastatic growth. Arrest at any step will prevent completion of the process. In the current view, the metastatic process starts at the primary tumor site, where a genetic alteration leads to initiation of the steps of metasatasis. Sub-populations of tumor cells gain an invasive genotype that enables them to escape the confines of the primary tumor and start the journey toward secondary colonization. These cells will detach from neighboring cells and invade the local stroma by secreting enzymes such as collagenase and other proteases. Then they will penetrate the nearby lymphatic or blood vessels and intravasate. These disseminated cells must overcome the turbulence of circulation and evade immune cells in order to survive and continue the journey to the ultimate site of metastasis. Cells that survive circulation will stop at the capillary bed of the host organ and then extravasate. Once they are in the secondary site, they start to proliferate and establish micro-metastasis [36, 37, 30, 38, 39, 31, 40]. Although primary tumors shed million of cells per day for every gram of primary tumor into circulation, only a very small fraction (0.01%) of shed cells successfully establish secondary growths. Metastasis is thus a highly inefficient process [1, 36, 31].

Despite great advancements in molecular biology and the understanding of human genome, metastasis remains a mysterious process [41, 31]. To tackle metastasis, we need to define target systems that are critical to steps in this process. According to postulated model, two critical components (targets) of metastasis are unique: 1) the primary tumor-derived cells that have full metastatic potential (seeds) and 2) the microenvironment of the host organ (soil). The seeds, either circulating tumor cells (CTC) or disseminated tumor cells (DTC), have been thoroughly isolated and examined using bioinformatics approaches. However, there are no conclusive findings yet because of their unstable genetic profile [42, 43, 44]. Recently, the host tissue microenvironment (the soil) has become a fo-

cus of attention and studies reveal that it is a more stable therapeutic target, though it is much more poorly understood [40, 36, 45].

Is Metastasis a Late or Early Event?

Metastasis was originally believed to be a linear process. In this model, primary tumor cells undergo rounds of mutations that create genetically heterogeneous clones of cells where some clones acquire ability to metastasize. Since a series of mutations is required, cells randomly accrue the correct series of mutations only with time and as the primary tumor reaches a considerable size. Scientists adopted this linear model when they noticed a correlation between the size of the primary tumor and frequency of metastasis. Further supporting the linear model, resecting small primary tumors was found to limit chances of metastasis [46]. More recently, a newly proposed parallel model has gained favor over the linear model in explaining cellular events of cancer metastasis. It is based on studies that concluded that metastatic lesions are too big to have been initiated late in cancer progression. The parallel model states that cells start to disseminate from the primary tumor and settle in multiple secondary organs very early in the disease progression. Only at a later stage subsequent genetic mutations occur. Each model has strong supportive evidence, but recent findings favor the parallel model [47, 35, 33, 36].

The Parallel (Stromal) Model of Metastasis

In 2012, Sleeman and his colleges built a more comprehensive model of metastasis [47, 33]. This "stromal model" combines aspects of both the parallel and the linear model. The stromal model says that there is very early crosstalk between the primary tumor and the host organ through secreted biomolecules, which they called pre-metastatic microenvironment conditioning factors. Researchers have alluded to the hypothetical presence of factors derived from the primary tumor for many years. Initially such tumor-derived factors were envisioned when researchers believed that cancer spread through a poisonous or

an infected juice that drained from the primary tumor [48]. Since we learned body circulation, the idea was abandoned with the conclusion that tumor-derived factors were instead the common elements of body circulation (blood and lymph). Scientists turned their focus to the seed and soil as the main elements driving cancer metastasis and the idea of circulating tumor factors promoting metastasis was forgotten. Sleeman pointed out that such factors might enable communication between primary and secondary sites, thus accounting for issues with the parallel and linear models and helping to uncover puzzle of metastasis at the molecular level [47, 33]. Sleemans model allows for bidirectional communication using tumor-derived factors in circulation, suggesting that there are signals from the secondary site that are delivered back to the primary tumor via circulation, and hence stimulate or facilitate a re-seeding process [47, 33].

With communication occurring through circulation, blood is considered as a reservoir of many biological molecules that are potentially involved in cancer growth and progression [49, 50, 51]. Analyzing cancer patients blood components (serum/ plasma) may allow identifications of molecules that can be a signature of a particular cancer type or cancer stage. From the standpoint of prognosis, such factors could be used to screen for patient metastasis in a clinical setting or even be targeted to arrest microenvironment fertilization.

Premetastatic Niches

Tremendous research efforts focused on the seeds (metastatic cells in transit) and their genotypic and phenotypic signatures. The secondary site microenvironment's role (the soil) in disease progression has been given less attention until recently [40, 52, 3, 53, 38, 33]. The prepared soil at a secondary site was referred to as a pre/pro metastatic niche by Kaplan and colleagues in 2005 [54, 55]. Subsequent studies found that microenvironments at metastatic sites undergo significant molecular changes in response to factors secreted by the primary tumor and that the condition of the premetastatic niche will either

support or suppress the growth of the DTCs [56, 57, 58, 59]. As a result, escaped DTCs will either proliferate to establish a secondary growth, stay dormant as a micrometastasis, or die without producing metastatic disease [52, 59, 47]. The main contributors to the formation of premetastatic niches are tumor-derived secreted factors (TDSFs) and bone marrow derived cells (BMDCs) [60]. TDSFs recruit BMDCs from bone marrow, which subsequently start conditioning the secondary organ to build the fertile niche [60].

Microenvironmental Responses to TDSFs

The distant site microenvironment responds to TDSFs by increasing or decreasing the expression of certain extracellular matrix proteins and recruits accessory cell types that will help to condition the environment [30, 36, 55]. For example, matrix metalloproteinase 9 (MMP-9) is upregulated in premetastatic lungs in mice [61]. The increase in MMP-9 expression is stimulated by activation of vascular endothelial growth factor receptor 1 (VEGFR1). Furthermore, BDMCs recruited into the lung niche were found to be VEGFR1 positive. Surprisingly, challenging mice with tumor conditioned media (containing TDSFs) prior to tail vein injection of metastatic tumor cells resulted in formation of the premetastatic niche in the lung. These findings support the significance of TDSFs in conditioning the distant microenvironment and initiation of subsequent metastasis. Since these factors are circulating in the body, they can be identified in the blood or the lymph [62, 61, 63, 36].

Liquid Versus Tissue Biopsy

In 2012, Gerlinger and his team published a very shocking finding after they sequenced DNA from kidney tumors [64]. They found that intra-tumor heterogeneity is significantly more extensive than previously believed. Within one single tumor, cells expressed a variety of mutations and only one third of the studied mutations were shared across the entire mass. If this much of heterogeneity accumulates in one single mass of pri-

mary tumor, then secondary site tumors are expected to have a completely different and highly varied mutational genotype [64]. The use of a single treatment modality that targets primary and/or secondary cells with a particular genotype would therefore miss other cells with accumulations of different mutational signatures [64, 21, 49, 65]. For example, Gefitinib is an epidermal growth factor receptor (EGFR) inhibitor that is used in different chemotherapy combinations [66]. Although patients initially respond very well to Gefitinib, resistance eventually develops and the tumor evolves to be more aggressive. This response results from new mutations occurring inside the tumor where cells continue to accumulate mutational diversity that results in new phenotypes allowing them to survive treatment [64].

Considering cancer as a static mutational environment is an obstacle to cancer cure. Patients instead need to be frequently monitored for dynamic changes that emerge as a response to treatment or other factors. Monitoring requires tissue samples, which is a very invasive process, and since samples only represent a small portion of the tumor mass, it can miss some mutations due to heterogeneity and its localization within the tumor. Furthermore, the patient may have multiple site secondary growths that are inaccessible for biopsy exacerbating this problem. Finding biomarkers in the blood that reflect the status of the tumor as it progress is a promising step towards overcoming some of the obstacles in cancer therapy. Considering the uniformity of the contents of the serum and being a readily available specimen makes it appealing medium for clinical investigations.

In the clinic, cancer patients are exposed to different treatment regimens. Most guidelines support the use of combination therapy such as surgery followed by adjuvant chemo-radiotherapy [67, 68]. Chemotherapy by itself is usually composed of two or more drug combinations. Cancer patients also often receive sequential cycles of chemotherapy and need to be evaluated for response after almost every cycle. The current evaluation and assessment of patients to determine the next course of treatment is either through biopsy or radiological imaging, which lack sensitivity, are invasive, and cannot be done frequently.

Detecting circulating biomarkers that reflect response to treatment would be a watershed development in non-invasive treatment monitoring [69, 70, 71, 72].

Definition of Biomarker

There are several definitions for the term biomarker. World Health Organization (WHO) and in coordination with the United Nations and the International Labor Organization, has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [73]. Biomarkers can be one of or a combination of nucleic acids (DNA, RNA or miRNA), proteins, peptides, sugars, lipids, or whole cells (such as CTCs or DTCs) [74]. Different types of human biospecimens can be used to detect biomarkers, such as blood or its components (plasma or serum), body secretions (sputum, urine, saliva, stool), or tissue from organs [75].

Cancer Biomarkers

Cancer biomarkers have been studied intensively and are divided into prognostic, predictive, and pharmacodynamic biomarkers according to their clinical uses. Prognostic biomarkers are the ones that predict the course of particular tumor. Their level can distinguish patients with poor prognosis from those with good prognosis. The presence or absence of prognostic biomarkers is useful to determine the treatment options for the patient, but they do not predict response [76]. Oncotype DX, a series of Breast Cancer gene-expression signatures, is a good example of a prognostic biomarker. It is used to predict the recurrence rate of the primary tumor after surgical removal. It also can determine which patients will benefit from adjuvant chemotherapy after surgical resection and who will not [77]. Another Breast Cancer prognostic biomarker is BRCA1, where high expression of BRCA1 is linked with poor prognosis in untreated patients [76].

Predictive biomarkers are the biomarkers that predict patient response to a particular therapy. For example, Breast Cancer patient are tested for tumor hormone receptor status because that will determine the treatment to which the patient will respond [77]. Breast Cancer primary tumors that express estrogen receptor will respond to tamoxifen, but not necessarily to trastuzumab that will only benefit patients who are HER2 receptor positive [76, 67].

The third biomarker category is the the pharmacodynamic biomarkers that are mainly used in clinical trials to adjust the dose of new drugs, to measure drug activity on biological targets, and to calculate their maximum tolerated dose. They are subsequently used to calculate the immediate effect of a treatment on the tumor [77].

Biomarker Assay Clinical Validation

Once an empirical biomarker is identified, a clinical assay for that marker needs to be constructed. In order to implement any biomarker assay for clinical use, it should be tested in term of its analytic validity, clinical utility and clinical validity. First, the analytic validity is usually evaluated after the biomarker assay is commercially manufactured. It is evaluated by the assay accuracy, reliability, and reproducibility. The clinical validity of a biomarker assay refers to the ability of the marker to further divide the group of patients into subtype according to its level of expression. Finally, a biomarker assay will be considered for clinical utility only if its application will results in clinical benefits more than harm when compared to clinical practice without its use. Benefits here means a better clinical decision making than other practice or the same decision at a lower cost, less invasive or lower risk. [18].

Genomic Versus Proteomic Biomarkers

Advancements in -omics (genomics, proteomics, lipidomics and metabolomics) allowed for high throughput studies to mine for specific cancer biomarkers circulation [78,

79, 80]. Each of the -omics field has advantages and limitations for biomarker development. Genomics and proteomics complement each other, though proteomics is considered theoretically superior. Although genomics provides the blueprint of life, proteins are the ones that enact cellular functions and better reflect body physiology [81]. Although nucleotide-based approaches can be used to assess mRNA levels in order to gauge the levels of their products, the abundance of proteins in the body often does not correlate with corresponding mRNA levels. Therefore, protein concentration can not be determined by studying the level of mRNA alone because the translation rate varies among different mRNAs. Beyond this, there are different isoforms of proteins generated from the same gene; it has been estimated that almost 100,000 proteins are encoded by only 20,235 genes in human [82]. Furthermore, translated proteins undergo post-translational modifications that creates further complexities to the protein structure that do not exist at the mRNA level. Post translational modifications, such as phosphorylation, acetylation and glycosylation, result in different copies of the same protein having its own physiological function. Therefore, in order to study a rapidly changing physiology, we need to examine proteins directly in laboratory experiments [81, 83, 80].

The study of proteins is complicated by the complex network of interaction between them. Scientists were surprised with the complexity of protein interactions found in yeast Saccharomyces cerevisiae, despite it being a simple model organism. One study revealed the presence of a single large network of 2,358 interactions between 1,548 proteins [84]. The human protein interaction network cannot be uncovered by genomic studies [81, 85]. Therefore, proteomic studies provide a wider understanding of molecular pathways and interactions in physiological systems than genomics. Fortunately, proteomic studies are increasingly prevalent due to rapid advancement in high throughput technology and in response to the sore need for disease specific biomarkers [86].

Clinical Proteomics in Biomarker Discovery

The goal of clinical proteomics is to characterize protein pathways, interactions, localization, and signaling events that are relevant in disease. It have been applied extensively in BC research and provide a treasure of information that explain or partially define cellular pathways. Recently BC metastasis has gained a focused attention and efforts were directed towards completing the molecular picture of metastatic process. Here, we focus on the proteomic analysis of Breast Cancer progression and discuss some of the key issues.

Techniques of Proteomics

Proteomic studies are divided into many categories such as expression, functional, modification, localization and protein-protein interaction depending on the aim of the study. They complement each other to provide the complete picture of a particular protein or a cellular pathway. Here, we focus on the expression proteomics that aims to quantify the protein content in the biospecimens that represent BC metastasis. Expression proteomics studies encompasses different techniques with distinct advantages and limitations. The choice of the proteomic techniques depends on the biological system under study and the aim of the study (quantitative or qualitative). In fact, some proteomic techniques can only be applied in specific biological samples but not in others. For example SILAC needs a prior culture of the studied protein with isotopically labeled amino acids which makes it impractical in human bio-fluid [87].

In general, proteomic analyses are divided into Mass Spectroscopy (MS)-based and non-MS based techniques [88]. The non-MS proteomic approaches—also called targeted proteomics— use specific antibodies or other ligands against a particular, and known, protein, requiring prior knowledge of the protein under study. This can be done by enzyme-linked immuno sorbent assay (ELISA), immunohistochemistry (IHC), Western blotting, tissue microarray (TMA) and Protein microarray (PMA) [89]. On the other hand, in MS based proteomics there is no need for foreknowledge of proteins under study. It is more

informative as it might reveal previously unstudied proteins and even show unexpected interactions despite being technically challenging [90].

Previously, proteomic studies were only capable of analyzing few samples and therefore could not provide an accurate protein quantification. The recent advancement in mass spectroscopy (MS) based proteomics greatly improved protein quantitation even in complex samples such as human specimens. The innovations in sample preparation, separation, and computational proteomics together with the implementation of hybrid mass spectrometers that combine high resolution, high mass accuracy and wide dynamic range all enable more confident protein identification and quantification at a large scale. As a result, the MS-based proteome analysis has grown exponentially in the last decade yielding plethora of identified proteins (biomarkers) that represent different diseases [86].

Quantitative Analysis by Mass Spectroscopy

Mass spectroscopy based proteomics are capable of providing absolute and relative quantitation of proteins in samples under study. The quantitative MS approaches are either labeled or label free (Table 1.2). The label free quantitation methods includes the spectral counting approach and the Peptide peak intensity approach. In spectral counting, proteins are quanrified according to the number and abundance of MS/MS spectra that is produced by the fragmentation. This is based on the concept that highly abundant protein will provide more spectra with higher intensities than less abundant proteins. Comparing the number and intensity of the MS/MS spectra of a particular protein between samples will provide relative quantitation. Peptide peak intensity measurements could provide quantitation of the protein under study through the extraction of the chromatographic peak of the corresponding peptide in the ion chromatograms from a capilary Liquid Chromatography Mass Spectrum (cLC-MS). Comparing the peak areas of the same ion/peptide between different runs and samples will provide a relative quantitation. The labeled quantitation methods include the use of multiple labeling methods such as metabolic labeling

(e.g SILAC), isotopic labeling (e.g.ICAT) and isobaric labeling(e.g. iTRAQ). They provide relative or absolute quantitation and vary in their proteomic coverage as described in table 1.2.

Bottom Up vs Top Down Proteomics

There are two types of MS-based proteomics, referred to as bottom up and top down approaches. In the bottom up, or shotgun approach, a protein is identified through detection of peptides that result from the proteolytic breakdown of that particular protein [91, 92, 93]. In a typical bottom up proteomic experiment, specimens containing intact proteins are treated with a proteolytic enzyme to break them into their corresponding peptides and then loaded into cLC-MS/MS. The proteins are later identified by comparing the fragmentation pattern of the daughter peptides with a protein database. This approach does not identify post-translational modifications and is complicated when a single peptide is shared by more than one parent protein [82, 94]. These limitations are overcome by top down proteomics, where intact proteins are analyzed. Therefore, top-down has the advantage of being able to detect post translational modification and distinguish between protein isoforms. A further advantage over the bottom up approach is its ability to characterize small low molecular weight (LMW) proteins [95, 96, 94, 97]. The major challenge in the top down approach is the wide dynamic range of protein abundance in human samples. This has been overcome by the implementation of multiple separation steps prior to introducing the samples into the instrument [95, 96], as well as by enrichment of LMW during precipitation of high abundance proteins [97, 96, 94, 98].

Mass Spectrometry-Based Biomarker Discovery Workflow

With advances in proteomics techniques, it is becoming more feasible to study hundreds or even thousands of proteins in a single experiment [85, 99]. High throughput proteomic studies usually involve mass spectrometry (MS) or microarray technology [86]. The

Table 1.2: Summery of the Quantitative MS Approaches.
Reprinted with permission from [100].

Quantitation method	**Dynamic range**	**Quantitation Nature**	**Number of samples**	**Proteomic Coverage**
Labeled				
SILAC	12	Relative	2 to 3	Medium
ICAT	12	Relative	2	Poor
ITRAQ	2	Relative/Absolute	2 to 8	Medium
Label-free				
2D gels	1 to 4 (stain dependent)	Relative	many	Medium
Ion intensities	3	Relative	many	Good
Spectrum count	3	Relative	many	Good

general workflow of a global mass spectrometry-based biomarker discovery experiment occurs in 4 steps [86]. The first step is choosing the most appropriate disease-related samples, which are then processed to deplete the high abundance large MW proteins. Next, processed samples undergo a pre-fractionation step, using either gel electrophoresis or chromatography. At this stage, samples are then loaded into the MS instrument and the levels of individual proteins are quantitatively assessed for comparison between sample groups. The last step is validation of identified proteins or protein panels using independent analytical approaches.

Low Molecular Weight Peptides as Disease Signature

Recently, proteomic studies have been directed toward the low abundance and low molecular weight (LMW) proteins that are less than 30KDa [98]. It has been found that disease specific molecular signatures, including central regulators of biological pathways, are usually of low MW and present at low concentration [101]. Furthermore, It has been estimated that almost 50% of the human peptidome is composed of peptides that are less than 26.5 kDa [102]. Low MW proteins are categorized into cytokines, peptide hormones, small signaling proteins, or fragments of a larger cellular proteins. Previously, these low MW proteins were considered as cellular trash, though they are now known to have physiological significance [103, 81, 104]. Low MW proteins were hard to detect in gel-based proteomic studies because they stain very poorly and they migrate rapidly during electrophoresis [102].

The potential diagnostic significance of these molecules was revealed when the serum proteome of ovarian cancer was examined using MALDI-MS and significant biomarkers were detected in the Low MW range [105, 106]. Discovering the low MW range of human proteome is ongoing, but is incomplete and no examples of low MW biomarkers have yet reached diagnostic utility in clinical settings.

Challenges in Serum Proteomics

Proteomics can be applied to any biological samples: cell lines, clinical tissue samples and biofluids such as serum, tears, urine and organ aspirates. The complexity of the human proteome depends on the samples used for the study. Body fluids, such as blood, urine and saliva, are the most complicated and challenging samples for proteomic study, but are also the most clinically relevant [86]. Human serum/plasma is considered the most accessible body sample for clinical biomarker investigation; and therefore it is appealing clinically as a minimally invasive source for diagnosis and screening. It contains a vast number of molecules that are shed out from almost every tissue of the body, all of which vary in their molecular weight. This makes the serum a vast and diverse reservoir of signals that reflect body physiology [107, 108].

Despite being readily accessible, human serum proteomics is technically challenging. The biggest challenge is the wide dynamic range of proteins in human serum, which reaches a magnitude as high as 12 units. This is because more than 90% of serum proteins are of large molecular weight and these mask the visualization of more divers low MW proteins that are believed to be disease specific. These low MW serum proteins are called the "hidden proteome" for this reason. The hidden proteome is mainly composed of proteins of less than 50 kDa, which are either small circulating proteins or fragments of cellular proteins that have diffused into circulation. Low MW protein fragments in serum have their own physiologic function and the diagnostic impact can be very different than the parent protein. Data contradict the original belief that circulating protein fragments represent the level and function of their parent [104]. For example, low MW hyaluronan (LMW-HA) levels in serum, but not the parent HA levels, were found to correlate with Breast Cancer metastasis. Although total HA levels are elevated in primary Breast Cancer, it has no role in Breast Cancer progression. [109].

In order to uncover these low MW proteins in serum, we need either to carefully separate them from the large MW or to deplete the large MW from the sample before

conducting proteomic study. Large MW protein in human samples suppress the low MW protein signals in the MS and makes them difficult to quantitate [110, 111, 112, 113, 114]. Several separation methods, such as gel electrophoresis [115, 116, 117], immunodepletion [118, 117], and chromatography [119, 117] have been implemented and all give a better sensitivity in mass spectrometry analysis. Furthermore, the use of enrichment strategies have been shown to be effective in depleting the large MW serum proteins. Enrichment strategies could be through organic solvents precipitation, ultrafiltration, solid phase extraction and others [120, 121, 122, 123, 124, 125, 126, 120, 110, 127, 111, 128]. Acetonitrile precipitation is considered superior to other enrichment approaches [122, 128, 127, 110].

Acetonitrile Precipitation

Acetonitrile is an organic solvent that has been used to precipitate high abundance, large MW proteins. It has been found that it also dissociate the low MW protein from their carrier proteins making them available for proteomic study [110]. When cLC-MS chromatogram of acetonitrile treated serum sample compared with untreated serum sample cLC-MS chromatogram, the total-ion chromatogram (TIC) of the treated sample had more numbers of molecular species than the untreated samples [128]. This reflect the power of acetonitrile precipitation in enriching the low MW proteins pool for further proteomic study.

CHAPTER 2: Literature Review

Biomarkers for BC Progression

The application of the high throughput screening techniques in BC cancer research provided several putative biomarkers that needs further evaluation for their analytical validity, clinical validity and clinical utility. Table 2.1 summarizes the most recent potential proteomic biomarkers that have been identified and found to play a role at one or more stages of BC progression and invasiveness. The list is providing examples of the most recent identified proteins and it is not an exhaustive list.

Metaphorically speaking, "the water is everywhere, but little is yet ready to drink", describes the current state of the amount of identified proteins that are associated with BC progression but are not yet ready for clinical utility. Despite the long list of empirical proteins that claims success, FDA has not yet approved the use of any MS-based protein biomarker in the clinic [86, 18]. It is worth mentioning here that there are some BC metastasis empirical biomarkers are in their way toward the clinic. For example, there are accumulating evidences toward the use of Ki67 as a prognostic indicator for the progression of BC but not as a predictor of treatment benefit as indicated in Table 1.1 [129]. Additionally, urokinase-plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1) have been recently recommended by the ASCO for the determination of prognosis in lymph node negative Breast Cancer [130]. The proposed biomarkers are promising and even if they are not sensitive to predict outcome, they might add an important piece of information towards the understanding of BC metastasis.

Table 2.1: List of Most Recent Protoemics Studies on Breast Cancer Progression.

Experimental methodology	**Proteomic technique**	**Findings**	**Reference**
Breast cancer tissues	LC-MS/MS	Significant up-regulation of PDI A3 was detected only in the metastasized breast cancer	[135]
Breast cancer cell lines	Nano-UPLC-MS-MS	EDIL3 on exosomes promotes breast cancer invasion via integrin-FAK signaling	[136]
Breast cancer cell lines	iTRAQ-nano-HPLC-MS/MS, IHC in tissue	RAB1B is significantly down-regulated in highly metastatic breast cancer cells	[137]
Breast cancer cell lines	2DE-MALDI-TOF/TOF, Wastern blot	Downregulation of RhoGDI in MCF7 and MDA-MB-231 cells increased the invasion and migration of BC cells	[138]
Breast cancer cell lines, animal study	2DE-MALDI-TOF/TOF Wastern blot	STC1 is positively associated with the development, progression and metastasis of BC especifically to the lung	[139]
Breast cancer cell lines, animal study	nano LC-MS/MS	High expression of LTBP3 and SNED1 correlates with poor outcome for ER(-)/PR(-)breast cancer patients	[140]
Breast cancer cell lines and animal study	SILAC-LC-MS/MS, IHC	LRRC59 positively correlate with BC aggressiveness and metastasis, CD59 and CSPG4 inversely correlate with metastasis	[141]
Breast cancer cell lines and animal study	LC-MS/MS , IHC	hnRNPM is associated with aggressive BC	[142]
Breast cancer cell lines and tissues	SILAC-LC-MS-MS, IHC	Reduction of lung metastasis when CYB5R3 expression was significantly reduced	[143]

Experimental methodology	Proteomic technique	Findings	Reference
Breast cancer cell lines and tissues	HPLC- LTQ-Orbitrap	High levels of IDH2 and CRABP2 and low levels of SEC14L2 are prognostic markers for overall breast cancer survival	[144]
Breast cancer cell lines and tissue	Discovery: 2D-DIGE-LC/MS/MS, validation: IHC	composite biomarker comprising CAPG and GIPC1 was associated with development of bone metastasis and reduced survival	[132]
Breast cancer serum samples	Recombinant antibody microarray platform	21-candidate protein biomarker signature associated with metastasis, included cytokines (IL-6, IL-18) involved with cell migration, infiltration and angiogenesis	[145]
Breast cancer serum samples	Chemiluminescent assay, IHC	High serum HER-2 extracellular domain (ECD) levels associated with a worse disease-free survival and overall survival in primary operable BC patients	[146]
Breast cancer serum samples	SELDI-TOF-MS, Wastern blot	High expression levels of SAA in sera of almost all advanced-stage cancer patients	[147]
Breast cancer serum samples	Luminex liquid protein chip	Decreased expression of CP-1 and IP- 10 significantly correlated with patients who had more positive lymph nodes	[148]
Breast cancer serum samples	ELISA	Serum amyloid A (SAA) protein indicated presence of lymph node metastases and distant metastases	[149]
Breast cancer serum samples	ELISA	Serum cadherin-5 discriminated patients with recurrent BC from those with no sign of recurrence (90% specificity)	[150]

Experimental methodology	Proteomic technique	Findings	Reference
Breast cancer serum samples and cell lines	HPLC followed by UV/refractive index dual detection	Elevated serum levels of LMW-HA, but not total HA, correlate with BC metastasis	[109]
Breast cancer serum samples and cell lines	ELISA	Higher serum CIP2A levels positively associated with the aggressive BC in vivo and ivitro	[151]
Breast cancer tissues	iTRAQ-2DLC-MS/MS, IHC	CPB1, PDLIM2, RNF25, RELA, STMN1, TMSB10, TRAF3IP2, and YWHAH as proteins correlating with lymph node positivity of low grade breast cancer	[152]
Breast cancer tissues	HPLC- ESI-LTQ-Orbitrap screen, IHC validation	ATPIF1, CK17, thrombomodulin and tubulin -chain are part pathways involved in cell adhesion, migration pathways and immune response	[153]
Breast cancer tissues	2DE-MALDI-TOF/TOF/MS/MS	1433G, 1433T, K1C19, K2C8, PSME2, SNAA, TPM4, TRFE and VIME were up-regulated in PBT while ALDH2, GDIR2 and K1C19 were up-regulated in LNM	[154]
Breast cancer tissues	iTRAQ sets for LC-MS/MS , SRM, IHC	High expression of DCN was associated with higher risk for lymph node metastasis, high expression of HSP90B1 was correlated with higher risk of developing distant metastasis	[155]
Breast cancer tissues	2DGE- MALDI-TOF, wastern blot	Deregulation of multiple S100 protein members is associated with breast cancer progression	[155]
Breast cancer tissues	2D-PAGE-LC-MS/MS	Higher expression of Stat1 and the HLA II gamma subunit CD74in LN +ve tumor	[135]

Experimental methodology	Proteomic technique	Findings	Reference
Breast cancer tissues	nLC-MS/MS	10 proteins were downreg- ulated (CMPK1, AIFM1, FTH1, EML4, GANAB, CTNNA1, AP1G1, STX12, AP1M1, and CAPZB), whereas one protein was upreg- ulated (MTHFD1) in TNBC	[156]
Breast cancer tissues	IHC	Significant correlation between the cytoplasmic expression of VCP and adverse prognosis in breast carcinoma	[157]
Breast cancer tissues	IHC	C6orf106 promote tumor progression in the invasive breast cancer	[158]
Breast cancer tissues	IHC	Data support a role of SIRT1 protein as tumor suppressor in luminal A breast cancer	[159]
Breast cancer tissues	IHC	Overexpression of G6PD protein predicted a high risk of recurrent metastasis and poor PFS during follow-up	[160]
Breast cancer tissues	Western blotting, IHC	Down-regulated CDK10 expression frequently occurs in BC and correlates with disease progression and poor sur- vival	[161]
Breast cancer tissues	TMA	CCR7 and CXCR4 were expressed more often in BC bone metastases than in visceral metastases	[131]

Organ Specific Distant Metastasis

Biomarker Associated with Bone Metastasis

We notice from the above list in table 2.1 that in general, couple of studies were focusing in organ specific BC metastasis while others were mining for protein that associate with BC progression and invasiveness in general. Liu found that the expression of CCR7 and CXCR4 combinedly is more predictive of bone metastasis than their sole expression [131]. Another composite biomarker comprising of CAPG and GIPC1 has been implicated to the development of BC induced bone metastasis as well [132]. Bone is a common destination for BC tumor. It has been estimated that up to 75% of patients with metastatic BC will have a bone metastasis [133]. Bone metastasis is considered a sign of poor prognosis because of the severe skeletal complication that impact patients life [134]. Developing a signature biomarker to predict the risk of developing bone metastasis will help to improve BC patient management and prognosis.

Biomarker Associated with Lung Metastasis

The second common destination for BC metastasis is the lung. Approximately 60% of Breast Cancer patients have secondary growth in the lung. In fact, about 21% of BC patient have lung metastasis as the sole secondary growth. Several biomarkers have been proposed to associate with lung metastasis in BC. For example, CYB5R3, LDHA, NPC1, NRH2 protein expression has been linked to lung metastasis. Moreover, inhibition of CYB5R3 was highly associated with reduction in lung metastasis [143]. In addition, Olkhanud suggested that CCR4 is a metastasis-associated receptor that when targeted, it decreases lung metastasis [162]. Furthermore, STC1 as a single biomarker found to positively associate with BC metastasis to the lung. [131]. The role of MMP9 on metastasis was examined using an animal study. It showed that suppression of MMP9 markedly reduced lung metastasis [61].

There are other proteins that are associated with BC aggressiveness and metastasis in general with no organ preference. For example serum amyloid A (SAA) protein was highly expressed in sera of BC metastatic patients with a metastasis in multiple distant sites [149]. Interestingly, Carlsson conducted a longitudinal study where he screened BC sera at different intervals of the disease for a period of 3 years to identify proteins that can predict recurrence. He built a serum protein signature, which contain couple of cytokines and are involved in cell migration, infiltration and angiogenesis [145]. It is very important to stress that previously mentioned protein biomarkers are not an exclusive list but rather reflects the most recent published studies in literature.

Multiple Markers Reflect Complicated Pathway

The striking observation here is that vast number of proteins have been identified to be associated with BC progression. This might reflect the complex pathway and protein interaction involved in this process. Therefore, all the identified markers could add a new understanding to the mechanism of metastasis and if they are not good biomarkers per se, they could direct further research to reach a better biomarker model. In addition, the study findings are highly dependent on the used specimen and the proteomic techniques utilized. An ample number of cell types, BC tissues, and biological fluids have been studied in an effort to identify biomarkers that can cross their way to the clinic. The specimen used in proteomic studies has a critical role in determining the clinical relevance of the detected protein. As a matter of fact, the proteomic profile complexity is increasing as we move from cell lines study, animal studies, tissue biopsy to blood and biofluid. Although human blood and biofluid are the most challenging and their proteomic profile is very complicated, they are providing the most clinically relevant findings [86].

Conclusion

The use of biomarkers could only replace other practiced diagnostic and prognostic guidelines if they only provide better insight towards the disease progression or the similar insight at a lower cost and less invasiveness. BC metastasis biomarker studies will be easier if a comprehensive understanding of the metastatic process exists. Since Breast Cancer is very dynamic and heterogeneous, there are always emerging new molecular mechanism and pathway. Therefore any identified potential marker will need continuous re-evaluation to get the best practice [163].

CHAPTER 3: Methods

Study Population

Proteomic studies can be applied in any biological specimen. The most commonly used samples for cancer research are cell lines, tissue biopsies, animal model and biofluids. The study of any of these samples can uncover a molecular aspect related to cancer initiation, cancer progression or response to therapy which can be considered as biomarkers for clinical use. Technically, biomarkers that are identified and validated in a less invasive samples are more enviable [164]. Serum/plasma are the least invasive and readily available samples and therefore considered as optimum biospecimens for biomarker discovery (see Table 3.1). In our project, we used commercial serum samples that represent metastatic and non-metastatic stages of BC for both the initial and confirmatory studies. Table 3.2 is showing the clinical stages of Breast Cancer and at which stages it is considered metastasized. For our study samples, we considered all patients classified in stages 0, I, IIa with no local (lymph node) or distant organ metastasis as Non-metastatic, and all patients in other stages as metastatic.

Experimental Pipeline

This study was conducted through 3 phases, the discovery phase, training phase and testing phase. The biomarkes that have been discovered in the serum samples in the initial discovery study were tested for persistent significance in independent serum samples set in the training set. A confirmatory testing set were then conducted using a new serum sample to further confirm and validate the findings. We aimed to get more confidence that the peaks identified are truly associated with the experimental condition, rather than a statistical expectation from having a lot of peaks to examine.

Table 3.1: Summery of Sources of Biomarkers for Discovery Platforms. Reprinted with permission from [86].

Sample	**Advantage**	**Disadvantage**
In vitro cell culture	Easy to obtain; no ethics; abundant sample quantity; good for characterizing cell-specific responses	Lack of heterogeneity; may not represent clinically relevant results
Tissue biopsy/core sample	Accessibility to samples stored, long term; direct comparison to standard diagnosis; tissue-level representative profiling	Potential for sample degradation; require large validation datasets; invasive sample collection
Urine/blood	Easy to obtain; express representative protein and gene expression of a large number of cell types	Low marker concentration; high sample complexity; technically difficult to detect

Table 3.2: Clinical staging of Breast Cancer.
Reprinted with permission from American Joint Committee on Cancer 7th edition, BC staging.
N1: Metastasis to movable ipsilateral level I, II axillary lymph nodes.
N2: Metastases in ipsilateral level I, II axillary lymph nodes that are fixed or matted.
N3: Metastases in ipsilateral infraclavicular (level III axillary) lymph nodes.

Stage	**Primary tumor**	**Lymph node**	**Distant metastasis**	**Survival rate**
0	$\leq$2 cm	None	None	100%
I	$\leq$2 cm	None	None	98%
IIa	$\leq$2 cm	N1	None	88%
IIb	>2 cm,$\leq$5 cm	N1	None	88%
IIa	>2 cm,$\leq$5 cm	N2	None	52%
IIb	>5 cm	N2	None	52%
IIc	Any size	N3	None	52%
IV	Any size	Any involvement	Detectable	16%

Initial Study

For the initial study, six samples of each stage (stage I and stage III) were purchased from Proteogenex. We used the following criteria to match these patients as closely as possible between the two groups: race, age and absence of comorbid diseases and tumor subtype. The provided serum samples lacked vital information such as time of sample collection (whether pre or post cancer therapy). Table 3.3 shows demographic, clinical history and diagnosis of patients in the the initial study.

Confirmatory Study

For the confirmatory study, we purchased serum samples from Conversent Bio. It included 19 samples in stage I and 20 samples in stage III. A variety of sample types were included, as we aimed to detect biomarkers of metastasis in a way that is not dependent on factors such as treatment regimens, race or age. Table 3.4 demonstrate the demographics, diagnosis and treatment of the patients used in the confirmatory study. All samples in both studies were coded without personal information. According to our conversations with both companies, samples in both studies were exposed to the same environment from collection to storage with no prior thaw and freeze cycle. Specimens were maintained at -80 C during pre-processing and post-processing.

Samples Preparation

Despite the high clinical prevalence of serum/plasma proteomics and its easy accessibility, it is technically challenging. The challenges in serum proteome is due to the wide dynamic range (upto 12 orders of magnitude) of its protein content [165] which can not be covered by the current available proteomic approaches that are limited to linearity of just over 3 orders of magnitude [166]. It has been very confronting to uncover this hidden serum proteome because of the masking effect of the high abundance low MW which constitute more than 90% of serum proteome [104]. Identification of these cancer-causing pro-

Table 3.3: Demographics of the Initial Study Samples.
IDC: Infiltrating ductal carcinoma, ILC: Infiltrating lobular carcinoma.

Stage	**Histological Diagnosis**	**Age**	**Ethnicity**	**Family History**	**Personal History**
I	IDC	63	Caucasian	No	No
I	IDC	63	Caucasian	No	Ischemia; hypertension
I	IDC	67	caucasian	No	Ischemia
I	IDC	46	Caucasian	No	No
I	ILC	46	Caucasian	No	Chronic cholecystitis
IIA	IDC	51	Caucasian	No	No
IIIA	IDC	46	Caucasian	No	No
IIIA	IDC	51	Caucasian	No	No
IIIA	IDC	55	Caucasian	No	No
IIIA	IDC	39	Caucasian	Uterus Cancer (Grandmother)	No
IIIC	IDC	60	Caucasian	No	Chronic Cholecystitis
IIIC	ILC	45	Caucasian	No	No

Table 3.4: Demographics of the Confirmatory Study Samples.
IDC: Infiltrating ductal carcinoma; ILC: Infiltrating lobular carcinoma; Tx: Treatement history, TAC: Taxotere+Adriamycin+Cyclophosphamide, AC: Adriamycin+Cyclophosphamide.

Stage	**Histological Diagnosis**	**Treatment Status**	**Age**	**Race**	**Ethnicity**
I	IDC	Taxol	54	Black	Non-Hispanic
I	IDC	Taxol	77	White	Non-Hispanic
I	IDC	Taxotere; Herceptin; Carboplatin	53	White	Non-Hispanic
I	Undefined	TAC	56	White	Non-Hispanic
I	IDC	Reclast	70	White	Non-Hispanic
I	IDC	Post Tx	80	Black	Non-Hispanic
I	Undefined	Post Tx	56	White	Non-Hispanic
I	IDC	Active Hormone Tx	85	White	Non-Hispanic
I	IDC	Active Hormone Tx	65	White	Non-Hispanic
I	ILC	Active Hormone Tx	66	White	Non-Hispanic
I	Undefined	Active Hormone Tx	42	White	Non-Hispanic
I	IDC	Active Hormone Tx	64	White	Non-Hispanic
I	Undefined	Active Hormone Tx	75	White	Non-Hispanic
I	IDC	Active Hormone Tx	53	Black	Non-Hispanic
I	IDC	Active Hormone Tx	72	White	Non-Hispanic
I	IDC	Active Hormone Tx	59	White	Non-Hispanic
I	Undefined	Active Hormone Tx	60	White	Non-Hispanic
I-A	IDC	Taxotere; Cytoxan	55	Black	Non-Hispanic
I-A	IDC	Cytoxan; Taxotere	71	White	Non-Hispanic

I-A	IDC	AC; Taxotere	73	White	Non-Hispanic
III	IDC	Active Hormone Tx	36	White	Non-Hispanic
III	IDC	Adriamycin; Cytoxan; Taxotere	65	White	Non-Hispanic
III	IDC	Taxotere; Cytoxan	69	White	Non-Hispanic
III	IDC	Adrimyacin; Cytotoxan; Taxotere	49	Black	Non-Hispanic
III	IDC	Carboplatin/Taxol	54	White	Non-Hispanic
III	IDC	Taxol; Adriamycin; Cytoxan	75	White	Non-Hispanic
III	IDC	Taxotere	65	White	Non-Hispanic
III	ILC	Doxorubicin; Cyclophosphamide	63	White	Non-Hispanic
III	IDC	Active Herceptin	56	White	Non-Hispanic
III	Undefined	Active Hormone Tx	47	Black	Non-Hispanic
III-A	IDC	Active Hormone Tx	55	Black	Non-Hispanic
III-A	IDC	Active Hormone Tx	53	White	Non-Hispanic
III-A	IDC	Unknown	30	Black	Non-Hispanic
III-A	IDC	Unknown	60	White	Non-Hispanic
III-A	Undefined	Active Hormone Tx	56	White	Non-Hispanic
III-A	ILC	Unknown	71	White	Non-Hispanic
III-A	Undefined	Active Hormone Tx	82	White	Non-Hispanic
III-A	IDC	Herceptin; Tamoxifen	48	Black	Non-Hispanic
III-C	IDC	Unknown	75	White	Non-Hispanic

tein biomarkers in the human serum proteome is like "looking for a needle in a haystack". One of the strategies designed to overcome the issue of dynamic range involved the use of protein pre-fractionation coupled with depletion methods to remove abundant proteins in the plasma proteomes. Different methods have been used for sample pre-fractionation and biomolecule separation such as immobilized dyes (cibacron blue), immunoaffinity-based techniques, solid phase fractionation, liquid chromatography, or low-molecular weight fraction enrichment [167, 168, 169, 170, 171, 172, 165]. In our study, we applied a unique proteomic approach that composed of a simple depletion method by acetonitrile (ACN) coupled with a liquid chromatography separation interfaced with Quadropole Time of Flight Mass Spectroscopy (Q-TOF/MS).

ACN Precipitation

We washed out the high-abundance proteins (those with MW less than 20 kDa) by adding acetonitrile in a ratio to serum of 2:1; following an established acetonitrile precipitation protocol [173, 127, 122]. Although acetonitrile precipitation sacrifices most proteins, it does dissociate the small unobservable peptides from their carriers making them available for MS analysis [110, 111]. A BCA Protein Assay (Pierce Microplate BCA Protein Assay Kit; Thermo Scientific) was used to determine the apparent protein concentration of ACN treated samples. An aliquot containing 4 µg protein was lyophilized to 10 µL (CentriVap Concentrator Labconco Corporation, MO, USA) and then acidified by adding equal volume (10 µL) of 88% Formic acid. The samples then were loaded into capillary liquid chromatography mass spectroscopy (cLC-MS).

Liquid Chromatography Mass Spectroscopy

Several proteomic approches have been applied for biomarkers dicovery including two-dimensional (2D) gel-electrophoresis, liquid chromatography coupled with mass spectrometry (cLC-MS), and protein- and antibody-based microarray. In fact, cLC-MS based

proteomic technologies is considered the method of choice for proteomic profiling of human complex biospecimens [174] because of its highly sensitive analytical capabilities and a relatively large dynamic range of detection. The Chromatographic Reversed-phase capillary liquid chromatography (cLC) was operated with an LC Packings Ultimate Capillary (HPLC) pump system, with a Famos autosampler (Dionex, Sunnyvale, CA, USA). The cLC system composed of a 1 mm (16.2 µL) dry packed MicroBore guard column (IDEX Health and Science, Oak Harbor, WA, USA), coupled to a 15 cm×250 µm i.d. capillary column, slurry-packed in-house with Poros R1 reversed-phase medium (Applied Biosystems, Foster City, CA, USA). To generate a gradient mobile phase, we used 98% HPLC-grade water, 2% acetonitrile, 0.1% formic acid as an aqueous phase and 98% acetonitrile, 2% HPLC-grade water, 0.1% formic acid as an organic phase. The gradient was set as follows: 3 min of 95% aqueous and 5% organic phase, followed by a linear increase in organic phase to 60% over the next 24 minutes. The gradient was then increased linearly to 95% organic phase–5% aqueous phase over the next 7 minutes, held at 95% organic phase for 7 minutes and returned to 95% aqueous phase.

The cLC system was interfaced to Agilent 6530 Accurate-Mass Q-TOF LC/MS system. The ESI source was operated in positive ion mode. The scans were collected at 8 spectra/s and the mass spectra were within the range of m/z 500 to m/z 2500. The samples were run in sets that are randomly assigned with an approximately equal number of stage I and stage III samples. The same instrument was used for tandem MS in order to identify the promising markers where we used targeted MS/MS, and scans were collected at 1 spectra/s. Agilent MassHunter Qualitative Analysis B.06.00 software was used to extract the data and ion intensities.

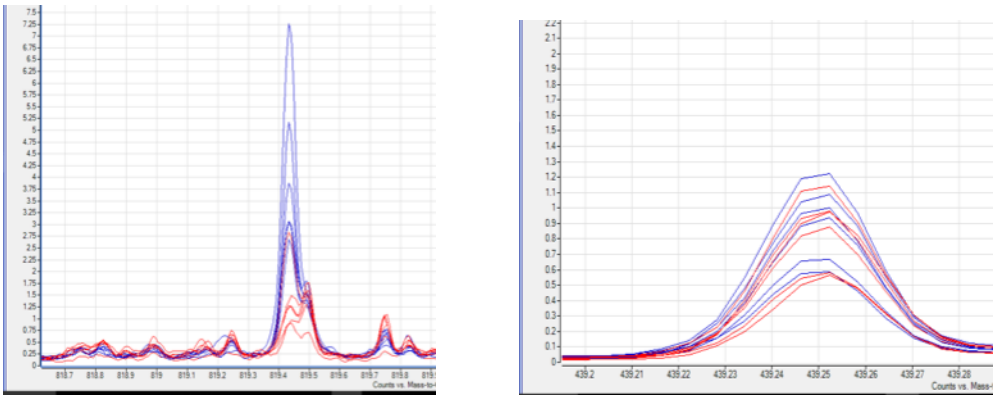
MS Data Analysis and Time Normalization

In any proteomic study there are several biological and non-biological variations in mass spectra that are not reflective of disease status. One way to minimize elution time

variability between samples is through normalizing time markers and peak alignments [122, 173, 127]. The time-marker normalization concept assumes the existence of ten endogenous species that elute every 2-3 minutes in the cLC chromatogram of human sera. The compounds in the series elute at approximately two-minute intervals from each other. This allowed for the same time windows to be considered for each specimen run. Data were organized in two-minute windows centroided on each marker or where there were gaps between two time markers. The time markers allowed for time normalization, providing uniformity in chromatographic elution windows over the important chromatographic region (˜15 to ˜35 minutes) as described in detail in [128]. We applied the time-marker normalization to our data and created 10 2-minute time windows for each sample. The m/z of the ten time markers were: 733.3 (z = +2), 721.3 (z = +2), 1006.0 (z = +2), 1013 (z = +5), 547.3 (monoisotopic) (z = +1), 547.3 (z = +1), 1047.7 (z = +1), 637.3 (z = +1), 781.5 (z = +1), and 1620.2 (z = +1), details are in Table 3.5. Then, the samples' mass spectra were overlaid after we color-coded them. Interesting peaks were picked upon visual assessment of the difference in their heights between stage I and stage III samples (Figure 3.1). Then peak heights were extracted from the ion count using the instrument software.

Data Normalization and Initial Marker Filtering

Peaks that appeared quantitatively different between stage I and stage III were considered for further analysis. We extracted their ion counts in every sample and recorded the peak height that corresponds to the marker intensity in each sample. To minimize non-biological variation that may arise from different runs on the instrument, we normalized the extracted intensities by calculating the ratio of each intensity value to the mean across all samples for the same marker. Normalized intensities were evaluated statistically using Students t-test. All markers with a p-value less than 0.05 in the initial study were evaluated for their predictive power in the confirmatory study. We assumed that some



(a) (b)

Figure 3.1: Visual Assessment of the Mass Spectra.
Different colors indicate sample stage (blue for I and red for III). (a) m/z 819.43 is a potential marker; (b) m/z 439.25 shows no visually significant difference.

Table 3.5: Details of the 10 time markers.
Reprinted with permission from [128].

Marker #	Reference Peak *m/z*	Charge State	Monoisotopic	Relative Elution Time (min)	XIC Window
1	733.3	+2	Yes	0.0	733–734
2	721.3	+2	No	2.2	721–722
3	1006.0	+2	Yes	3.7	1006–1007
4	1013.5	+5	Yes	6.3	1013–1014
5	547.3	+1	Yes	8.7	547–548
6	547.3	+1	No	10.9	547–548
7	1047.7	+1	Yes	12.8	1047–1048
8	637.3	+1	Yes	17.3	637–638
9	781.5	+1	No	19.8	781–782
10	1620.2	+1	Yes	22.1	1620–1621

markers might have modest predictive power in isolation but that this predictive power would increase when combined with other markers in our subsequent machine learning analysis.

Evaluation of Potential Batch Effect

It is almost inevitable in a high throughput study to introduce batch effect due to the splitting of samples into batches that are run under different conditions [175]. This may lead to biases that confound with the original study design [176]. We used two approaches to minimize such biases: 1) we analyzed data from the initial study (first 12 samples) separately from the data from the confirmatory study, and 2) we applied the ComBat algorithm [177] to data from the confirmatory study, which had been loaded into the MS on sequential days due to the long run time in the mass spectroscopy (one hour run per sample followed by 45 minutes wash).

Confirmatory Study Workflow

An arbiter who was not part of the analysis team blinded the 39 samples from the confirmatory study by assigning generic labels to each sample. These labels did not indicate whether the samples were stage I or stage III samples. The samples were processed on cLC-MS in batches that included an approximately equal number of stage I and stage III samples, according to the order created by the arbiter. Candidate markers intensities were extracted, normalized by the mean marker value and then evaluated by the analysis team. At this stage, the 39 samples were randomly divided into training (n = 24) and testing (n = 15) sets, each with an approximately equal number of stage I and III samples. At this point in time, the analysis team was made aware of the stage status of the training samples but not of the testing samples.

Multi-marker Model

The previously believed concept that the risk of metastasis can be determined by the presence or absence of one particular molecular marker is far from reality [178]. The very complicated interaction between several different molecules within a single pathway and the overlap between different molecular pathway within the same cells can explain the single marker concept incompetence [90]. Additionally, histo-pathologically identical tumors might express different biological behaviors, they respond differently to treatment and they progress at variable rates. This has been explained by the tumor heterogeneity at different levels such as molecular heterogeneity from patient to patient, intra-tumor heterogeneity where a single tumor mass found to contain cells at different proliferation stage, and the patient immune reaction to the tumor [104, 179]. Recent studies showed that cancer cells utilize multiple pathways to maintain their uncontrolled growth [180]. Therefore targeting one pathway might slow down their growth but will never stop them. Therefore, future cancer therapy should target combined pathways to arrest cancer cell growth. This is vital in particular due to the fact that some pathways are common among different cancer types. Hence, studies showed that combined biomarker expression increased the accuracy of the prediction. For instance, CCR7 expression has been related to lymph node metastasis prediction, however, combined CCR7, CXCR4 and HER2-neu biomarkers expression was more accurate in predicting lymph node metastasis than CCR7 expression alone [131]. Panels of protein biomarkers typically will transcend the tumor heterogeneity and have higher sensitivity and/or specificity for population-based screenings compared to a single biomarker model [178]; see figure 3.2 for illustrations.

Statistical Analysis

We used machine learning algorithms to derive multivariate models that predicted whether each sample was stage I or stage III sample. We applied two-fold cross validation to the training data (to avoid over-fitting) and used a forward-selection approach to

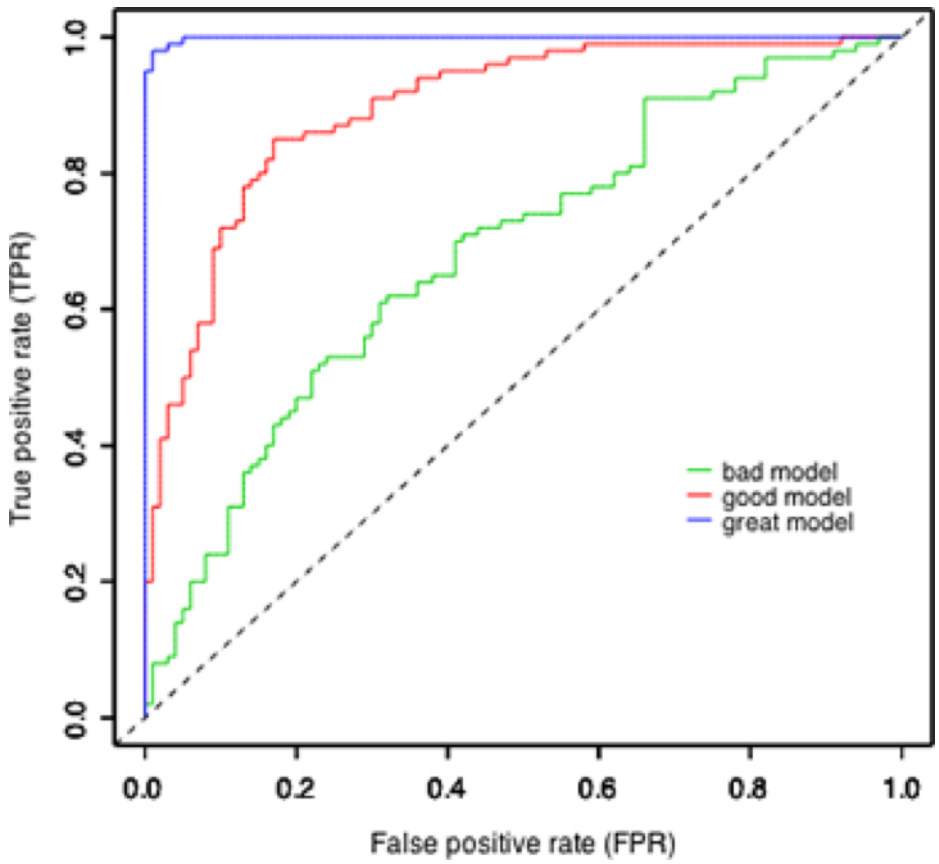


Figure 3.2: ROC Curve Improvement by Multimarker Model.
The performance of A+C markers combined is better than the performance of biomarker A alone.
Red line: marker A performance, green line: markers A+B performance, blue line: markers A+C performance.

identify combinations of markers that were most predictive of cancer stage. Initially, we identified individual markers that attained an area under the receiver operating characteristic curve (AUC) greater than 0.65. We then evaluated each pairwise combination of these markers and identified combinations that attained an AUC greater than 0.70. We continued adding one marker at a time to the model, only considering markers that increased the AUC value at each step. We used AUC thresholds of 0.75, 0.85, 0.90, and 0.95 for 3-way, 4-way, 5-way, and 6-way marker combinations, respectively. We incremented the number of markers in our models until adding more markers no longer exceeded these thresholds. Based on these results on the training data, we selected five marker combinations, which we further verified using the blinded samples from the testing set. We applied the following classification algorithms to the training data: Support Vector Machine (SVM), Random Forests, k-nearest neighbors, Naive Bayes classifier, and logistic regression. [181, 182, 183]. Random Forests gave the most consistent and robust results; therefore we used this algorithm exclusively in our analysis of the testing data. For our testing data analysis, we trained the Random Forests algorithm on the full training set and applied the resulting multimarker combinations to the full testing set. After un-blinding the class labels, we used receiver operator characteristic (ROC) curves and AUC metrics to assess sensitivity and specificity.

Identification of Significant Markers

The markers that showed consistent significance across the studies were further evaluated through tandem mass spectroscopy with collision-induced energy (CID). We took 7 µL of the sample that had high concentration of the marker to be identified, and treated it with 3 µL 88% formic acid, then injected it into the tandem MS with different collision energy. The fragmentation was produced by ion collisions with nitrogen or argon. The fragment patterns of every marker at different collision energy were summed for a complete fragmentation coverage. The fragmentation spectrum was inspected visually

for any mis-assigned charge state. Peak charges were corrected to their +1 m/z values using the formula

$$+1\ mass = m/z\ value \times charge - (charge - 1H^{+})$$

The corrected charge peaks were submitted to a Mascot database search to determine the amino acid sequence of the protein and the parent protein. Some of the markers were of very low abundance despite serum lyophylization and therefore they did not provide an informative fragmentation pattern.

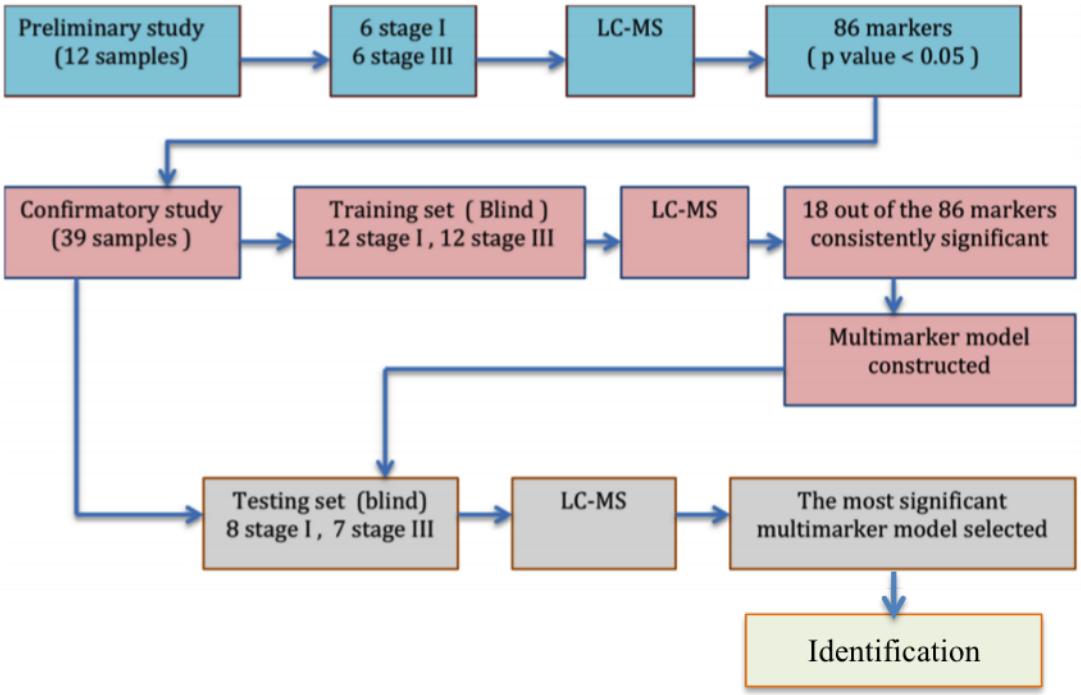


Figure 3.3: Pipeline Used in the Laboratory Experiments.

CHAPTER 4: Results

Preliminary Study: Significant Biomarkers

The goal of our study is to identify novel biomarkers in human serum that would indicate the presence of metastatic disease for patients of Breast Cancer. We used our serum proteomics approach to analyze low abundance low molecular weight (LMW) species, primarily peptides, in serum samples from Breast Cancer patients. The high diversity of these species gives a high probability of identifying biomarkers whose expression is specifically associated with the presence of metastasis. In order to determine differential expression, we compared serum samples from stage I and II Breast Cancer patients with no metastatic disease to patients with stage III disease and documented metastasis. For this preliminary study we used commercially available samples for which there is limited patient information, but we sought to avoid samples where there was co-morbidity. Though the serum samples in each group could not be matched perfectly, the diversity in each group is expected to be sufficient to eliminate many biomarkers that are associated with some unrelated trait in one group.

Serum samples were all processed simultaneously and then frozen prior to analysis using mass spectrometry (MS) instrumentation. Each sample was then separated by reverse phase chromatography (the liquid chromatography or LC step) and subsequent MS analysis, referred to as LC-MS. Separation of individual species in the sample is achieved using the chromatography step, while the subsequent MS analysis provides mass information of separated species, including the relative abundance of each species. For each data set, sample processing was performed to align species between different samples and to determine the relative abundance of each species within that sample. Thus, the relative abundance of individual species in each sample could be directly compared to those in all other samples subjected to LC-MS instrumentation.

With data generated for each sample, we then directly compared the abundance of each species detected across all samples. Our preliminary study revealed the feasibility of identifying candidate markers using this peptidomic approach on BC serum samples. Interestingly, the results from this study showed also that some biomolecules could differentiate the stages of Breast Cancer. About 2500 peaks were visually examined in every time window (total=25,000 peaks in 10 time windows), and their intensities were extracted across all the samples. Students t-tests on the total mean normalized intensities identified 65 candidate biomarkers with statistically significant (p-value less than 0.05) differences between stage I and III samples (Table 4.1) and another 21 biomarkers that had a nearly significant statistical difference ($0.05 <$ p-value < 0.10). Among the significant markers (n=65), 9 markers were up regulated in the metastatic sera, while 56 were unexpectedly and surprisingly down-regulated. According to the proposed stromal model by [47], freely circulating factors (tumor-derived serum factors, or TDSFs) are released by the primary tumor as the tumor progress. They proposed that the TDSFs levels increases with time. Conversely, our study showed that most differentially expresses LMW peptides are down regulated as disease progression occurs. Nonetheless, our preliminary study of small groups reveals that proteomic analysis of highly divers and low abundance species in serum could yield biomarkers or biomarker profiles that indicate the presence of metastatic disease in Breast Cancer patients.

Confirmatory Study

We used data from our preliminary study to evaluate the predictive power of candidate biomarkers for detecting Breast Cancer metastasis. We procured an additional set of 39 samples (24 used for training; and 15 used for testing) of Breast Cancer patients with either stage I/II disease with no metastasis or with stage III disease and documented metastasis. In order to prevent any bias in our evaluation of these samples, we had a third party give new identification numbers to the samples, such that the patient status of each

Table 4.1: Details of the Most Significant Markers in the Initial Study.
Markers are defined by their m/z and charge status. P-value is calculated using the markers intensities following normalization with the overall mean calculated across all the samples. Markers status describes the level of the marker as the disease progress. D: downregulated in stage III; U: upregulated in stage III.

Serial #	**Marker (m/z)**	**Charge (z)**	**P-value**	**Marker Status**
1	403.23	2	0.008	D
2	409.2	2	0.04	D
3	414.23	2	0.003	D
4	415.67	2	0.01	U
5	421.22	2	0.002	D
6	425.25	2	0.002	D
7	430.28	1	0.01	D
8	436.24	2	0.004	D
9	442.23	2	0.001	D
10	443.23	2	0.01	D
11	447.26	2	0.012	D
12	450.3	1	0.007	D
13	451.2	2	0.006	D
14	458.25	2	0.004	D
15	459.24	1	0.03	U
16	464.31	1	0.03	U
17	465.74	2	0.014	D
18	472.3	1	0.007	D
19	473.21	2	0.029	D
20	482.29	1	0.005	D
21	487.25	2	0.003	D
22	488.29	1	0.017	D

23	494.3	1	0.006	D
24	495.23	2	0.014	D
25	497.26	1	0.006	D
26	522.28	1	0.03	D
27	524.26	2	0.01	D
28	537.27	1	0.03	D
29	541.29	1	0.01	D
30	543.31	3	0.019	D
31	546.27	2	0.004	D
32	555.27	1	0.0002	D
33	568.27	2	0.001	D
34	571.25	1	0.0014	D
35	572.63	3	0.008	D
36	575.33	1	0.04	D
37	582.3	2	0.03	U
38	585.3	1	0.006	D
39	590.3	2	0.007	D
40	599.29	1	0.002	D
41	605.28	2	0.03	D
42	629.3	1	0.003	D
43	643.3	1	0.003	D
44	649.32	2	0.01	D
45	656.33	2	0.008	D
46	673.33	1	0.0018	D
47	687.35	1	0.002	D
48	700.36	2	0.003	D
49	709.4	1	0.02	U

50	713.47	1	0.02	U
51	717.35	1	0.002	D
52	721.37	2	0.005	D
53	721.42	1	0.008	U
54	722.37	2	0.002	D
55	731.37	1	0.002	D
56	743.37	2	0.02	D
57	744.3	2	0.04	U
58	747.3	1	0.02	D
59	761.37	1	0.004	D
60	792.58	1	0.019	U
61	819.43	1	0.003	D
62	835.38	1	0.005	D
63	863.43	1	0.002	D
64	879.43	1	0.015	D
65	923.45	1	0.0085	D

samples was blinded during processing, instrumentation, and analysis. We processed all the samples for LC-MS instrumentation in parallel and froze all samples prior to instrumentation. During LC-MS instrumentation we only detected the species that were differentially expressed in metastatic and non-metastatic groups in the preliminary study with at least near statistical significance ($p < 0.10$), yielding a total of 86 species. The peaks representing these species were aligned and the relative abundance determined to allow comparison between each sample. Then, the differential expression between all samples was determined. We applied the Random Forests algorithm (using a two-fold cross validation design) to the 24 training samples. Initially, using a single-marker approach, we confirmed that 12 of the 86 candidate markers provide a relatively high predictive accuracy (AUC > 0.65; see Table 4.2). The best serum biomarker was of m/z 497.26 with an AUC of 0.79, sensitivity of 0.79 and specificity of 0.64 as shown in Table 4.2.

Multi-Marker Model Construction

Given that individual species have limited statistical power to predict the stage of metastasis, we sought to assess how evaluation of multiple markers in a single evaluation would improve predictive power. Using these 18 individual markers we constructed a number of multimarker models using the Random Forests algorithm. We used a forward-selection approach as described in the methods chapter to identify marker combinations that provided the best accuracy in the training set. In short, we randomly combined 2 or more species together into all possible combinations and assessed how the predictive power of the combination improved over any individual species. The best five multimarker panels are shown in Table 4.3, with their respective AUC values.

Multi-Marker Model Validation

With partially developed multi-marker models in hand, we sought to assess their predictive power on a set of samples that had not been previously analyzed. We procured

Table 4.2: List of Markers that Attained an AUC > 0.65 in the Training Data Set. 425.25a is marked with * because there is another marker with m/z 425.25 but different charge state and different elution time.

Serial #	**m/z**	**Sensitivity**	**Specificity**	**AUC**
1	497.26	0.79	0.64	0.79
2	923.45	0.65	0.66	0.72
3	761.37	0.63	0.7	0.7
4	425.25a*	0.66	0.66	0.69
5	722.37	0.58	0.6	0.66
6	585.3	0.59	0.55	0.65
7	458.25	0.55	0.64	0.65
8	747.3	0.67	0.63	0.63
9	555.27	0.63	0.6	0.63
10	442.23	0.59	0.7	0.62
11	546.27	0.56	0.56	0.62
12	879.43	0.5	0.56	0.61

Table 4.3: Performance of the Top 5 Multimarker Models in Training Data Set.

m/z	**AUC**
497.26 585.3 722.37 923.45	0.915
425.25a 497.26 585.3 722.37 923.45	0.919
458.25 497.26 585.3 722.37 923.45	0.913
497.26 585.3 722.37 761.33 923.45	0.912
425.25a 497.26 585.3 722.37 761.37 923.45	0.915

15 serum samples from the confirmatory study (the test set). We got relatively equal number of both stages (8 of stage I/II serum and 7 of stage III). The stage of the serum sample was blinded by an arbitrator who is not involved in the study. All samples were processed in parallel in the same day. They were run in the cLC-MS in a random manner. Evaluation of the predictive power of the best 5 Multi-marker models on the 15 blinded samples in the testing set is outlined in Table 4.4. As might be expected, accuracy levels on the testing set were lower than were obtained with the training set. However, the multimarker models still attain accuracy levels that indicate promise for further evaluation as predictive biomarkers in a clinical setting. For example, the best-performing model which consisted of markers with m/z 497.26; z = +1, 458.25; z = +2, m/z 585.3; z = +1, m/z 722; z = +2 and m/z 923.45; z= +1 attained an AUC of 0.84, a sensitivity of 43% and a specificity of 88% as shown in Figure 4.1. The box plots for each biomarker in this model across the confirmatory dataset are plotted below (Figures 4.2–4.6). In summary, development of the multimarker models described here have been validated with a small blinded test set and are ready for a more robust validation with a very large sample set.

Biomarker Identification

We sought to identify the species found in peaks used in the multuimarker models. To do this, individual peaks are subjected to LC-MS under conditions where the species is fragmented into smaller species whose individual masses are measured by the MS instrument. For peptides, fragmentation usually occurs between individual amino acids and allow for the determination of the precise sequence. This approach is particularly challenging for low abundance species. A species that is low in abundance could by suppressed by other species and not be picked by the instrument for fragmentation. We used a concentrated serum for those species that appear low in abundance in the sample. Furthermore, low MW species especially those with +1 charge will provide a very short combination of amino acid that could be shared by several parent proteins. However, they could give hints

Table 4.4: Performance of the Top 5 Multimarker Models in Testing Data Set.

m/z	**AUC**
497.26, 585.3, 722.37, 923.45	0.84
425.25a, 497.26, 585.3, 722.37, 923.45	0.76
458.25, 497.26, 585.3, 722.37, 923.45	0.804
497.26, 585.3, 722.37, 761.37, 923.45	0.77
425.25a, 497.26, 585.3, 722.37, 761.33, 923.45	0.77

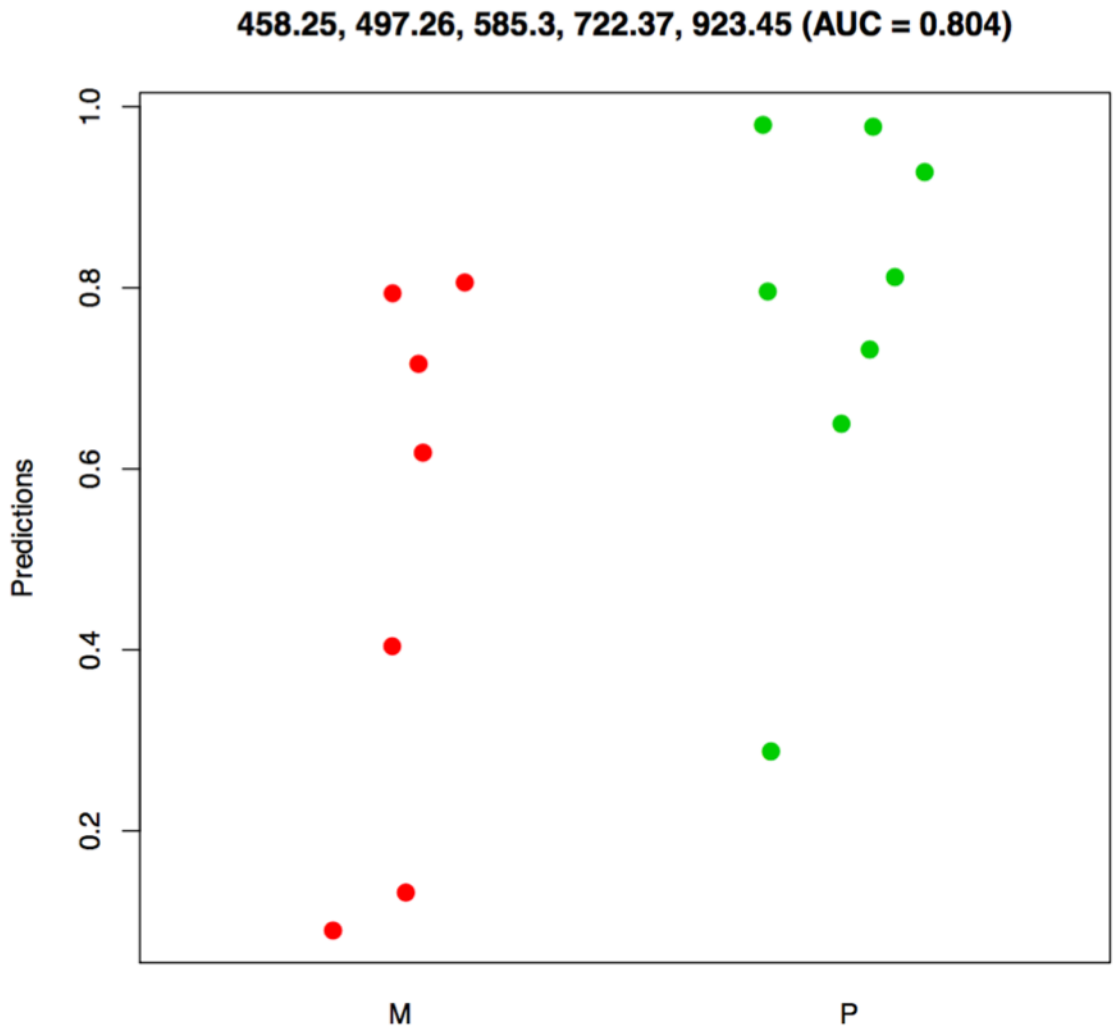


Figure 4.1: Performance of the Top 5 Multimarker Models in Testing Data Set. M: Metastatic, P: Pre-metastatic.

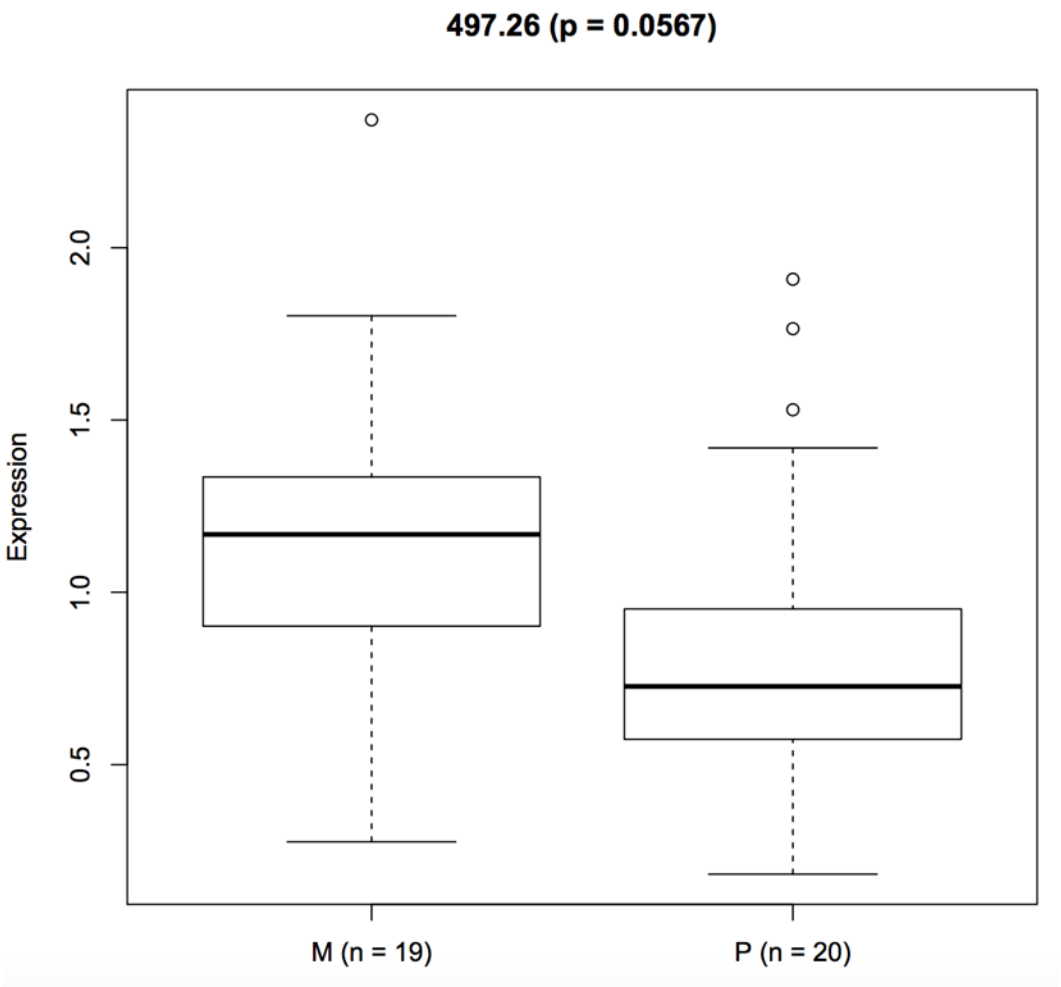


Figure 4.2: Box Plot of Biomarker with m/z 497.26

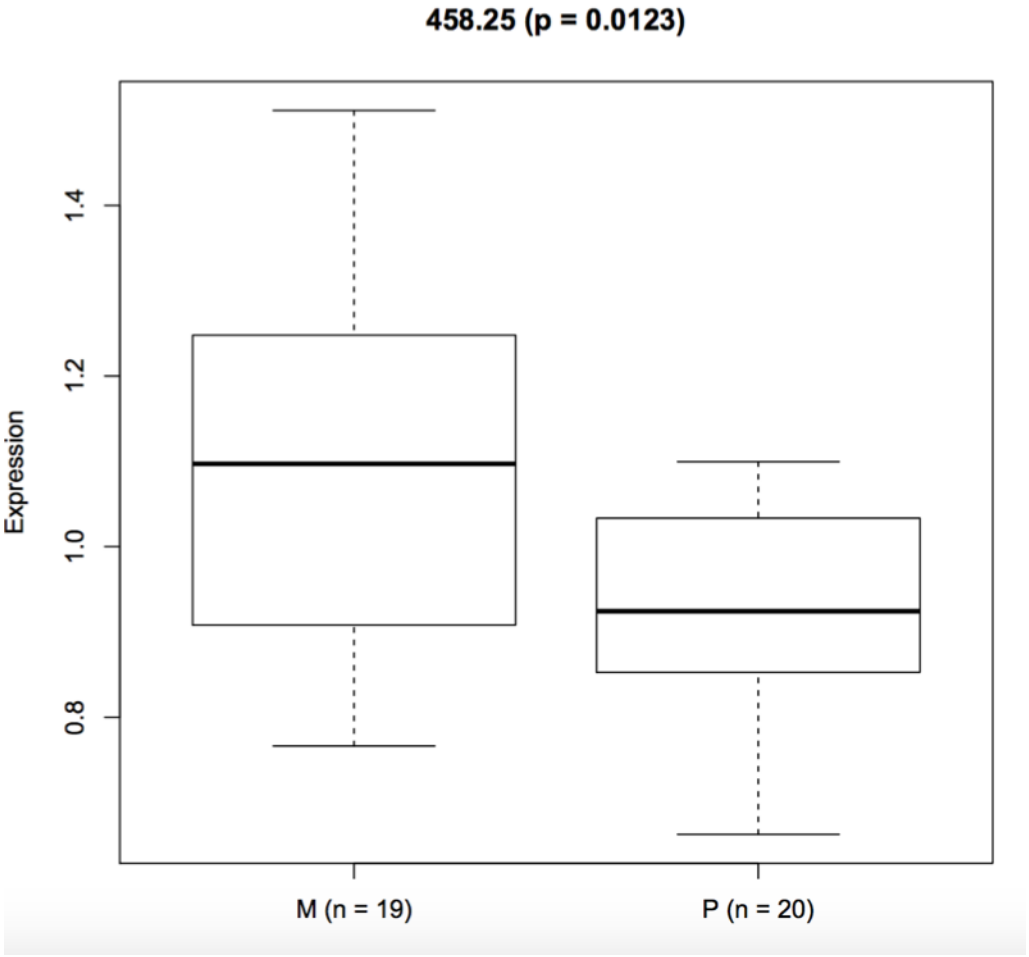


Figure 4.3: Box Plot of Biomarker with m/z 458.25

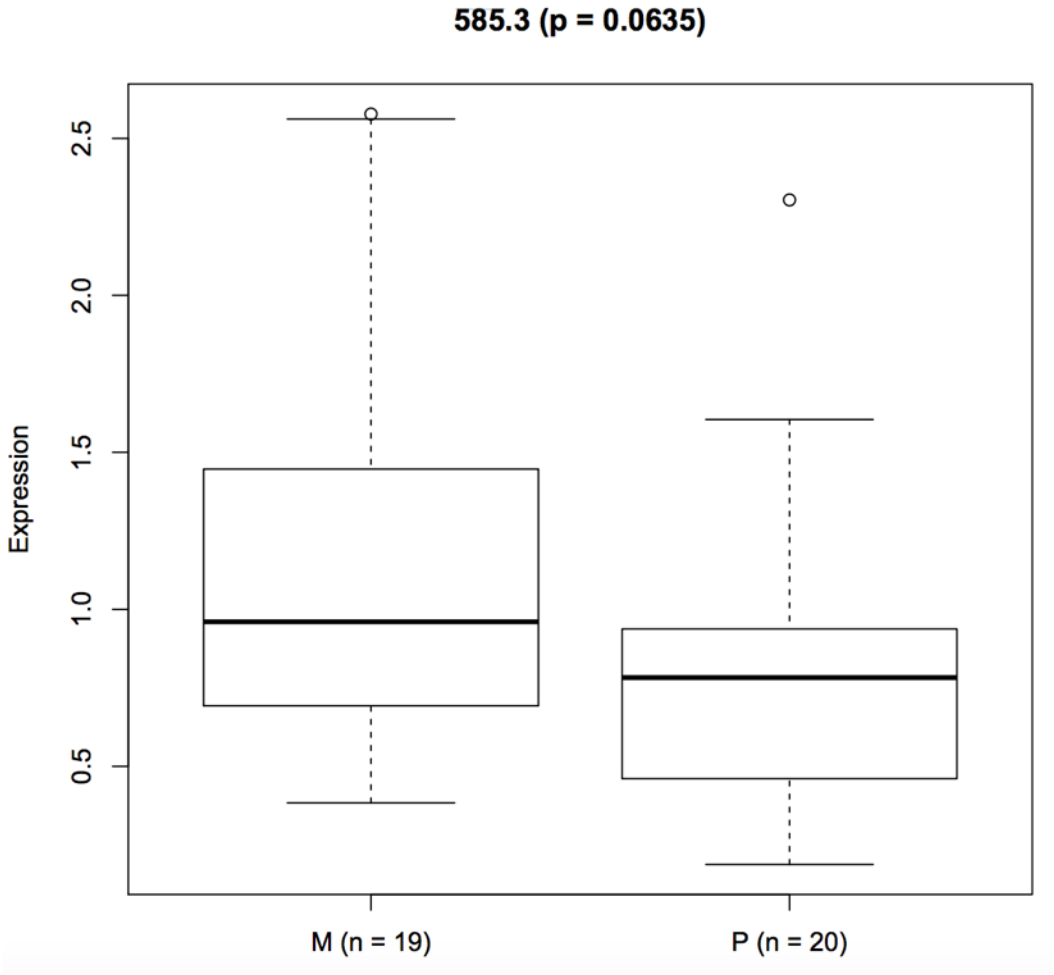


Figure 4.4: Box Plot of Biomarker with m/z 585.28

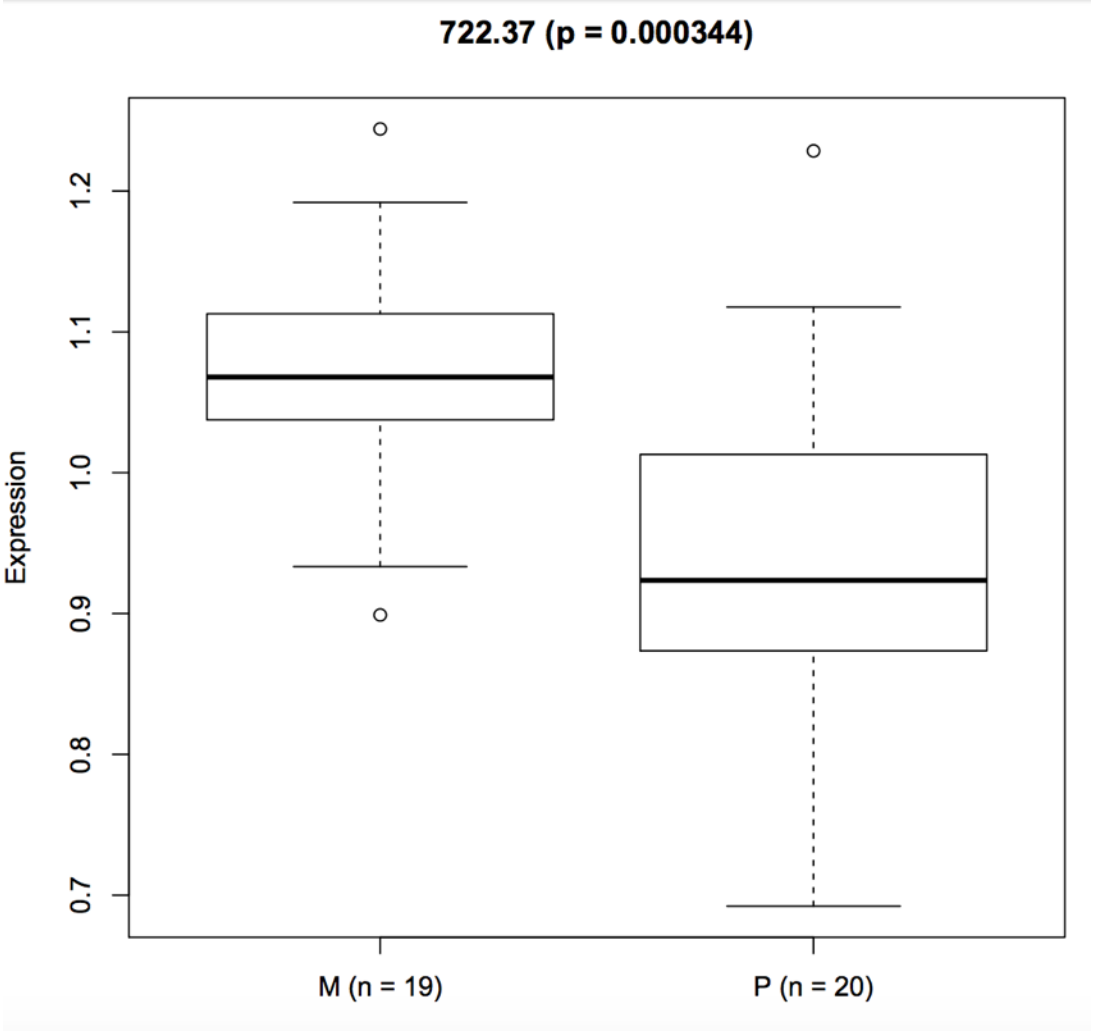


Figure 4.5: Box Plot of Biomarker with m/z 722.37

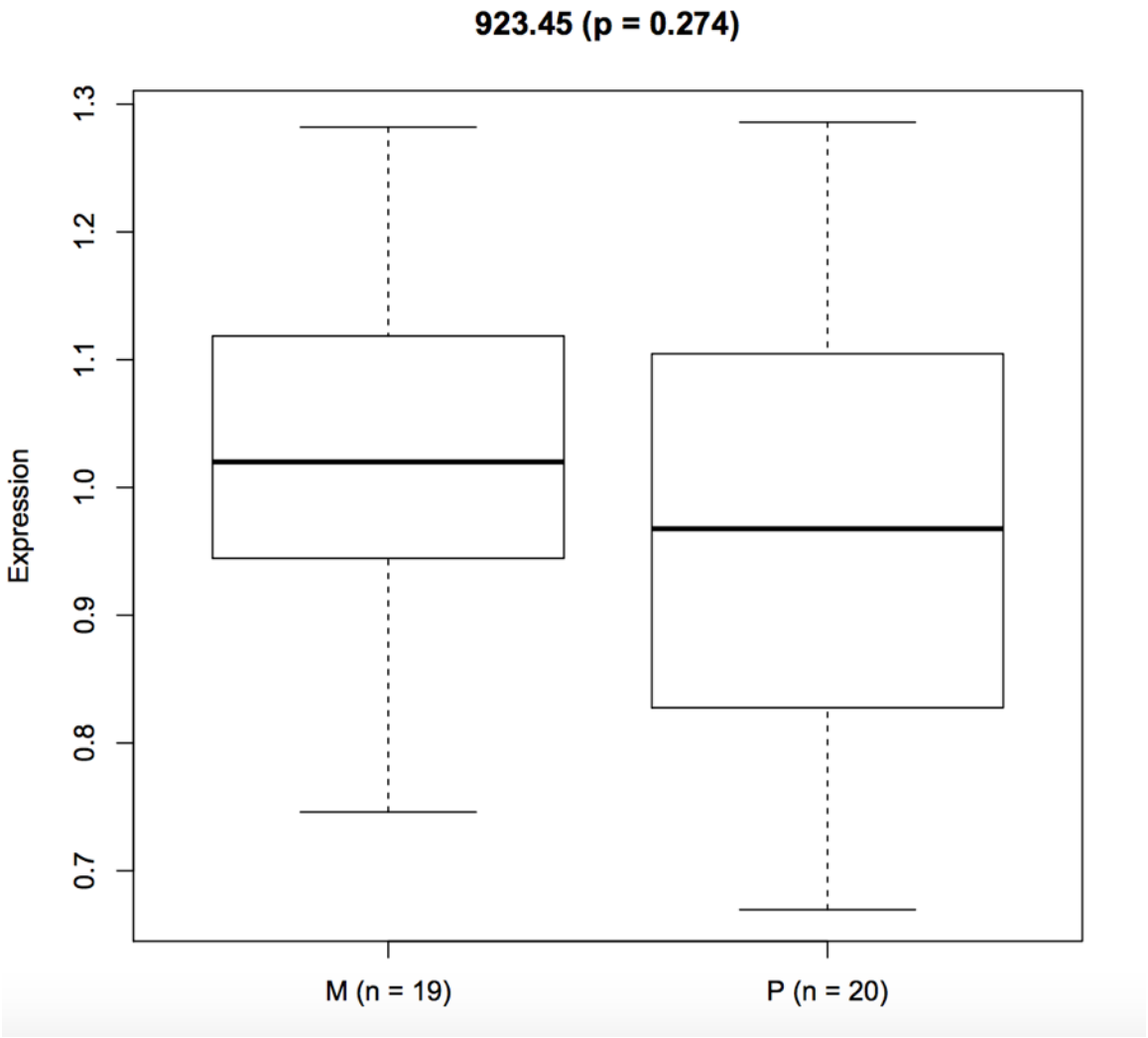


Figure 4.6: Box Plot of Biomarker with m/z 923.45

to the identity of the parent protein and therefore exclude other possibilities. Most of the biomarkers that built the multimarkers models were having an adjacent overlapped peak. Therefore, the fragmentation pattern of these biomarkers might contain fragments of both peaks that were hard for the system to distinguish. We tried to fragment the biomarker m/z 497.26 which was showing a consistent significance across the initial and confirmatory data set. Its mass spectrum is showing a overlapped peak with m/z 497.24 (Figure 4.7).

Surprisingly, its fragmentation profile did not appear to be consistent with the species being a peptide because there was no evidence of immonium ions and no fragments occurred at intervals consistent with amino acids, with or without modifications. Instead, the fragmentation spectrum of this biomarker was consistent with choline containing lipid species, primarily due to the presence of a high abundant peak at m/z 184.07 that indicates the presence of a phosphocholine head group. We tried to fragment this biomarker with collision energies that range from 15 to 35 V and we noticed that the fragmentation spectrum of this particular species yielded a consistent fragmentation profiles with different intensities. In addition, the m/z 184.07 fragment was always observed as the collision energy changed. In general, the fragmentation profile of phosphocholine lipid species such as phosphatidylcholine (PC) , sphyngomyeline (SM) and lyso PC display the product ion with either m/z 59, 104 or 184 corresponding to trimethylamine, choline and phosphocholine moieties, respectively. The m/z 104 and m/z 184 fragments are displayed in the m/z 497.27 fragmentation spectra (Figure 4.8). The complete structure of the phosphocholine species could not be determined but the nitrogen rule can suggest what species it is. The nitrogen rule states that, if M^+H^+ has an even m/z (i.e., having odd neutral mass), then it should correspond to the presence of odd number of nitrogen atoms. Conversely, if M^+H^+ has an odd m/z (i.e., having even neutral mass) then it should represent even numbered nitrogen atom containing species. In other words, protonated PC molecules appear at even m/z values, whereas protonated molecules of SM exhibit odd m/z values. This is due to the presence of an additional nitrogen atom in SM. The appli-

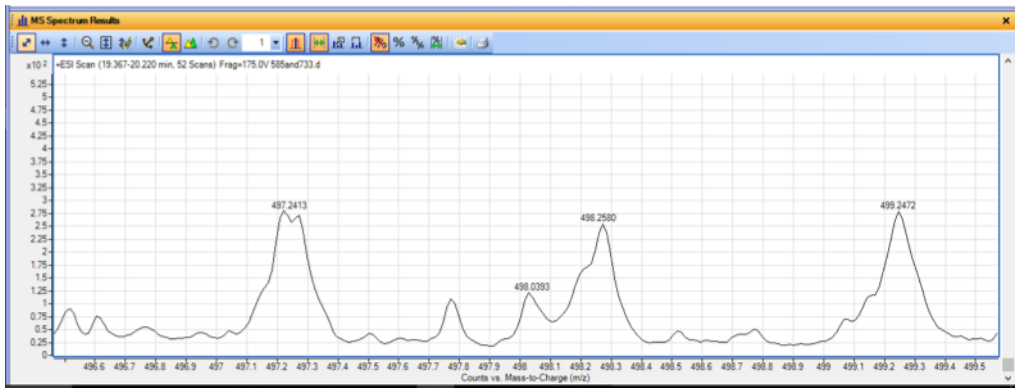


Figure 4.7: Overlapping Peaks at m/z 497.27.

cation of this rule suggests that 497.27 is a SM species of lipid. Further supporting this, we observed a peak of m/z 69 that is indicative of sphyngomyelin lipid group. Taken together, this biomarker fragmentation profile indicates that this critical biomarker is a SM lipid species. Alternately, the consistent presence of the fragment with m/z 479 indicates water loss is occurring in this molecule. Therefore, this SM species could be oxidated. On the other hand, the product ion at m/z 147 corresponds to sodiated five-member cyclophosphane [184], which is displayed in the m/z 497.27 fragmentation spectra. Therefore, this biomarker could also be a sodiated SM. In fact, fragment ion of m/z 147 has been seen in the fragmentation profile of non-sodiated SM, which results from some overlap.

Despite the ability to identify this molecule as an oxidated SM, we can not be sure that it is corresponding to m/z 497.27 because of the co-eluting peak with m/z 497.24 that is more prominent as shown in Figure 4.7. Unfortunately, all the other biomarkers that built the multimarker model were having an overlapping peak that is interfering with the fragmentation profile of the biomarker of interest (Figures 4.9–4.13). Therefore, they did not yield interpretable MS/MS fragmentation spectra. There are several factors contributed to the difficulty in identifying these other biomarkers using the fragmentation approach. The use of the microcapillary column for peptide separation, which is packed with POROS R1 slurry of 10 µm particle size could be one of the main factor because it gave wide peaks and overlapped spectra. Other factors that could be responsible for the low resolution will be discussed in the discussion.

We tried a new run where we changed the flow rate in the experiment from 5.0 µL/min to 10 µL/min and we were able to get a better resolution for the biomarker with m/z 761.38. Fragmentation of this biomarkers was possible because of the absence of co-eluting peaks.

The fragmentation profile of the marker with m/z 761.38 was showing immonium ions of some amino acids. Through scanning of the fragmentation profile we could see

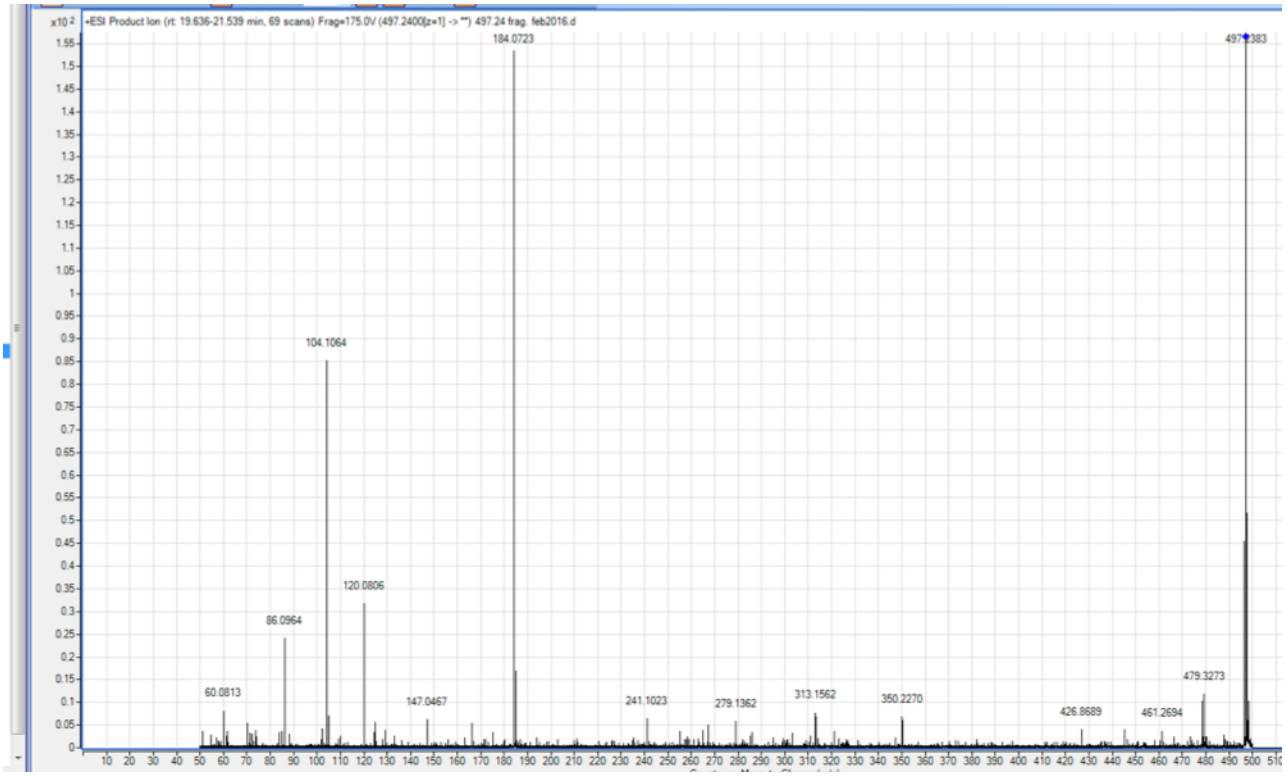


Figure 4.8: The Fragmentation Spectra of Biomarker with m/z 497.27.

some mass differences that correspond to the mass of amino acids and the proposed sequence is DLVPGNF, see Figure 4.14.

Doing a blast search for this sequence showed some suggested proteins that have DLVPGNF partial sequence. Using Fragment Ion Formula Calculator, we got a list of expected b and y ions series that should be seen in the ms/ms spectra of m/z 761.38 according to the proposed protein. It turned out to be a fibrinogen alpha chain (FAC) isomer 2 as the proposed b and y ions corresponding to the FAC by Fragment ion formula calculator were all present in the m/z 761.38 fragmentation profile, see Figure 4.15.

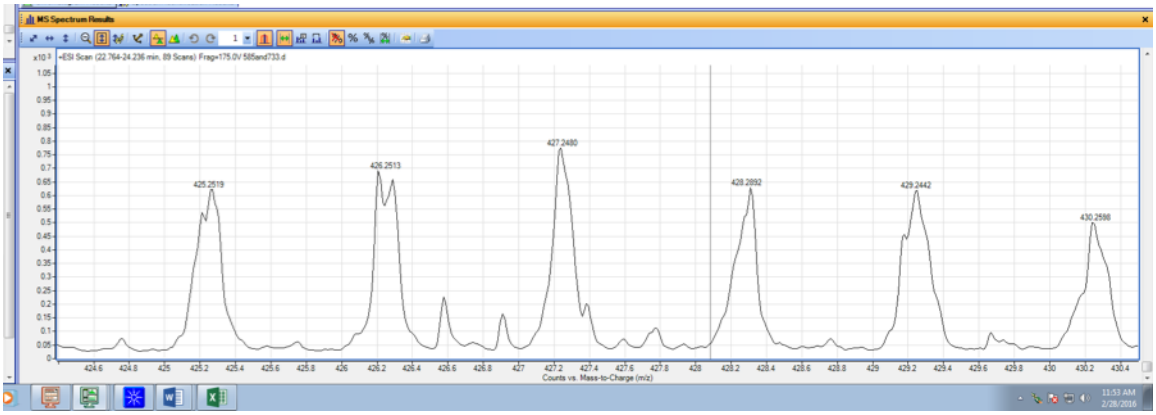


Figure 4.9: Overlapping Peaks at m/z 425.25

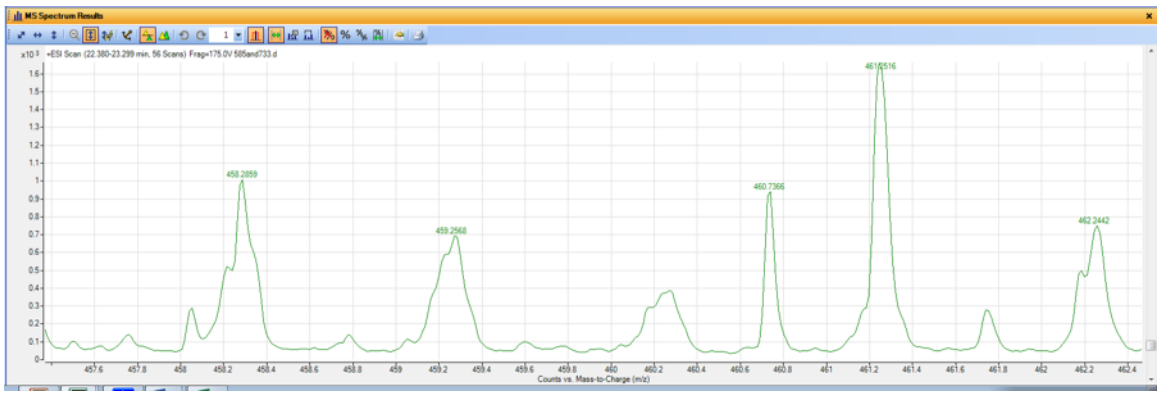


Figure 4.10: Overlapping Peaks at m/z 458.25

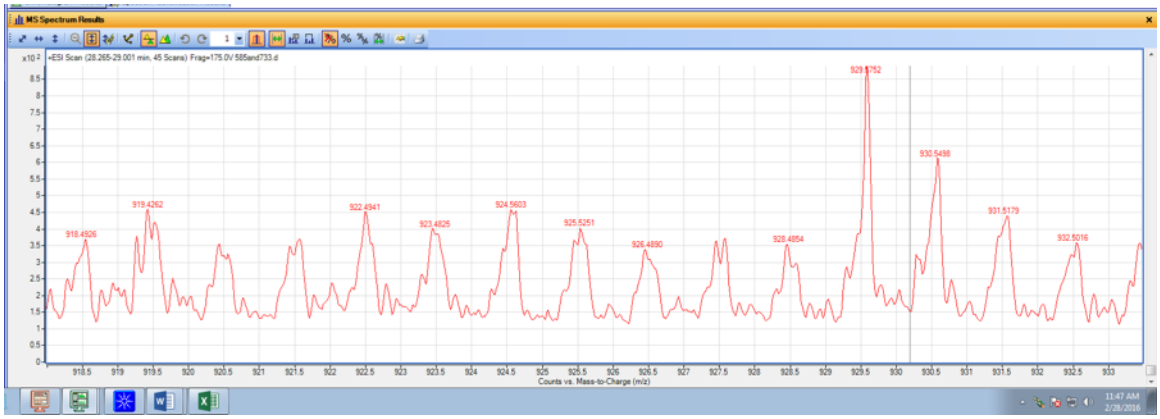


Figure 4.11: Overlapping Peaks at m/z 923.45

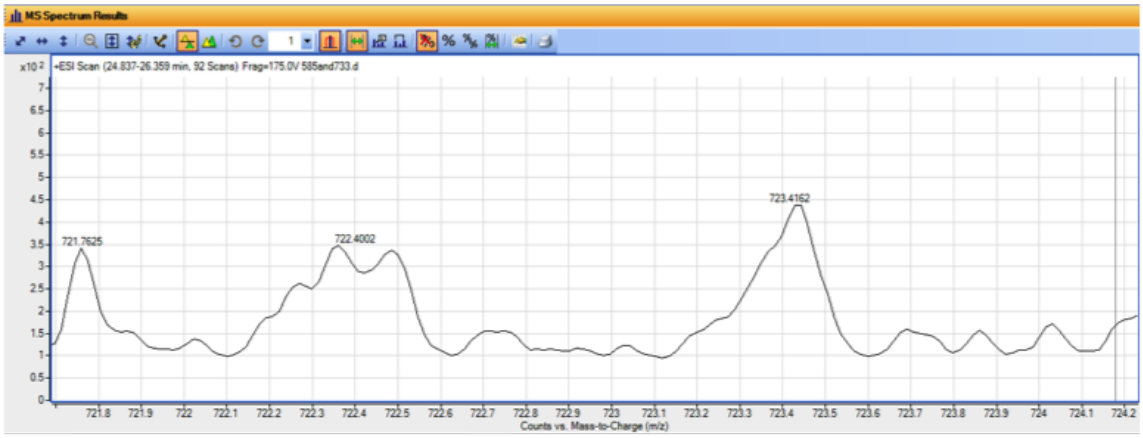


Figure 4.12: Overlapping Peaks at m/z 722.37

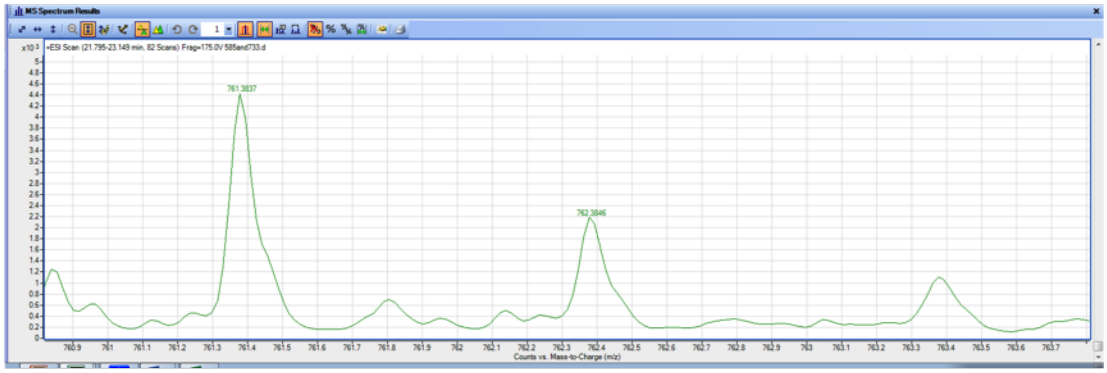


Figure 4.13: Overlapping Peaks at m/z 761.38

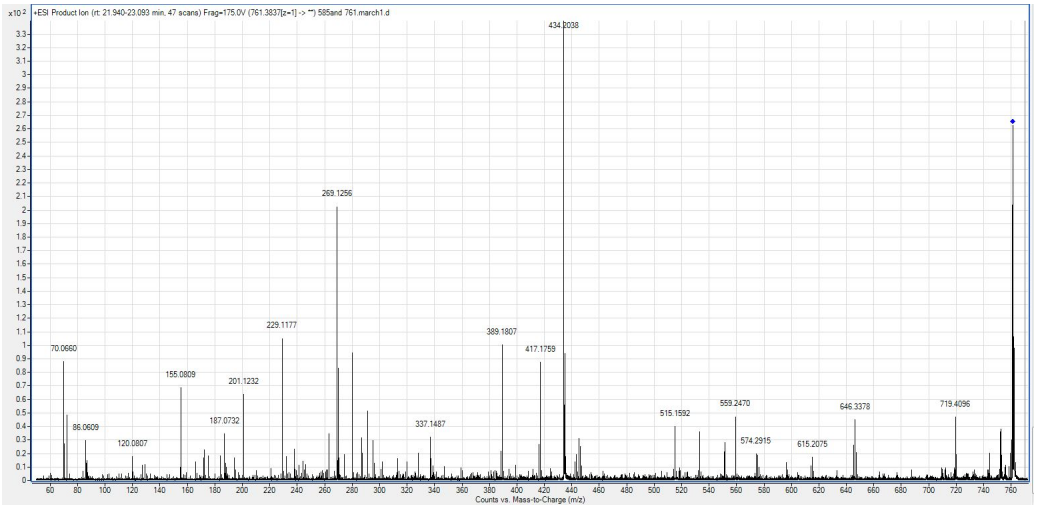


Figure 4.14: Ms/Ms of m/z 761.38

Fragment Ion Calculator Results

Sequence: **DLVPGNF**, pI: **3.79981**

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
D	1	116.03481	761.38342	7
L	2	229.11888	646.35648	6
V	3	328.18729	533.27242	5
P	4	425.24005	434.20400	4
G	5	482.26152	337.15124	3
N	6	596.30444	280.12978	2
F	7	743.37286	166.08685	1

Figure 4.15: Fragment Ion Calculator Result

CHAPTER 5: Discussion

The advancement in early Breast Cancer detection led to a 16% increase in early diagnoses, while the mortality rate of BC remained unchanged [5]. Metastasis is the main cause of death among Breast Cancer patients. Therefore, determining the tumor potential to spread before the onset of metastasis will pave the road toward personalized therapy and save many patient from being overtreated and improve their quality of life [185].

Breast Cancer metastasis is currently monitored by radiological imaging that suffers from detection limitations [9]. While tumor metastatic potential is largely determined by evaluating the tumor size, grade and involvement of regional lymph nodes, molecular biomarkers are currently used in this evaluation. The complementary effect of the biological markers such as estrogen and progesterone hormone receptors, HER-2, and plasminogen activator inhibitor 1 should not be underestimated [9, 18]. It is worth mentioning that all of these prognostic factors rely on tissue specimens from primary or secondary site, obtained either by biopsy or surgical resection. Therefore, they can not by definition be used for a inexpensive, reproducible and noninvasive screening assay [186]. Ca 15-3 and CEA are the two serum markers that are widely used to predict recurrence and metastatic potential of breast, ovarian, and uterine cancers. [18]. These serum markers suffer from low sensitivity and specificity that necessitates the search for a more powerful prognostic and metastasis predictive approach [187]. There is a soaring need for more robust markers that have diagnostic and predictive power across Breast Cancer subtypes [11] and circulating markers are more clinically desirable [188].

The rapid advancement of high throughput proteomics in recent decades have not uncovered any novel serum biomarkers that can displace currently used tissue or replace existing low-predictive serum markers. This is a result of the inherent limitation of the conventional 'bottom-up' approach and the complexity of human serum. In addition, subtypes and subpopulation heterogeneities of tumors complicates markers discovery, which

could be overcome by combining markers, as proposed by some researchers [189]. Our goal was to evaluate the applicability of a proteomic approach in BC serum samples and its quantitative power to differentiate between metastatic and non-metastatic Breast Cancer. The proteomic approach we have applied in this project showed success in previous studies done on preterm birth [190], preeclampsia [173, 191] and Alzheimer's disease [192]. The uniqueness of this approach is its capability to detect biomarkers from an array of low abundance but highly divers species found in different human biospecimens. This study demonstrated that this 'top-down' approach is effective in identifying differentially expressed biomarkers from low abundance low molecular weight (LMW) molecules in serum.

We were able to detect biomolecules in serum samples that significantly differentiate between the two serum sample groups. Surprisingly, the majority of identified molecules were down-regulated in stage III sera, rather than upregaultes, as expected. Previously identified circulating peptides were mostly up-regulated with disease progression. Lv et. al. studied the role of circulating cytokine in BC metastasis and found multiple cytokines that are positively associated with BC progression. However, they also found two serum cytokines, MCP-1 and IP- 10, that were down-regulated with disease progression. Their decreased expression levels were significantly and inversely correlated with patients who had more positive lymph nodes[148].

Downregulated Metastatic Biomolecules Identified in Virto

There are other peptides that are found to be downregulated in BC tissues and cell lines studies. For example, RAB1B expression was low in highly metastatic cells and could be considered as a metastasis suppressor in triple negative BC (TNBC) [137]. In another study, the migration and invasion of a highly metastatic BC cell line were dramatically reduced by RhoGDIα upregulation [138]. Furthermore, CD59 and CSPG4 were found to be inversely correlated with BC metastasis [141]. Li et. al. looked at the BC cell line secretome using a bottom up approach and all the identified peptides were upregulated

in the metastatic cell line secretome compared to the non-metastatic ones. Mesothelin (MSLN) was the only peptide found to be inversely correlated with tumor aggressiveness [11]. LIFR is an example of a metastatic suppressor that is found to be down-regulated in Breast Cancer tissue [193]. As an alternate theory to explain the large number of peptides whose expression is down-regulated in metastatic patients, perhaps the presence of metastatic cells results in the depletion of specific peptides and proteins from serum. Torosian detected some circulating peptides that were suppressing tumor metastasis which could not be synthesized by tumor-bearing animals on protein depleted diet due to absence of amino acids subunits [194].

Metastatic Suppressors

Metastasis suppressors are molecules that prevent the dissemination and growth of tumor cells in the secondary organ but has no or minimal effect on the primary tumor [195]. The first identified metastatic suppressor was Nm23 where it provided functional evidence for the existence of specific genes that control metastasis [32]. Today, more than thirty metastasis suppressors have been identified [196] that vary widely in term of cellular localization where some are produced intracellular and some are in the extracellular matrix. They vary in their mechanism of action, some promote cell-to-cell adhesion that will slow cell migration out of the primary tumor such as E-cadherin. Other metastasis suppressors act by inhibiting cell motility and invasion such as Nm23, tissue inhibitors of metalloproteinases (TIMP), SseCKS, caspase-8, BRMS1, KAI1 inhibit metastasis by reducing cells survival while they are in the way toward the secondary site. KISS1, MKK4, p38, MKK7 act on the disseminated cells at the secondary site to prevent their proliferation [196, 195, 197, 198].

Metastatic Suppressor Genes

It is likely that the discovered metastatic suppressor genes did not provide a complete understanding of the mechanism of metastatic and its suppression [197]. That is because the protein products of these genes varies and each will have a different mechanism of action. For example, serum kisspeptin levels (product of metastatic suppressor gene KISS1) were significantly lower in the infertile male compared to fertile male who have no malignancy [199]. Therefore, identifying metastatic suppressor proteins will be more functional and applicable. Since the downregulated biomarkers that we detected were associated with invasive stage of BC, further understanding of their identities, origin, and fate during cancer progression is clearly needed.

Peak Overlapping in Mass Spectroscopy

Mass spectroscopy based serum proteomic studies are prone to show ion suppression due to the variation of the serum peptide MW despite the use of acetonitrile precipitation. Isobaric species tend to co-elute and provide a convoluted mass spectrum. This could affect the quantitation of the co-eluted species and more importantly interfere with fragmentation profile of the peak of interest because the eluted peaks will be fragmented together. The identification of the biomarker that built the multimarker model was challenging because of the existence of overlapping peaks. Several technical parameters of the chromatographic methods could contribute to these findings which can be summarized in the resolution equation

$$R_s = 1/4\ [k/(1+k)]\ (1-\alpha)\ (N)^{1/2}$$

where R_s is the resolution of two closely eluting peaks, k represents retention; α is the ratio of the retention for closely eluting peaks; and N represent column efficiency (plate number). These three parameters need to be adjusted in order to get a better peak

spacing. For example, increasing column efficiency could solve moderately overlapped peaks. Column efficiency could be enhanced by increasing the column plate number that is a achieved by increasing the column length or the use of smaller particles. Columns with smaller particle sizes results in a higher plate numbers which subsequently give sharper peaks. The size of the particles also depends on the size of the studied molecules because large MW species dont show a good separation in the small pore size packings [200]. Due to financial constraints, we could not replace the column we implemented in our studies with a longer column or using different packings. Another way to improve column efficiency is to elevate column temperature. Higher column temperatures lead to reduce mobile phase viscosity that subsequently increase column efficiency due to increase solvent diffusion [200]. Since we are dealing with proteins which are thermolabile, increasing column temperature might degrade them.

Changing the strength of the mobile phase has been found to solve minor overlaps. What could be seen in our mass spectra is that we have major overlap that would be unlikely to be solved by this intervention. Additionally, it has been shown that changing the mobile phase will not help to solve the co-elution if the sample has a large number of components or if the co-eluted peaks are isomers that crowd the chromatograph which is the case with human serum samples that we study [201]. In fact, we did several runs with different mobile phase gradient and were unlucky getting the overlap solved.

Flow rate is another factor that could exert a change in the peak signals as increasing the flow rate results in large signals and therefore a greater amount of sample mass is reaching the instrument per unit. We noticed biomarker m/z 761.38 was represented with a very clear peak when we increased the flow rate from 5 µL/minutes to 10 µL/minutes.

Sphingomyelin Species are Potential Biomarkers for Cancer

It is worth mentioning here that the fragmentation of one of the best markers in our study was somehow consistent with oxidated sphingomyelin (SM). SM is a polar lipid

which is composed of an alcohol portion (sphingosine), a long-chain fatty acid that is connected by an amide bond to the amino group and a phosphorylcholine head group. The combination of a fatty acid and sphingosine is called ceramide, see Figure 5.1.

Sphingomyelins are one of the major membrane phospholipids that are mainly localized to the outer leaflet of the plasma membrane. Several studies link SM to multiple cellular pathways such as cell migration, cell proliferation, apoptosis, autophagy and growth arrest [202]. In fact and to be more specific, it is SM metabolites, such as sphingosine, sphingosine 1-phosphate (S1P), and ceramide, that control these pathways. Ceramide has a pro-apoptotic property while S1P has anti-apoptotic property and it induces cell proliferation and growth [184]. The dynamic balance between these two SM metabolites usually determines cell behavior.

Cancer cells exhibit altered metabolic activity that aims to maintain the rapid proliferation of cancer cells [203]. SM is one of the lipids that is highly involved in cancer development and progression. SM levels are found to be significantly higher in highly metastatic cancer cells compared to those in less metastatic cells [204]. Unexpectedly, in our study, we found that this specific SM lipid is down-regulated in metastatic serum samples. We propose four possible explanations for the reduction of serum SM in the metastatic stage: 1) the up-regulation of Ceramide SM metabolites, 2) up-regulation of sphingosine-1-phosphate SM metabolites, 3) the high prevalence of lipid rafts in cancer and 4) the production of extracellular membrane vesicles from tumor cells.

Upregulation of Ceramide in Cancer

Ceramide is a sphingolipid with sphingosine backbone that is generated de novo by condensation of serine and palmitoyl-CoA or through hydrolysis of sphingomyelin by sphingomyelinases, known as the "sphingomyelin cycle". Ceramide is a pro-apoptotic molecule; it induces cell death and arrests growth. Doria et al. found that regular intake of SM was associated with a reduction in colon cancer in animals [205]. Researchers proposed that

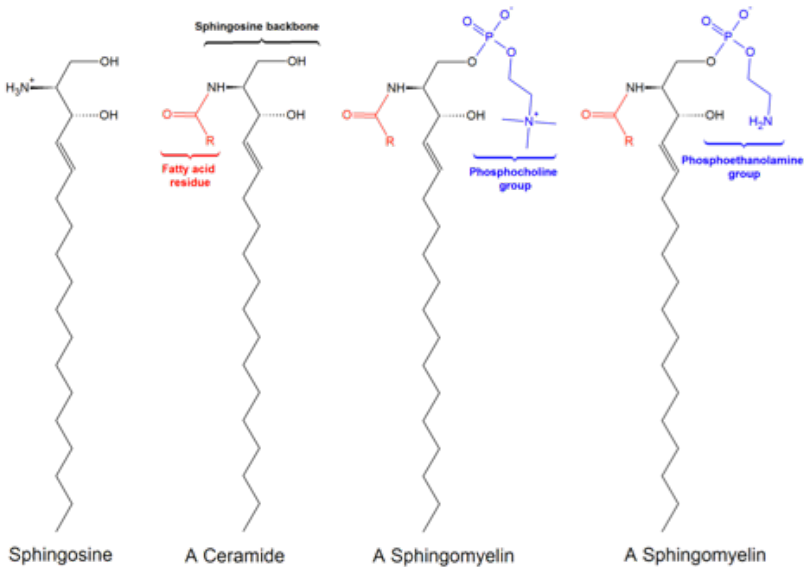


Figure 5.1: Chemical Structure of Sphingomyelin.
SM is comprised of sphingosine backbone (mainly C-18). (A) A long chain fatty acid attached to sphingosine through amide linkage forms ceramide. (B) SM is produced by replacement of hydrogen group of ceramide (H*) with various functional head groups phosphocholine in (C) and phosphor-ethanolamine in (D).

one possible mechanism for this inhibition is through the generation of ceramide from SM hydrolysis, which will exert an apoptotic effect on colon cancer cells [206].

Several studies revealed high expression of different species of ceramide in different cancers, such as nodal positive pancreatic cancer, squamous cell carcinoma of the head and neck, BC and prostate cancer. Theoretically, ceramide should be down-regulated in cancer [206]. The up-regulation of ceramide was explained by the presence of several distinct species of ceramides, which differ according to length of fatty acid chain, saturation level, and sites of double bonds. Different ceramide species also differ in their functionality. For example, the accumulation of C16-ceramides found to have a proliferative properties whereas C18-ceramides have apoptotic/growth arresting properties. Thus, up-regulation of some ceramides could be associated with cancer progression. The generation of such species necessitates increased SM hydrolysis, which could explain low levels of serum SM in metastatic patients.

Upregulation of Sphingosine-1-phosphate (S1P)

S1P is produced by phosphorylation of sphingosine by sphingosine kinases, see Figure 5.2. It can be produced either in the inner part of the plasma membrane and then transported elsewhere or in the plasma. Plasma production may occur by either the same biochemical steps as occur in the membranes of cells [207] or by the hydrolysis of sphingosylphosphorylcholine by the enzyme autotaxin [208]. S1P is well known to induce cell migration, proliferation, invasion, and angiogenesis [209, 210]. Analysis of plasma from nodal positive pancreatic cancer patients reveals high expression of S1P compared to nodal negative pancreatic cancer patients [211]. Additionally, it is up-regulated in BC and is associated with poor prognosis and resistance to chemotherapy [212]. Further, Ogretmen et. al. found that disseminated cancer cells into the blood stream induce the elevation of serum S1P. They found that systemic S1P generated in circulation, but not primary

tumor-derived S1P, controls cancer metastasis. Clearly serum SM is utilized extensively in order to produce more S1P, which in turn drives cell proliferation and migration.

Lipid Raft-associated SM

Lipid rafts are specialized cholesterol-enriched microdomains of the plasma membrane that are formed by the assembly of cholesterol, sphingolipids and certain types of proteins. They have been implicated to play a role in different cellular pathways needed for cell survival, proliferation, and migration [213, 214, 215, 216]. The composition of lipid rafts differs from that of the surrounding bilayer membrane. Studies show that they contain 3-5 fold more cholesterol than the plasma membrane. The sphingomyelin content of the lipid raft is 50% more than that of the adjacent part of the plasma membrane. Since rapidly proliferating tumor cells have more rafts and require cholesterol for new membrane synthesis, their need for SM and cholesterol is highly elevated [213]. In fact, researchers found that patients with advanced cancer have hypocholesterolemia that is associated with hyposphingomyelinemia [217].

Extracellular Membrane Vesicles from Tumor Cells (EMVTCs)

Studies showed that the level of extracellular membrane vesicles are elevated by five times in cancer patients compared to levels in normal patients [218]. Their number in the blood is positively correlated to the invasiveness of the tumor [219, 220]. EMVTCs are believed to have angiogenic activity, which can promote the growth of disseminated cancer cells. These EMVTCs are enriched with SM, which is considered the active component that stimulates angiogenesis. As the tumor progress, angiogenesis increases and could deplete the plasma of EMVTCs that contain SM. It is worth mentioning that cancer tissues need more cholesterol and SM than they are capable of generating by their own lipid synthesis pathways. In fact adipocytes were found to enhance cancer cell migration and invasion through the continuous supply of lipids, which cancer cells use for structural assembly

and energy for rapid growth [221]. Several studies showed that cancer mortality increases if the patient has low plasma cholesterol [222]. Whether low cholesterol drives cancer aggressiveness or the progression of cancer results in low plasma cholesterol is unclear and needs further study.

Oxydated Sphingomyelin in Cancer

It is well known that cancer cells experience a metabolic shift from oxidative phosphorylation to aerobic glycolysis, which is known as Warburg effect [223]. They use different metabolic pathways to meet their increased energy demands. Lipid metabolism is a vital source of energy for cancer cells and results in changes in lipid synthesis, lipid degradation and catabolism, and fatty acid (FA) oxidation [224]. It has been estimated that almost all tumors will gain lipogenesis capability, meaning cancer cell are able to the synthesize fatty acids de novo in a rate comparable to liver cells. The increase in FA oxidization in cancer cells could explain the detection of oxidated SM in the serum of BC patients.

Despite the evidence linking SM to cancer initiation and progression, it is too early to speculate as to the physiological significance of downregulation of circulating SM species as a predictor of tumor progression. However, coupling this marker with other clinicopathological parameters might increase the predictive power of these combined markers.

Fibrinogen Physiological Function

The fragmentation profile of the biomarker with m/z 761.38 was consistent with fibrinogen α chain (FAC). FAC is one of the chains that build up fibrinogen molecule. Fibrinogen is a plasma protein produced by hepatocyte with a molecular weight of 340-kDa. It consists of two pairs of three polypeptide chains α, β, and γ that are connected by disulfide bonds. It is involved in the last phase of the coagulation process. It circulates in the blood in insoluble form and need to be activated into its active form by the protease thrombin. Thrombin cleaves four specific Arg-Gly bonds at the N termini of both the α

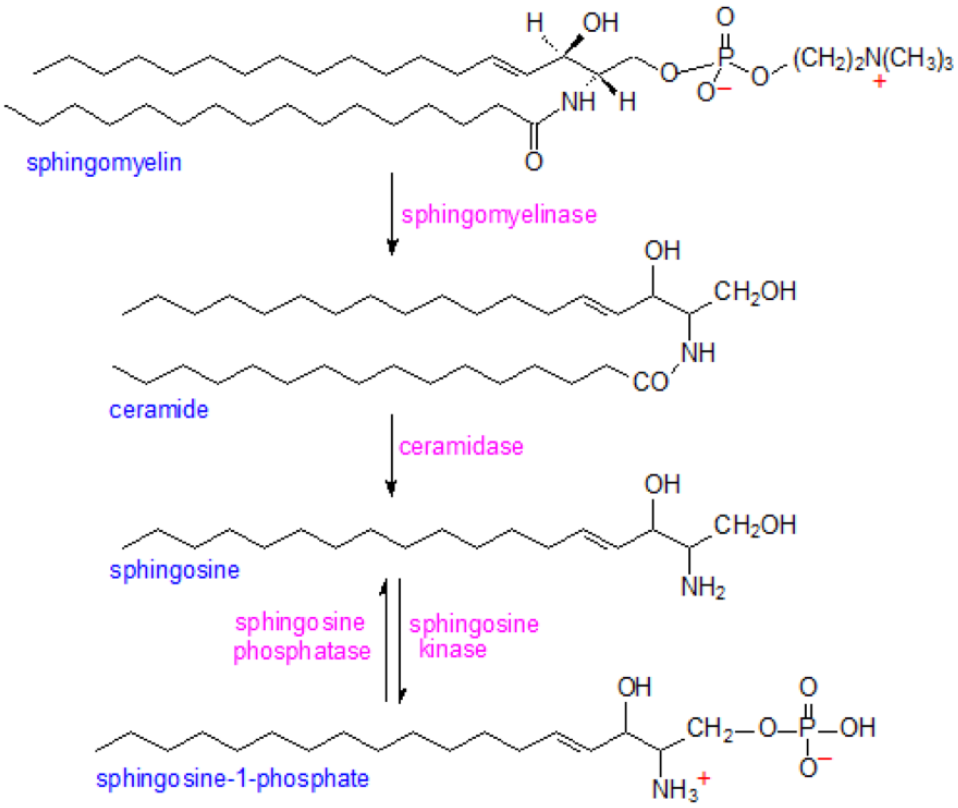


Figure 5.2: Sphingomyeline Hydrolysis.

and β chains, releasing fibrinopeptides A (FpA) and B (FpB) respectively, see Figure 5.3 [225]. These cleavages result in further arrangement of the molecule chains forming a network of fibres that stabilise the clot [226]. In fibrinolysis, fibrin will be cleaved by plasmin at two different sites to break down the clot. Fig 5.4 shows the cleavage sites of FAC by the three enzymes: thrombin, plasmin and hementin that are involved in coagulation process.

Fibrinogen and Fibrin in Metastasis

The relationship between tumor metastasis and coagulation process has been widely studied. Fibrin and fibrinogen in particular gained more focused interest. Cleavages of fibrinogen and fibrin yield various products that have been found to regulate several critical cellular pathways and functions such as chemotachtic activities, cell adhesion and vasoconstriction. For example, fibrin A (a cleavage product of fibrin clot formation), fibrinopeptide A and fibrinogen α found to be associated with the initiation of multiple solid tumor [227, 228]. Furthermore, Fibrinogen/fibrin have been found to play a role in tumor progression. Collectively, studies found that fibrin and fibrinogen products enhance tumor progression through three proposed mechanisms, inducing angiogenesis, protecting the tumour cells from the natural killer cell and serving as a bridging molecule between tumor cells and the surrounding micro-environment [229]. Studies found that breast cancer, lung carcinoma and malignant melanoma metastasis but not primary tumor growth was significantly reduced in fibrinogen deficient mice [230]. These findings suggest that therapy targeting the fibrinogen system might prevent or treat metastasis. In fact recent studies showed that anticoagulant therapy resulted in diminished metastasis and improved cancer outcome [229]. We found a decrease in the serum level of a fragment of fibrinogen α chain in the metastatic group. This fragment is not related to physiological cleavages of fibrinogen α chain by any of the enzymes thrombin, plasmin and hementin that are involved in coagulation, see Figure 5.4. There might be another break down mechanism of fibrinogen

α chain that release such fragment in malignency. Further studies are needed to understand the mechanism and regulation of the reduction of this fibrinogen α chain fragment in metastatic breast cancer.

Limitations

Potential pitfalls usually exist in any scientific approach and we faced several challenges which accounted for a number of limitations in this project. Starting with the design of our study and due to financial constraints, we used small numbers of samples in each experimental group. In our preliminary study, in which we identified initial peaks and validated the approach for use in differentiating between metastatic and non-metastatic patients, we only used 6 samples of stage I and 6 samples of stage III BC. The use of small numbers of samples reduces the ability to identify highly predictive biomarkers or biomarker profiles. Further complicating this issue is the fact that serum samples are considered a major source of false discovery despite the fact that they are more attractive for biomarkers studies. Variability in serum samples exists at different levels of collection and storage. Standardization of collection techniques, handling and storage will eliminate the non biological quantitative differences. We tried to confirm this with both companies for both samples sets. One of the inevitable factors is that these samples are collected at different phase of the tumor development and collected at different clinic visit so they reflect different points along the the course of the tumor development. We tried to get the best matching sample sets in every study, but we could not get the full details of each patients such as menopause, genetic subtypes and stage of treatment. All these factors make it very challenging to label an identified protein as BC specific marker that is produced by tumor secreted protease. They will need further validation studies that follow a rigorous sample collection protocols and using different methodology.

The subsequent validation of the preliminary biomarkers in a set of blinded and independent serum samples (our confirmatory study with 39 total samples) showed the

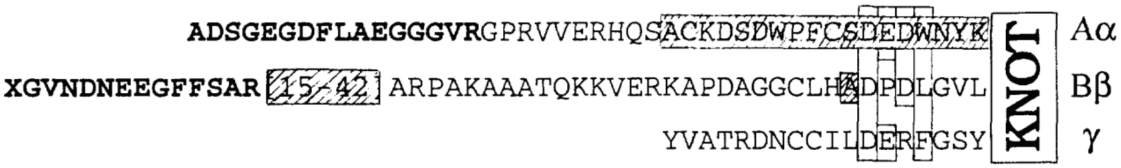


Figure 5.3: Primary Structure of the Fibrinogen Chains up to the First Disulphide Bond.
Shaded sequence: thrombin binding site, sequence with bold characters: fibrinopeptide A and B

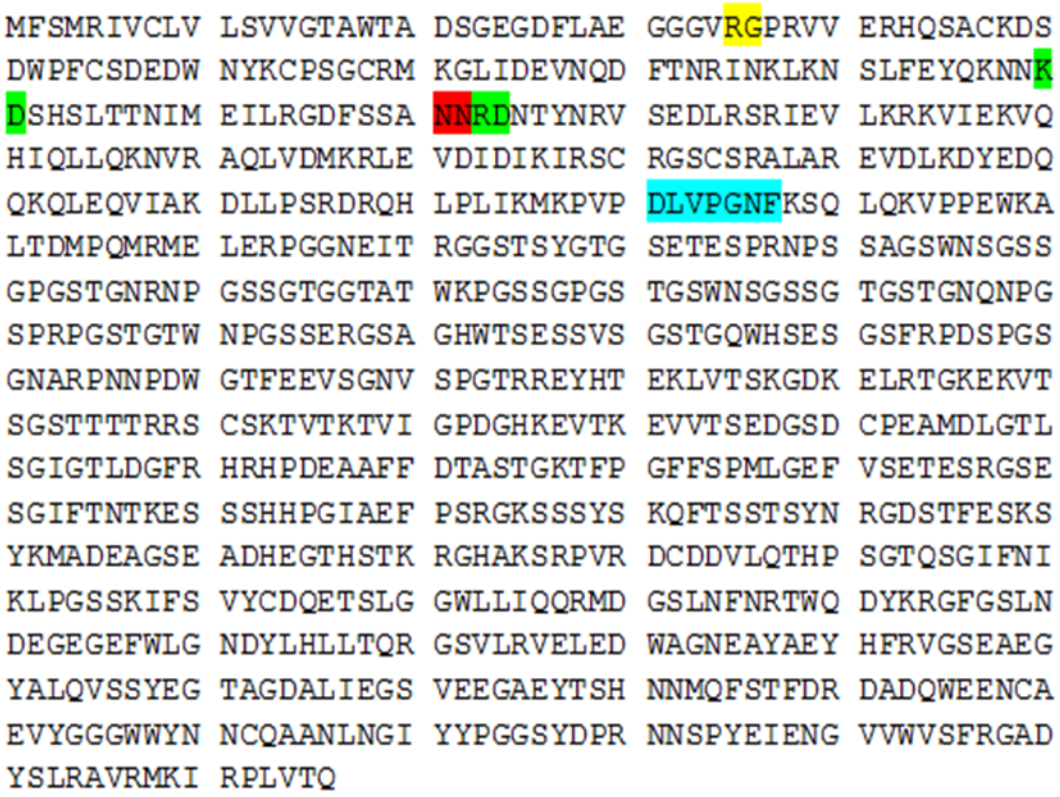


Figure 5.4: Full Length Sequence of Fibrinogen Alpha Chain.
Yellow: Thrombin cleavage site to release fibrinopeptide A.
Green: Plasmin cleavage site to break down fibrin clot.
Red: Hementin cleavage site to prevent coagulation.
Blue: Sequence of novel fragment (m/z 761.38).

potential significance of some of these biomarkers. The ability to replicate results in a blinded test set suggests that the use of best multimarker models could differentiate between samples based on the metastatic status and not based on artifacts of processing or on other confounding factors.

An additional issue comes from biomarker quantitation. Several factors should be considered that might contribute to the quantitative difference in proteins levels in any clinical samples [231]. Cancer heterogeneity and clinical samples variabilities are the two main critical and contributing factors that should be considered [231]. Breast cancer in particular is among the most heterogeneous cancer types. There is a complex heterogeneity at the inter- and intra-tumor levels. It is hard to predict that the same type of BC will have the same alteration in protein level. Furthermore, proteins undergo cancer specific posttranslational modification that are reflective of the tumor cells physiology and might interfere with analysis and identification of detected serum markers [232].

Additionally, the approach we used in this study was mainly built for the study of low M.W. peptides in biospecimens. However, the tandem MS studies that were designed to identify the potential biomarkers revealed that there are lipids components (m/z 497.27). The protocol calls for normalization of biomarker levels across all samples, but uses a bicinchoninic acid (BCA) protein assay that quantitates protein levels but does not detect lipids. Therefore, it is uncertain if the amount of each sample loaded into the MS was normalized for lipid levels. It will only become accurate if the levels of lipids relative to protein were constant.

Furthermore, the mass spectrum of the samples regularly showed overlapping or wide peaks. This can be explained by effects from the microcapillary column that we used for peptide separation, which is packed with POROS R1 slurry of 10 µm particle size. This effect results in difficulty in the identification of most of the biomarkers which have an overlapping peak adjacent to them. Therefore, their fragmentation spectrum included fragments from the overlapping peaks as well, making conclusions from the post collision

spectrum very challenging. For example, the fragmentation spectrum of biomarker m/z 497.27 displayed a product ion at m/z 184.06 that indicates a phosphocholine group, but this peak could be a fragmentation product of either the peak of interest or to the overlapping peak with equal probability.

Another challenge we faced is in lipid identification. Despite that the presence of a polar group that could hint at the lipid class, the complete structure of the identified lipid class could not be determined. Available databases, such as lipid map, do not not provide fragmentation data for comparison with fragmentation spectra generated from our experiment. Instead it only provides list of matches according to the accurate m/z. Therefore, further challenging lipid identification is the lack of a lipid database that includes any oxidized lipids.

One of the most challenging and tedious part of our study is the data analysis, which was performed manually. The output of LC-MS/MS of serum is very complicated, commonly has overlaps, and ion suppression must be accounted for during the visual analysis. The size of the file from one sample spectra is between 1 to 5 GB (2500 peaks in 10 time windows = 25000 total peaks), which makes it a tedious job in the absence of an automated software. Though we tried several software programs for automated analysis, it missed almost 80% of the potential biomarker that were detected manually. Technically, the development of computer programs that could allow faster and accurate data analysis will increase the efficiency and productiveness of analyzing many more samples within reasonable time. Efforts could be made in this direction in the future in developing software that can discriminate peaks, compare them and calculate the statistical differences between cases and controls automatically.

Future Research

In this study we were able to partially identify two of the low molecular weight serum biomarker for Breast Cancer that were detected through protoemics-based approach.

Further work focusing on identifying other biomolecules that are consistently present in our high performing multimarker models, such as m/z 458.25, 722.37, 923.45 and m/z 425.25, will be valuable. In addition, running an MS/MS experiment to fragment the marker m/z 497.27 at different chromatographic parameters to separate it from the co-eluting peaks would help to figure out if a reduction in serum SM could reflect the stage of Breast Cancer. It would also be very interesting to evaluate the performance of the detected markers in stage IV (distant metastatic) Breast Cancer sera. This will provide more details on the role of these molecules in distant metastasis and if cancer cell use different metabolites for their distant colonization. The proteomic evaluation of the changes in circulating molecules from stage I to stage II, stage III and stage IV could uncover the ongoing metabolism in the progression of the Breast Cancer.

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Conferences & Seminars

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